Universitat de Lleida

Estudi dels mecanismes moleculars implicats en la sensibilització a l'apoptosi en cèl·lules de carcinoma d'endometri.

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Índex

Abreviatures

Apaf-1: Apoptotic-protease-activating factor-1

BAFF: B-cell activating factor

Bcl-2: B-cell lymphoma/leukemia-2

BH: Bcl-2 homology domain

CARD: Caspase-recruitment domain

cDNA: Complementary deoxyribonucleic acid

ced-3: Cell-death abnormality-3

CK2: Casein kinase 2

DcR: Decoy receptor

DD: Death domain

DED: Death effector domain

DISC: Death-inducing signaling complex

DMAT: 2-dimethylamino-4, 5, 6, 7-tetrabromo-1H-benzimidazole

DR: Death receptors

EEC: Endometrioid endometrial carcinoma

EGF: Epidermal growth factor

ERK/MAPK cinases: Extracellular-signal-regulated kinase/mitogen-activated protein kinase

FADD: Fas-associated death domain containing protein

FLICE: Fas associated death domain-like ICE

FLIP: Fas-associated death domain-like inteleukin 1β converting enzime inbhibitory protein

ICE: Interleukin-1β-converting enzyme

IκB: Inhibitor of κB

IKK: Inhibitor of kB kinase

IQA: (5-oxo-5, 6-dihydroindolo (1, 2-a) quinazolin-7-yl) àcid acètic

KSR1: kinase supressor of ras-1

MI: Microsatellite Instability

NEEC: Non-endometrioid endometrial carcinoma

NF-κB: Nuclear Factor-κB

NK: Natural killer

OPG: Osteoprotegerina

PCD: programmed cell death

PTEN: phosphatase and tensin homolog (mutated in multiple advanced cancers 1)

Raf: Rapidly accelerated fibrosarcoma

RIP: Receptor interacting protein

TBB: 4, 5, 6, 7-tetrabromo-1H-benzotriazole

TBBz: 4, 5, 6-tetrabromobenzimidazole

TBCA: acid tetrabromocinnamic

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor- receptors

TRADD: TNF-receptor-associated death domain

TRAF: TNFR-associated factor

TRAIL: Tumor Necrosis Factor-related apoptosis inducing ligand

RHD: Rel homology domain

ROS: Reactive oxygen species

Introducció

1. ENDOMETRI

L'endometri és la capa més interna de l'úter. Histològicament també se'l coneix com a *túnica mucosa* i juntament amb el miometri (*túnica muscular*) i el perimetri (*túnica adventícia o serosa*) conformen les tres làmines que constitueixen la paret de l'úter.

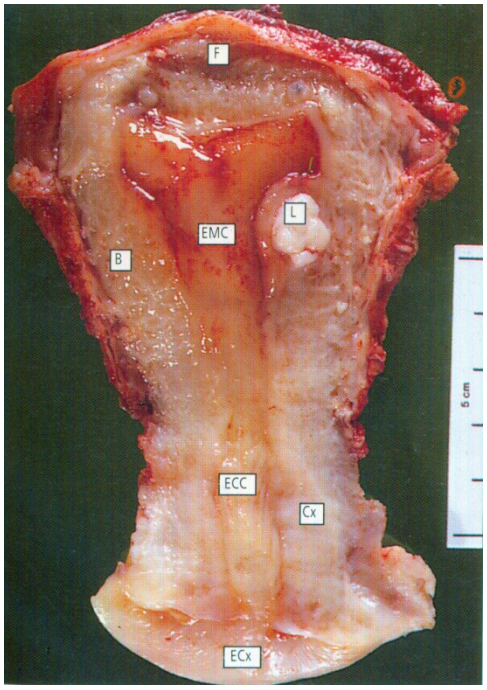


Figura 1: Tall sagital d'un úter d'una dona de 35 anys. S'aprecia el fundus (F), el cos (B) i el cèrvix (Cx). S'observa la cavitat endometrial (EMC), el canal endocervical (ECC) i l'ectocèrvix (Ecx). El múscul llis del cos conté un petit tumor, un leiomioma (L). *(Stevens, A & Lowe, J. Histología Humana, 1998)*[1].

1.1 Citoarquitectura de l'endometri

L'endometri està constituït fonamentalment per un epiteli columnar simple on s'hi troben dos tipus cel·lulars ben diferenciats: cèl·lules ciliades no secretores i cèl·lules secretores no ciliades.

D'altra banda, en l'estructura histològica de l'endometri també hi hem d'ubicar les glàndules tubulars que es situen en la part més basal de la túnica mucosa. Aquestes són simples i lleugerament ramificades però arriben a adquirir una complexitat remarcable en determinades fases del cicle menstrual. Finalment també hi trobem el còrion o component estromal, ric en vasos sanguinis i pobre en fibres nervioses.

Donat el caràcter altament dinàmic d'aquest teixit, podem classificar l'endometri en funció de les diferents fases del cicle menstrual en el que es troba. Així doncs podem parlar d'endometri proliferatiu, endometri secretor, endometri en fase descamativa, endometri atròfic o endometri gestacional.

1.2 L'endometri en el cicle menstrual

Comunament el cicle menstrual es coneix com a el procés durant el qual l'endometri sofrirà modificacions estructurals que el faran un receptacle apte per la implantació de l'òvul fecundat i posterior desenvolupament de l'embrió. Si finalment l'embaràs no té lloc, es produiran les variacions típiques del cicle menstrual que resultaran en última instancia en la menstruació.

El cicle menstrual va parell al cicle ovàric, essent aquest últim un element fonamental en la regulació de les diferents fases del cicle menstrual. Podem parlar de tres fases del cicle menstrual i de dos estrats de l'endometri. Les fases menstruals corresponen a la *Fase Proliferativa* (dies 6-14), *Fase Secretora* (dies 15-28) i *Fase Menstrual* (dies 1-5) mentre que els estrats endometrioides es divideixen en l'*Estrat Basal* (porció que roman després de la menstruació i que regenerarà l'estrat funcional) i l'*Estrat Funcional* (que es descama en cada cicle menstrual).

La Fase Proliferativa es caracteritza per un increment en la proliferació de l'endometri (còrion i epiteli) que resulta en el desenvolupament de les glàndules uterines. Aquest fenomen es deu a que, paral·lelament a aquesta fase, es desenvolupen en l'ovari els fol·licles ovàrics que secretaran i incrementaran la concentració d'estròg ens en el torrent sanguini que estimularan finalment la proliferació endometrial. El dia 14 es produeix l'ovulació, que es caracteritza per l'extrusió de l'ovari de l'oòcit secundari i que coincideix amb el pic de concentració de l'hormona luteïnitzant (LH) i hormona fol·liculo estimulant (FSH). Posteriorment a l'ovari les restes del fol·licle madur o fol·licle de Graaft formaran el que es coneix com a *cos luti* constituït primordialment per cèl·lules luteíniques de la granulosa i de la Teca i estroma vascular.

Durant la Fase Secretora (dies 15-28) es produeix un augment en la concentració de progesterona secretada pel cos luti. Aquest augment farà que les glàndules uterines es tornin tortuoses i que les cèl·lules secretores secretin productes rics en hidrats de carboni. Paral·lelament els vasos sanguinis també es tornaran més nombrosos per poder irrigar i nodrir així un endometri més complex compost ja per estrat basal i estrat funcional. Finalment el dia 28 es produeix la regressió del cos luti que es tradueix en un descens en els nivells de LH, FSH i progesterona (Fase

Menstrual) que provoca el col·lapse de l'estrat funcional que es trenca i descama. Els vasos sanguinis també es trencaran i es forma el flux sanguini.

Durant la Fase Menstrual es perd l'epiteli superficial (estrat funcional) i és el primer que es regenerarà a partir de les cèl·lules epitelials de les glàndules uterines localitzades en l'estrat basal. La regeneració es produirà en l'inici del següent cicle menstrual (fase proliferativa).

Val a dir que l'endometri humà té una gran capacitat de regeneració: en cada cicle menstrual l'endometri passa d'un gruix d'uns 0.5-1 mil·límetres després de la menstruació, fins un gruix d'uns 5-7 mil·límetres al final de la fase secretora.

2. EL CARCINOMA D'ENDOMETRI

El carcinoma d'endometri (EC, **e**ndometrial **c**arcinoma) és el carcinoma maligne més freqüent del tracte genital femení en països desenvolupats. Aproximadament el 75% està confinat a l'úter i s'associa a bon pronòstic. Tanmateix, en un percentatge significatiu de pacients, el tumor es presenta de forma disseminada o recidiva després del tractament; i en aquests casos el pronòstic és dolent.

Existeixen dues manifestacions clinicopatològiques del carcinoma d'endometri [2]: els de Tipus I són tumors de baix grau, estrogen dependents i de morfologia endometrioide (EEC, **e**ndometrioid **e**ndometrial **c**arcinoma), que generalment apareixen en dones peri-menopàusiques i coexisteixen o són precedits per hiperplàsia endometrial. Aquests són el tipus de carcinoma d'endometri objecte d'estudi en aquest treball.

Els de Tipus II són carcinomes de tipus no-endometrioide (NEEC, ***n***on-**e**ndometrioid **e**ndometrial **c**arcinoma). Aquests es comporten de forma agressiva, són independents d'estròg ens i succeeixen en dones post-menopàusiques a partir de pòlips endometrials o endometri atròfic. Els tumors de Tipus I (EEC) són més freqüents (85%) que els de tipus II (NEEC) (15%).

A

B

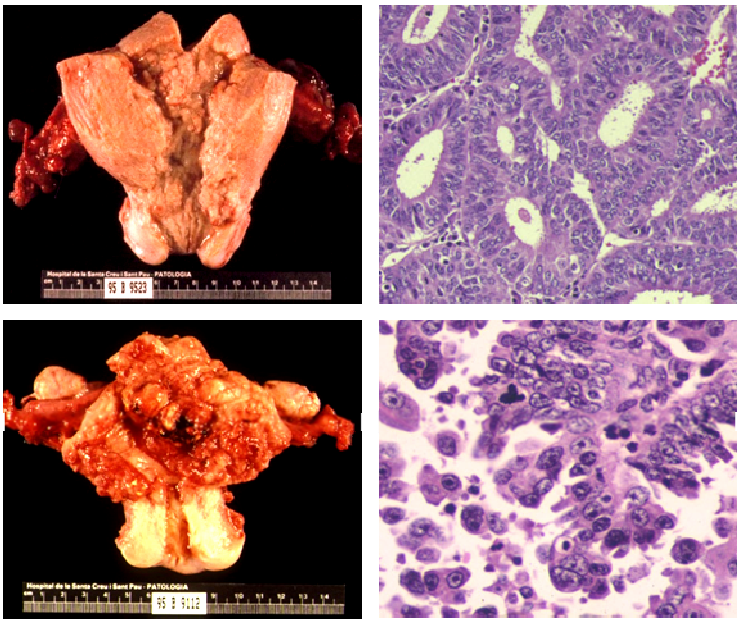


Figura 2: **A** (esquerra), representació d'un carcinoma endometrioide d'endometri (EEC). El tall histològic (dreta) mostra una citoarquitectura glandular normal, indicador de que el tumor és ben diferenciat (grau I). **B** (esquerra), representació d'un carcinoma d'endometri no endometrioide (NEEC). Dreta, tall histològic. S'aprecia una citoarquitectura aberrant típica d'un tumor d'alt grau (grau III). *(Modificat de: Prat, J; et al. Pathology, 2007. 39:72-87)*[3].

2.1 Alteracions moleculars en el carcinoma d'endometri

Les alteracions moleculars que participen en el desenvolupament dels EEC són diferents de les que participen en els NEEC. Clàssicament les alteracions més freqüents en EEC són la Inestabilitat de Microsatèl·lits, mutacions en PTEN, PI3KCA, K-RAS i CTNNB1. En canvi els NEEC mostren Inestabilitat Cromosòmica i mutacions en p53.

En el EEC, les alteracions moleculars anteriorment descrites probablement reflecteixin vies molecular diferents activades en diferents subtipus tumorals. De forma interessant aquestes alteracions poden coexistir en alguns casos: la Inestabilitat de Microsatèl·lits (MI, *M*icrosatellite *I*nstability) –alteracions en múltiples microsatèl·lits atribuïbles a inactivació de gens reparadors- té lloc en una freqüència de fins a un 20% dels casos, apareixent inclús en etapes inicials de la malaltia. Les mutacions activadores de CTNNB1, que codifica per la β-catenina, són altament

especifiques del EEC i succeeixen en el 30-40% dels casos. Les mutacions en CTNNB1 es solen associar a un patró d'inmunohistoquímica caracteritzat per l'acumulament de β-catenina en el nucli i citoplasma. Alteracions activants en K-RAS, fonamentalment en els codons 12 i 13, són una alteració precoç en el carcinoma d'endometri degut a que es detecten ja en les hiperplàsies.

D'altra banda, les mutacions inactivants de PTEN també són freqüents en el carcinoma d'endometri (33-85%), especialment en aquells tumors que presenten Inestabilitat de Microsatèl·lits i solament ocorren en aproximadament el 5% dels NEEC. Les alteracions de PTEN, juntament amb les mutacions activadores en K-RAS expliquen la freqüent alteració de la via PI3K/Akt en els EEC.

Per últim, les alteracions en p53 són considerades com un fenòmen tardà en el 10-20% dels EEC, mentre que es detecta en una freqüència de fins un 90% dels NEEC en estadis encara inicials.

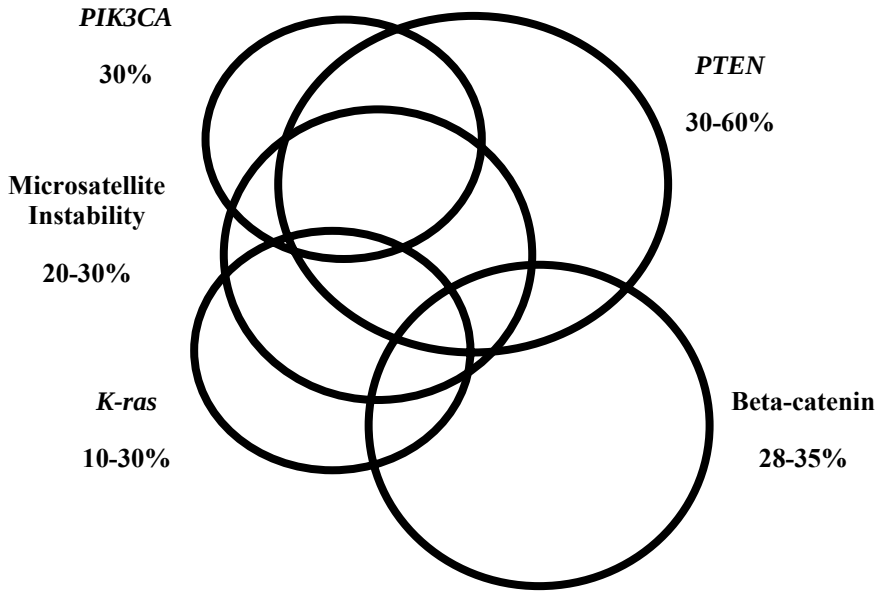


Figura 3: Principals alteracions genètiques trobades en els carcinomes endometrioides d'endometri. MI, inestabilitat de microsatèl·lits, mutacions en els gens PTEN, k-RAS i β-catenina. *(Modificat de: Matias-Guiu, X; et al. Human Pathology, 2001. 32:569-77)*[4].

Sembla evident que en el EEC existeix una progressiva acumulació d'alteracions moleculars en el procés de transformació neoplàsica i progressió tumoral. Això explica el solapament que s'observa per algunes de les mutacions més

freqüents (Fig3) i indica que per la formació i progressió d'un carcinoma es necessita més d'una alteració.

Aquestes alteracions ocasionen hiperactivacions de determinades vies de senyalització intracel·lular que resulten en una progressiva resistència a l'apoptosi i/o un increment de la proliferació cel·lular. Un exemple de la progressiva acumulació d'alteracions moleculars en el ECC es veu reflectida per les mutacions inactivants de BAX en els ECC que presenten Inestabilitat de Microsatèl·lits. BAX conté un tracte mononucleòtid en la seva seqüència codificant que està freqüentment mutat en els ECC amb MI. Tanmateix les mutacions secundaries de BAX succeeixen solament en subclones dels EEC amb MI, conferint resistència a l'apoptosi en aquestes clones i, per tant, una avantatge de creixement. Alteracions paregudes s'han observat en altres gens que contenen tractes mononucleòtids en els EEC amb MI.

3. MORT CEL·LULAR PROGRAMADA

La mort cel·lular és un procés irreversible que condueix a la pèrdua de la funció cel·lular. Existeixen diferents tipus de mort cel·lular i diferents nomenclatures per cada una d'elles. A continuació, però, descriurem el tipus de mort conegut com a apoptosi ja que és el tipus de mort en la qual es basa aquest treball.

La *mort cel·lular programada* (PCD, programmed cell death) és el principal tipus de mort implicat en processos de desenvolupament embrionari, proliferació o manteniment de l'homeòstasi tissular (sobretot en teixits amb una alta taxa de renovació i recanvi cel·lular com és el cas de la paret uterina) però també juga un paper clau en determinades patologies com és el cas de la neurodegeneració, patologies autoimmunes o càncer. Ja es tenia coneixença de la mort cel·lular molts segles abans que el laboratori del Dr. Currie proposés a principis dels anys setanta "batejar" aquest fenomen com a apoptosi [5]. Des d'aleshores mort cel·lular programada i apoptosi s'han usat indistintament per referir-se a un mateix fenomen.

L'apoptosi es caracteritza per ser un fenomen que actua sobre cèl·lules individuals i que, per tant, és molt específic i poc dràstic. Morfològicament l'apoptosi es caracteritza per una disminució del volum cel·lular, condensació de la cromatina, fragmentació nuclear, lobulació de la membrana plasmàtica (*blebbing*) i

la formació de cossos apoptòtics rodejats per membrana que seran finalment fagocitats per macròfags sense estimular cap resposta inflamatòria [6-8].

La existència d'un programa suïcida controlat genèticament va ser en primer terme descobert a través d'estudis en *Caenorhabditis elegans* [9]. En aquest treball Horvitz i col·laboradors demostren que la mort fisiològica de 131 cèl·lules, d'un total de 1090 cèl·lules generades durant el desenvolupament del citat nematode, està controlada per quatre gens: CED-3, CED-4, CED-9 i EGL-1 (Fig4). Mutacions inactivants en ced-3 o ced-4, o la sobreexpressió de ced-9 bloquejaven la mort cel·lular ocorreguda durant el desenvolupament. Estudis bioquímics subseqüents demostren que aquestes proteïnes actuen com un complex ternari on l'activació de la proteasa ced-3 a través de la proteïna adaptadora ced-4 és normalment reprimida per la proteïna antiapoptòtica ced-9. D'altra banda en aquelles cèl·lules que estan promocionades per morir, l'expressió de egl-1 promou el desacoblament de ced-9 sobre ced-4; llavors ced-3 podrà exercir la seva acció proteolítica.

Els components del citat programa apoptòtic s'han conservat al llarg de l'escala filogenètica i també els trobem en mamífers: CED-9 és homòleg als membres antiapoptòtics de la família de Bcl-2. CED-4 conserva homologia amb Apaf-1, CED-3 conserva homologia amb una sèrie de cistein proteases intracel·lulars conegudes amb el nom de caspases i finalment EGL-1 té homologia amb la família de proteïnes BH3-*only*.

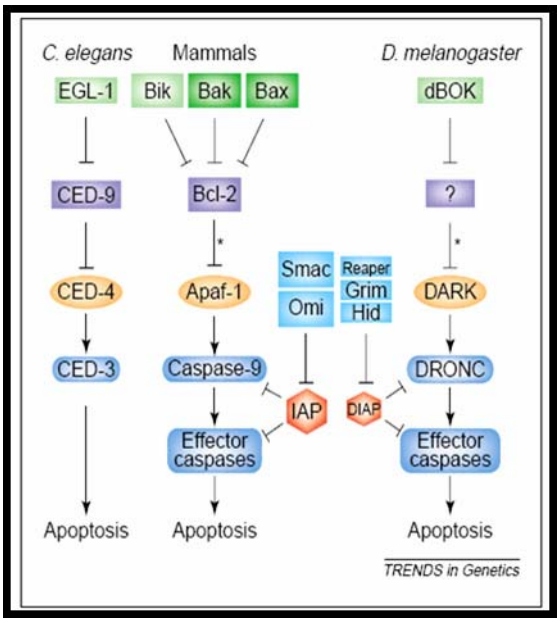


Figura 4: Conservació evolutiva de les vies apoptòtiques. Les proteïnes presents en mamífers que participen en l'activació i execució de la mort cel·lular, i les respectives proteïnes homòlogues en *Caenorhabditis elegans* i *Drosophila melanogaster*. *(Joza, N; et al. TRENDS & Genetics, 2002. 18:142-49)*[10].

L'apoptosi és, per tant, un mecanisme que ha d'ésser estrictament controlat en múltiples nivells dins la pròpia cèl·lula ja que alteracions que afectin tant a l'execució com a la captació dels estímuls adients poden tenir conseqüències greus en la viabilitat cel·lular.

3.1 La maquinària apoptòtica

L'observació de que la mort cel·lular és un fenomen que té lloc durant el correcte desenvolupament ja es va fer ara fa uns cent-cinquanta anys [11] però l'interés en aquesta àrea va romandre de forma més o menys marginal fins a les darreries del segle vint. L'any 1965 Lockshin i Williams proposen el terme mort cel·lular programada (PCD) per descriure la mort cel·lular durant la metamorfosi dels insectes i dels capgrossos com si, d'alguna manera, estigués programada intrínsicament dins les cèl·lules [12]. Tal com ja s'ha esmentat amb anterioritat, l'any 1972 Kerr, Wyllie i Currie introdueixen el terme "apoptosi" per descriure una mort cel·lular associada a una determinada sèrie de característiques morfològiques.

Posteriorment a finals de la dècada dels setanta els estudis en *Caenorhabditis elegans* per Horvitz i Sulston assenten les bases per a la caracterització genètica dels components moleculars de la maquinària apoptòtica. Aquests estudis van permetre identificar els gens que controlaven el mecanisme de suïcidi cel·lular en *C. elegans* [9,13] i van catapultar l'apoptosi de la relativa obscuritat a primera línia en la recerca biològica. A partir d'aquí, dècades d'intens estudi han permès durant els anys identificar i caracteritzar gran quantitat proteïnes i vies de senyalització implicades en l'apoptosi i descobrir que una inapropiada apoptosi també juga un paper important en el desenvolupament de patologies com, per exemple, malalties neurodegeneratives (Alzheimer, malaltia de Huntington), dany isquèmic, desordres autoimmunes o càncer.

En referència a aquest últim, la progressiva acumulació d'alteracions en vies apoptòtiques pot frustrar l'execució de l'apoptosi induïda per d'agents antitumorals; d'aquesta manera els tumors es fan progressivament resistents a les teràpies convencionals.

3.1.1 **REGULADORS DE L'APOPTOSI I:** Caspases

L'execució de l'apoptosi és efectuada, entre d'altres, per una sèrie de cistein-proteases conegudes amb el nom de caspases. Durant la mort cel·lular les caspases seran processades a partir de les respectives proformes catalíticament inactives en enzims actius que seran els responsables de la majoria de canvis morfològics i bioquímics propis de l'apoptosi [14,15].

Les caspases esdevenen d'aquesta manera els components centrals de la resposta apoptòtica i reben aquest nom perquè es tracta de **c**istein-protein**ases** que processen els seus substrats en una posició +1 adjacent a un residu **asp**àrtic, d'aquí el nom c-asp-ases.

Tot i que la primera caspasa fou identificada en humans (ICE, **i**nterleukin-1β-**c**onverting **e**nzyme) [16,17], el paper crític que juguen les caspases en l'apoptosi va ser descobert a partir de la caracterització en *C. elegans* de la proteïna ced-3 (**ce**ll-**d**eath abnormality-**3**), que conserva una alta homologia a ICE/caspasa-1. A partir d'aquí s'han identificat 14 caspases diferents en mamífers de les quals 11 s'expressen en humans.

La primera caspasa identificada en mamífers va ser identificada com un regulador de la resposta inflamatòria però al menys 7 de les 14 caspases conegudes participen en mecanismes apoptòtics. La primera evidència fou documentada pel grup de Horvitz [18] que observà com la sobreexpressió de ICE era capaç d'induir una resposta apoptòtica per si sola. Tanmateix, altres evidencies que reforcen el paper de les caspases en l'apoptosi ve donada per l'habilitat d'inhibidors específics de proteases de bloquejar l'apoptosi com són la proteïna del Cowpox virus CrmA [19-21] o la proteïna p35 del baculovirus [22].

3.1.1.1 Estructura i funció de les caspases

Les caspases es sintetitzen com a zimògens amb activitat nul·la o molt reduïda que s'activen per autocatàlisi o per acció d'una altra proteasa. Com a zimògens inactius les caspases comprenen un prodomini N-ternimal, una subunitat llarga (p20) que conté el centre cisteïna actiu i una subunitat petita (p10) (Fig5). El pas de enzim inactiu a actiu implica el processament de la pròpia caspasa en punts concrets de la seva pròpia seqüència aminoacídica i això permet establir una classificació en funció

del mecanisme d'activació per processament. Un cop processades i alliberada la forma catalíticament activa aquesta contindrà només les subunitats p20 i p10 [23-26].

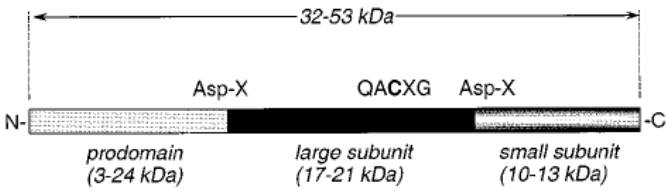


Figura 5: Representació esquemàtica d'una procaspasa. *(Nicholson, DW. Cell Death and Differentiation, 1999. 6:1028-42)*[24].

Les caspases reconeixen una seqüència de tall tetrapeptídica específica en les proteïnes diana. Essencialment les caspases requereixen que en la seqüència de tall es localitzi un residu aspàrtic en la posició 1 (P1). Les posicions P2 i P3 no necessiten estar altament conservades (tot i que la posició en P2 està normalment ocupada per un residu Glu) i permeten certa promiscuïtat, indicant que podrien determinar especificitat de substract per cada caspasa. Finalment el residu aminoacídic en posició P4 determinarà la seva classificació dins de tres grups genèrics.

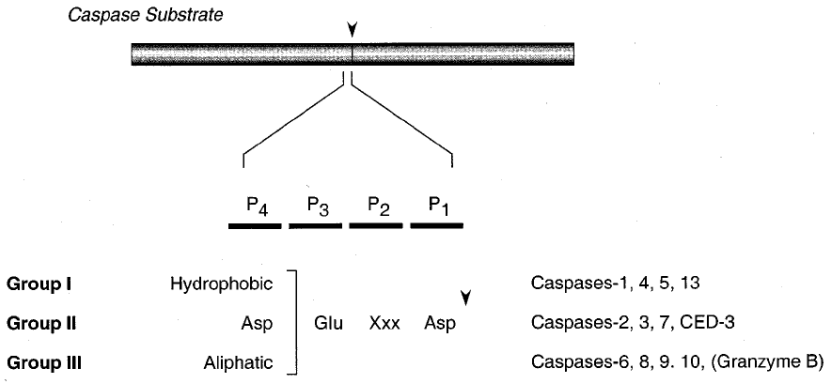


Figura 6: Representació esquemàtica del motiu tetrapeptídic identificat per les caspases i la classificació subseqüent en funció del residu en posició P4. El processament es produeix de forma adjacent al residu en posició P1. *(Nicholson, DW. Cell Death and Differentiation, 1999. 6:1028-42)*[24].

Per últim, algunes caspases contenen un prodomini curt (caspasa-3, -6 i -7) mentre que d'altres contenen prodominis grans (Fig6). Alguns d'ells com el prodomini DED (death-effector domain) o el prodomini CARD (Caspase-

recruitment domain) permet a les caspases-8 i -10 (DED) o a la caspasa-9 i -2 (CARD) associar-se a proteïnes adaptadores en receptors de membrana com CD-95 o TNFR-1 [27,28] o associar-se a proteïnes citosòliques com Apaf-1 (Apoptotic-protease-activating factor-1) respectivament. Aquest dominis, doncs, permeten a les caspases que els contenen posicionar-se en nivells estratègics pel que fa a la captació de senyals de mort i inici de la senyal intracel·lular i per això també se les coneix com a caspases inductores o iniciadores. D'altra banda aquelles que no comprenguin aquests dominis seran caspases l'activitat de les quals dependrà de la prèvia activació de les caspases iniciadores i reben el nom de caspases executores.

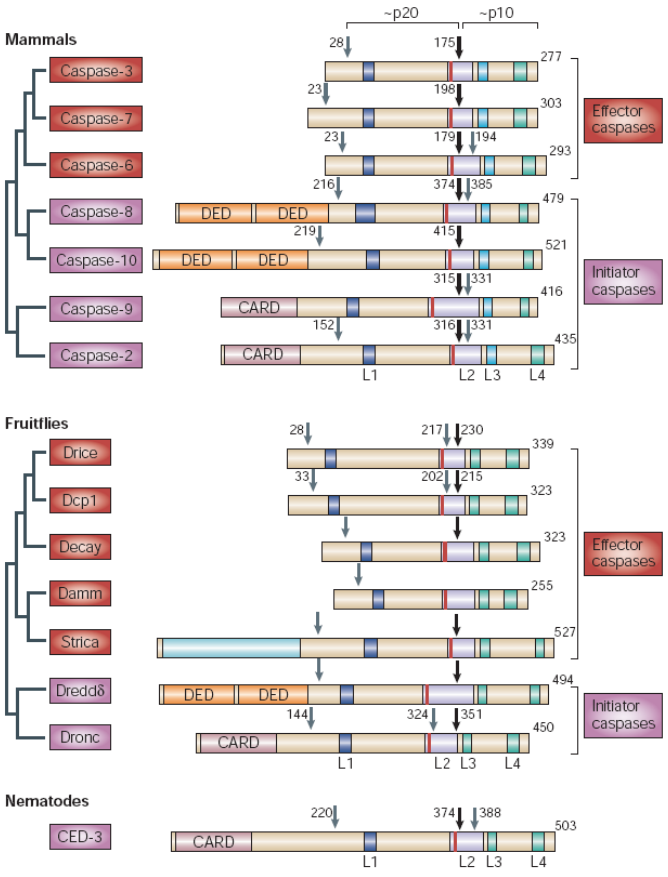


Figura 7: Caspases apoptòtiques en mamífers, mosca de la fruita i nematodes. S'aprecia la presencia dels prodominis grans en les caspases iniciadores (DED i CARD). *(Riedl, SJ and Shi, Y. Nature Reviews, 2004. 5:897-907)*[26].

3.1.2 **REGULADORS DE L'APOPTOSI II:** Bcl-2

Bcl-2 és l'acrònim pel gen ***B***-***c***ell ***l***ymphoma/leukemia-***2***. Tal i com indica el seu nom, aquest gen va ser descobert pel seu paper en malalties de limfòcits B, on una translocació cromosomal activava el gen en la majoria de limfomes fol·liculars de cèl·lules B no-Hodgkin. En aquestes translocacions, el gen bcl-2 és desplaçat des de la seva localització en 18q21 a una posició adjacent al locus de la cadena pesada d'immunoglobulines (IgH) en la posició 14q32, caracteritzada per presentar un promotor fort, amb la conseqüent sobreproducció de la proteïna bcl-2 [29].

A continuació, Vaux i col·laboradors van ser els primers en documentar el paper de bcl-2 en supervivència cel·lular a l'observar com l'expressió estable de bcl-2 perllongava la viabilitat cel·lular (però no la proliferació) de limfòcits B immadurs dependents de interleukina-3 quan aquesta no es trobava present en el medi de cultiu [30]. Aquests resultats suggerien que bcl-2 era capaç de bloquejar la mort cel·lular programada, una noció que va ser finalment demostrada pel laboratori de Korsmeyer l'any 1990 [31].

En contraposició a l'increment en supervivència cel·lular observada quan la proteïna bcl-2 era sobreexpressada, la seva reducció fent ús d'oligonucleòtids antisentit va demostrar una acceleració en el percentatge de mort cel·lular en un context de deprivació de factors de creixement [32,33]. De forma addicional, els experiments antisentit també demostraren que la reducció de bcl-2 feia les cèl·lules més sensibles a l'apoptosi però que en si mateixa era insuficient per causar la mort cel·lular [32-34], un descobriment que era consistent amb l'observació que alguns tipus cel·lulars no contenen nivells de bcl-2 detectables in vivo [35,36].

L'habilitat de bcl-2 quan era sobreexpressada de prevenir la mort cel·lular sense necessàriament afectar la proliferació cel·lular suggeria que el gen bcl-2 pertanyia a una nova categoria d'oncògens.

3.1.2.1 Família de bcl-2 i classificació

Bcl-2 donà nom a tota una família de proteïnes, amb aproximadament una vintena de membres identificats en mamífers, la principal característica de les quals és la presència d'un domini conservat homòleg a Bcl-2 (BH, ***B***cl-2 ***h***omology domain).

El clan inclou quatre proteïnes antiapoptòtiques més: Bcl-x, Bcl-W, A1/Bfl i Mcl1 i dos grups de proteïnes que promouen mort cel·lular: la família Bax i la família de les proteïnes BH3-*only*. Els membres de la família Bax presenten seqüències similars a les de Bcl-2, especialment en les regions BH1, BH2 i BH3 però la família també proapoptòtica BH3-*only* només conté un motiu BH (d'aqui el seu nom); un domini d'interacció que és necessari i suficient per la seva acció *killer*.

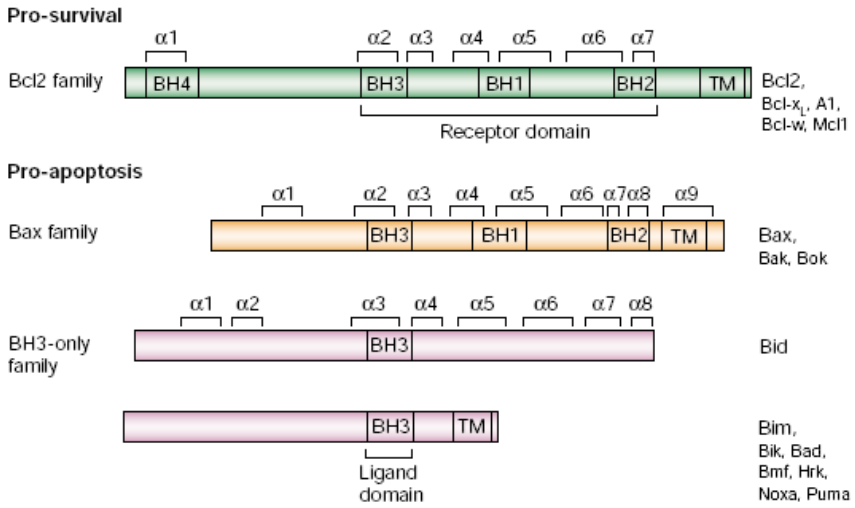


Figura 9: Les tres subfamílies de la família Bcl-2. S'aprecien els dominis d'homologia (BH). Molts dels membres presenten un domini hidrofòbic a l'extrem carboxil-terminal que els faculten per associar-se a membranes intracel·lulars. TM (transmembrane domain). *(Cory, S and Adams, JM. Nature Reviews, 2002. 2:647-56)*[37].

La subfamília antiapoptòtica Bcl-2

Bcl-2 i els seus homòlegs més propers Bcl-xL i Bcl-W inhibeixen potentment l'apoptosi en resposta a molts insults citotòxics com, per exemple, irradiació gamma i ultraviolada, drogues quimioterapèutiques o deprivació de factors de creixement [38].

El seu domini hidrofòbic localitzat a l'extrem carboxil-terminal (TM) les permet ancorar-se en la cara citoplasmàtica de tres membranes intracel·lulars: membrana mitocondrial, membrana del reticle endoplasmàtic i embolcall nuclear. Bcl-2 és una proteïna integral de membrana, inclús en cèl·lules sanes, mentre que Bcl-xL i Bcl-W

només esdevenen íntimament associades a membrana en resposta a una senyal citotòxica, senyal indicativa d'un canvi conformacional induïble [39].

Evidencies creixents semblen indicar que tota cèl·lula requereix la protecció de, com a mínim, un homòleg de Bcl-2 i que l'abundància d'aquests "guardians" regula l'homeòstasi tissular. La sobreexpressió de Bcl-2 en línies hematopoiètiques resulta en un excés de limfòcits B, T i cèl·lules mieloides que són refractàries a diversos estímuls citotòxics [40-44]. Contràriament, la inactivació dels membres d'aquesta família augmenta l'apoptosi en tipus cel·lulars específics presumiblement perquè les concentracions dels altres membres no són suficients per compensar. Així doncs, Bcl-2 per si sol és requerit per regular la supervivència de les cèl·lules mare de ronyó, melanòcits i limfòcits madurs [45], Bcl-xL per cèl·lules neuronals i eritroides [46], Bcl-W pels progenitors de cèl·lules espermàtiques [47,48], A1/Bfl per neutròfils [49] i Mcl1 durant l'implantació del zigot [50].

La subfamília BH3-*only*

Les proteïnes BH3-*only* són considerades com a "sentinelles" encarregades d'iniciar una senyal apoptòtica en resposta a dany intracel·lular. Actualment aquesta subfamília consta de més de deu membres, característica indicativa del refinament en el control de la mort cel·lular. Els seus membres provoquen la mort a través de la unió al domini receptor d'altres membres multidomini de la família de Bcl-2, inhibint-los (si són membres antiapoptòtics) o activant-los (si són membres proapoptòtics).

Una característica interessant d'aquestes proteïnes és que no poden desencadenar mort apoptòtica en absència de Bax i Bak (membres de la subfamília Bax) i, per tant, la seva funció l'hem de situar "aigües amunt" en la mateixa via de senyalització [51,52]. Alguns dels seus membres també contenen a l'extrem carboxil-terminal un motiu hidrofòbic que els permet associar-se a membranes intracel·lulars.

Individualment, les proteïnes BH3-*only* es mantenen a l'espera de forma inactiva per diversos mecanismes:

-Bim i Bmf són segrestades per la unió a la cadena lleugera de dineïna associada als microtúbuls (Bim) i citoesquelet d'actina (Bmf) [53,54].

-Bad, un cop fosforilada per proteïnes com Akt i la proteïna cinasa A és segrestada per la proteïna scaffold 14-3-3 [55] mentre que Bid es troba en estat relativament inactiu fins que és processada proteolíticament [56,57].

-Per acabar, proteïnes BH3-*only* com Noxa, Puma o Harakiri (Hrk)/DP5 són controlades principalment a nivell transcripcional [58-61].

La subfamília Bax

La família Bax consta de tres proteïnes: Bax, Bak i Bok. Mentre que Bax i Bak estan àmpliament distribuïdes, Bok es fa més patent en teixits reproductors.

Els primers estudis revelaren que la inactivació de Bax afectava l'apoptosi de forma poc dràstica i que la disrupció de Bak no presentava efectes apreciables mentre que la inactivació d'ambdós proteïnes impedia l'apoptosi en diversos teixits [52,62,63]. Es dedueix doncs, que la presència de Bax i Bak sembla essencial per l'execució de l'apoptosi en múltiples tipus cel·lulars.

Bax i Bak sembla que exerceixen la seva funció primordialment en la mitocondrial [64,65], però també se n'han documentat funcions en el reticle endoplasmàtic.

Bax és un monòmer en cèl·lules sanes però canvia de conformació durant l'apoptosi, s'integra en la membrana externa de la mitocondrial i oligomeritza [66-68]. D'altra banda, Bak és una proteïna integral de membrana mitocondrial però també canvia de conformació durant l'apoptosi i pot formar agregats.

Així, Bax i Bak poden oligomeritzar en la mitocondrial i formar porus que donaran lloc a la permeabilització de la membrana mitocondrial externa. Aquest procés es pot veure afavorit pel paper que poden jugar altre membres BH3-only com Bad o Noxa, que interaccionen amb proteïnes antiapoptòtiques com Bcl-2 i Bcl-xL [69].

3.2 Vies apoptòtiques

Existeixen dos vies de senyalització apoptòtiques principals, la via extrínseca o dels receptors de mort i la via intrínseca o mitocondrial (Fig10).

En la via dels receptors de mort, la senyal prové de la interacció entre el lligand i el receptor de mort. Els receptors de mort pertanyen a la superfamília dels receptors

de mort (TNFR, *T*umor *n*ecrosis *f*actor- *r*eceptors) mentre que els lligands pertanyen a la família dels factors de necrosi tumoral/factors de creixement nerviós.

D'altra banda, la via mitocondrial està regulada principalment per la família de proteïnes Bcl-2. Aquesta via s'inicia en resposta a diversos estímuls com, per exemple, estrés a reticle endoplasmàtic, dany al DNA, etc.

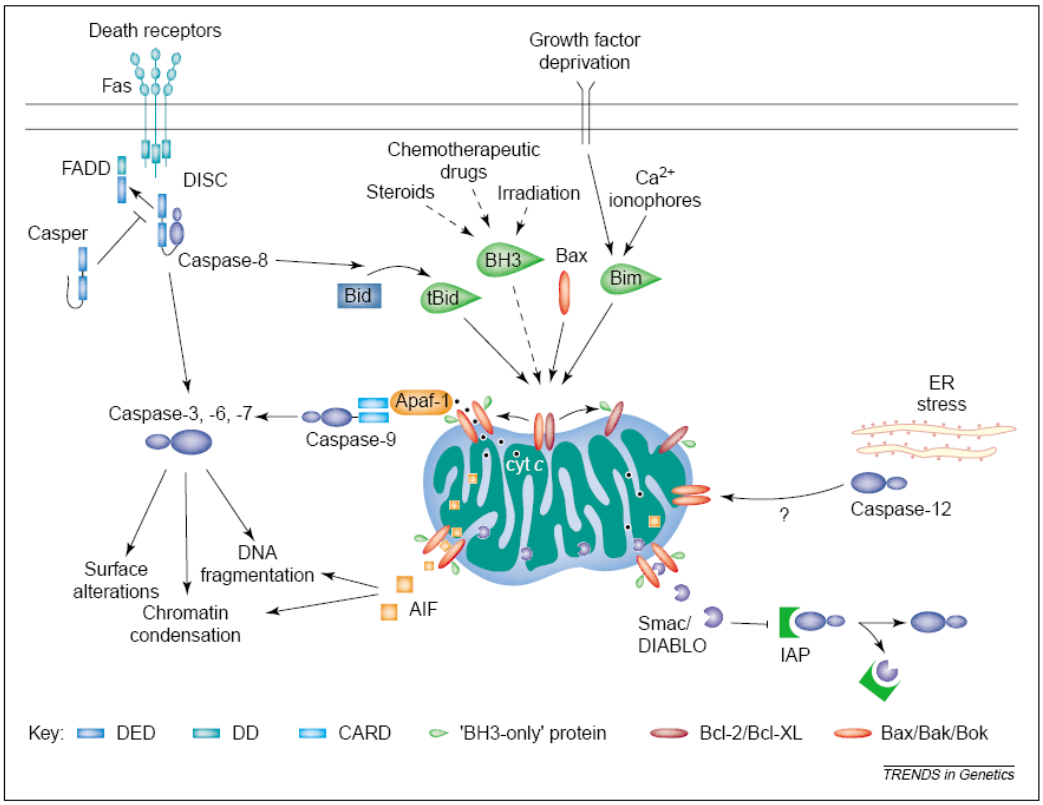


Figura 10: Representació esquemàtica de les principals vies de senyalització apoptòtica. *(Joza, N; et al. TRENDS & Genetics, 2002. 18:142-49)*[10].

3.2.1 Via intrínseca o mitocondrial

Alguns tipus cel·lulars entren en apoptosi a través de l'activació de l'anomenada via mitocondrial o intrínseca; caracteritzada per tenir la mitocondrial com a element central.

La mitocòndria pot ésser vulnerada en resposta a diversos estímuls com, per exemple, radiacions ultraviolada i gamma, dany al DNA, drogues quimioterapèutiques, estrés al reticle endoplasmàtic, etc [70,71].

En *Caenorhabditis elegans*, l'activació de la caspasa ced-3 requereix ced-4. Per contra, ced-9 actua bloquejant l'activació de ced-3 segrestant ced-4 a la mitocondrial [72,73]. Aquelles cèl·lules que rebien una senyal de mort responien produint egl-1, la qual s'unia a ced-9 i alliberava ced-4 per poder activar ced-3.

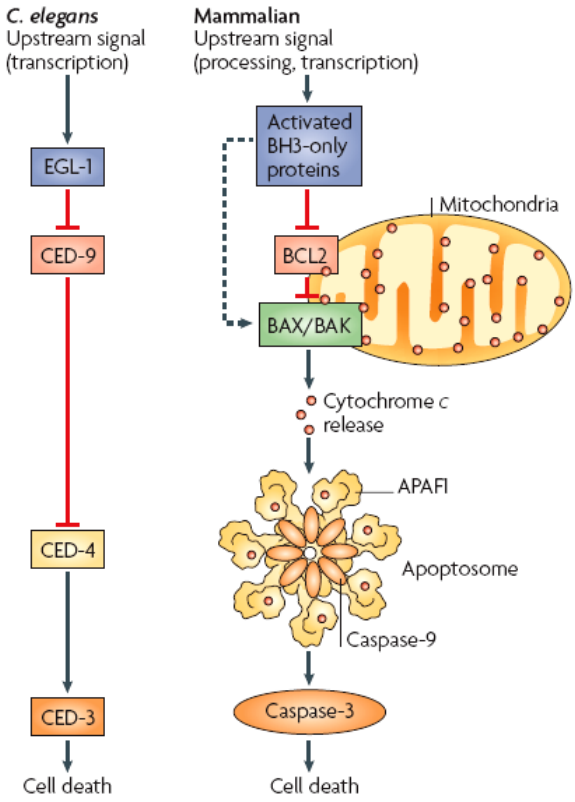


Figura 11: Expansió evolutiva de la maquinària apoptòtica des de C. elegans a cèl·lules de mamífer. (Degterev, A and Yuan, J. Nat. Rev. Mol. Cell. Biol., 2008. 9:378-90)[74].

La proteïna scaffold ced-4 té un domini amino-terminal anomenat CARD (**C**aspase **r**ecruitment **d**omain) que facilita l'interacció homotípica amb un domini CARD comparable present en ced-3. A més a més, ced-4 presenta un domini NBD (**N**ucleotide-**b**inding **d**omain) que permet l'oligomerització requerida per promoure el processament de ced-3 [75].

L'homòleg de ced-4 en mamífers Apaf-1 va ser descobert com un cofactor requerit per l'activació de la caspasa-3 a través de la caspasa-9 [76-78] però a diferència de ced-4, Apaf-1 és citosòlica [79], no s'uneix Bcl-2 o altres membres de la mateixa subfamília [80] i a més de presentar els motius CARD i NBD, conté dos dominis WD40 situats a l'extrem carboxil-terminal.

El domini WD40 és un motiu proteic conservat d'aproximadament 40 residus que permet unir el citocrom c provinent de la mitocondrial danyada [81,82] i possibilitar d'aquesta manera que Apaf-1 adopti una conformació més oberta. Com a resultat Apaf-1 multimeritzarà i s'associarà amb la caspasa-9 per formar un complex heptamèric d'uns 1.4 MDa (*M*ili *D*alton) anomenat apoptosoma. La formació de l'apoptosoma resultarà en l'activació de la caspasa-9 que, al seu torn, activarà les caspases efectores -3, -6 i -7 [83,84].

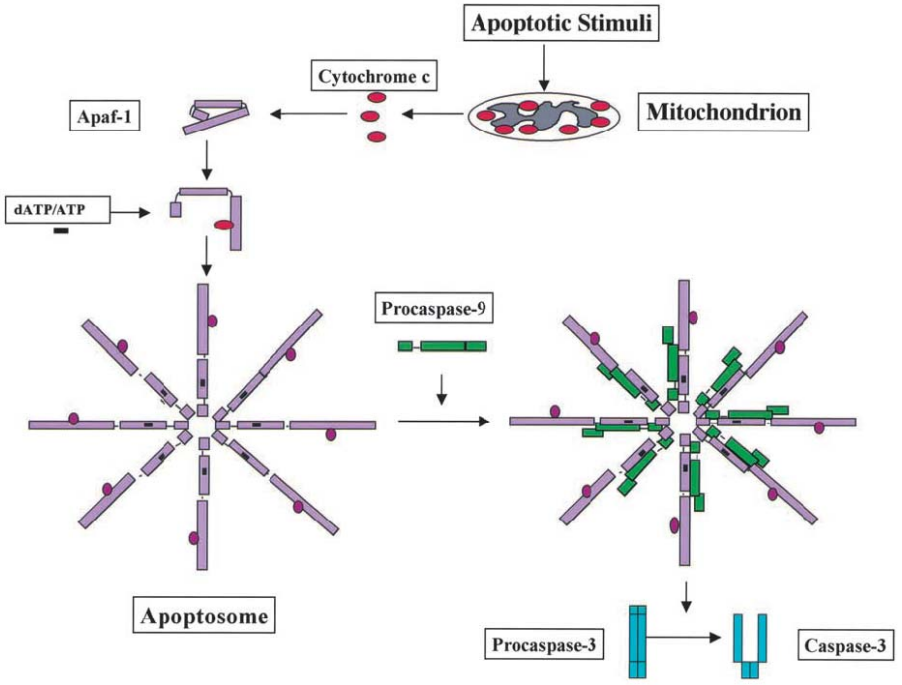


Figura 12: Formació de l'apoptosoma descrita per Xiaodong Wang al 2001. La sortida de citocrom c de la mitocondrial possibilita l'ensamblatge d'Apaf-1 amb la caspasa-9. La conseqüent activació de la caspasa-9 permetrà continuar el senyal apoptòtic cap a les caspases executores. *(Wang, X. Genes & Development, 2001. 15:2922-33)*[85].

La sortida del citocrom c de la mitocondria és el resultat en l'alteració de la permeabilitat de la membrana mitocondrial externa. Com a conseqüència, altres proteïnes com Smac/Diablo, AIF o l'endonucleasa G que normalment són retingudes dins aquest orgànul són alliberades al citosol [86-89].

La causa de l'alteració en permeabilitat mitocondrial apunta als oligòmers formats per Bax i Bak a la superfície de la membrana mitocondrial externa [64] (Fig11). Tanmateix, el mecanisme pel qual succeeix és controvertit però el model més

acceptat actualment és el que es basa en la semblança entre els membres de la família Bcl-2 i la toxina diftèrica i que postula que Bax i Bak poden formar canals. Aquesta hipòtesi s'implementa per l'observació documentada de que oligòmers de Bax poden formar porus en liposomes [90] i que les mitocòndries de les cèl·lules en apoptosi presenten un nou canal [91]. Alternativament, Bax podria interaccionar amb components del PTP (*P*orus *t*ransitori de *p*ermeabilitat) com per exemple la proteïna VDAC (*V*oltage-*d*ependent *a*nion *c*hannel) per crear un canal major [92,93].

La via mitocondrial, però, també pot ésser modulada per altres elements aliens a la família de proteïnes Bcl-2. Un exemple clar és p53, una proteïna supressora de tumors que respon a diversos estímuls d'estrès parant la progressió del cicle cel·lular a través de l'expressió de proteïnes com p21. En un context de dany cel·lular irreparable, p53 promou l'apoptosi via l'expressió d'altres gens clarament implicats en el control de l'apoptosi mitocondrial com, per exemple, puma, noxa, apaf-1, o bax [94,95] o inhibint l'expressió de gens antiapoptòtics com Bcl-2 o Bcl-xL [96].

Així doncs, l'acció reguladora de les proteïnes de la família Bcl-2 en la via apoptòtica intrínseca o mitocondrial està ben caracteritzada però és la formació de l'apoptosoma a partir d'Apaf-1/caspasa-9 l'únic mecanisme de mort apoptòtica? Si fos així, la pèrdua d'Apaf-1 o caspasa-9 hauria de ser tan efectiva com la sobreexpressió de Bcl-2 en la protecció cel·lular contra múltiples mecanismes d'estrès. Consistent amb aquest afirmació, la majoria dels animals deficients en Apaf-1 o caspasa-9 presenten cervells allargats a causa d'un dèficit en l'apoptosi durant el desenvolupament neuronal i moren poc després de néixer [97-100]. Tanmateix, Apaf-1 no és requerit per la inducció d'apoptosi en neurones post-mitòtiques [101] o l'eliminació de timòcits autoreactius [102]. Aquestes circumstàncies indiquen que en determinats contexts l'apoptosoma enlloc d'ésser essencial per la mort apoptòtica actua com un amplificador de l'activitat caspasa ja que determinats tipus cel·lulars (com les neurones en desenvolupament) però no d'altres (com els limfòcits) requereixen d'aquesta amplificació [103]. A més a més, aquests descobriments suggerien l'existència d'altres vies de senyalització apoptòtica que podien actuar de forma independent a l'apoptosi mitocondrial.

3.2.2 Via extrínseca o dels receptors de mort

Evolutivament els vertebrats han adquirit una nova ruta de mort cel·lular que és iniciada per receptors de superfície cel·lular i que resulta en l'activació de la procaspasa inductora 8 o el seu homòleg procaspasa-10 (present en humans però no en ratolins). Aquesta nova ruta rebrà el nom de via extrínseca o dels receptors de mort i com el seu nom indica serà executada pels receptors de la superfamília del TNF.

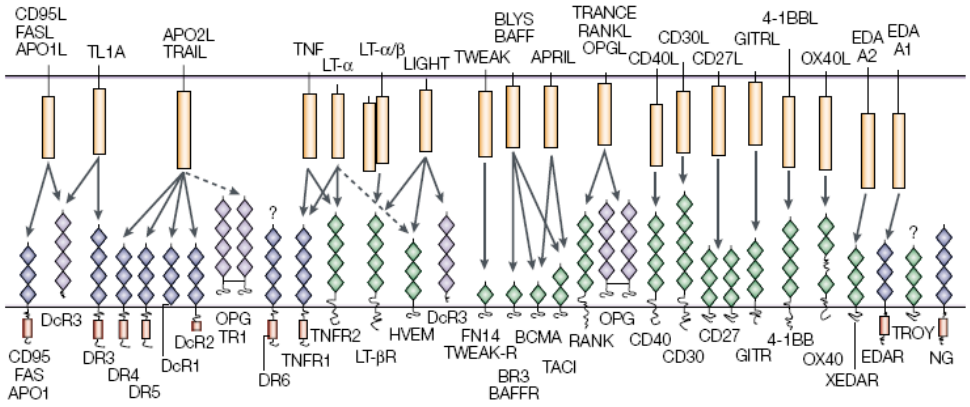


Figura 13: La superfamília de TNF i receptors corresponents. Les fletxes contínues indiquen les interaccions lligand-receptor d'alta afinitat mentre que les discontínues mostren unions de baixa afinitat. Els interrogants fan referència a receptors dels quals no es coneix lligand, els rombes representen els dominis rics en cisteïna i les caixes vermelles denoten els dominis de mort en posició intracel·lular. *(Ashkenazi, A. Nature Reviews, 2002. 2:420-30)*[104].

3.2.2.1 Introducció als lligands de mort. Aspectes generals.

El nom factor de necrosi tumoral entranya una connexió ja històrica entre els gens de la superfamília del TNF i la teràpia anticancerígena. El nexe es remunta a observacions que ja varen ésser documentades al 1868 per Brunes, qui observà que els tumors d'alguns pacients revertien espontàniament després d'una infecció bacteriana aguda. El 1894, Colley observà com extractes de bactèries –més tard anomenades toxines de Colley- podien causar la regressió tumoral. Posteriorment el 1944 Shear i col·laboradors aïllen un factor present en les bactèries gram-negatives anomenat endotoxina o lipopolisacàrid (LPS) que un cop injectat en tumors de ratolins era capaç d'induir necrosi tumoral.

El 1963, W. O'Malley va un pas més enllà i transfereix sèrum provinent de ratolins tractats amb LPS en tumors de ratolins i observa l'aparició de necrosi tumoral [105]. El 1975, E. Carswell, L. Old i col·laboradors confirmen aquesta observació i neix el terme factor de necrosi tumoral [106]; a més a més mostren com el LPS és capaç de matar cèl·lules tumorals en cultiu. El 1984, D. Pennica documenta el clonatge d'un cDNA (**c**omplementary **d**eoxyribonucleic **a**cid) que codifica per la proteïna TNF [107], mentre que P. Gray aconsegueix el clonatge d'una altra proteïna citotòxica anomenada ***l***imfo***t***oxina (LT) [108].

Sorprenentment, les proteïnes TNF i LT comparteixen un 30% d'homologia en la seva seqüència. Això va fer pensar en l'existència d'una família de gens. Les dues proteïnes van ésser renombrades TNF-alpha i TNF-beta però el subseqüent descobriment d'una altra proteïna molt semblant a LT [109] va obligar a tornar a l'antiga nomenclatura i reinstaurar el noms TNF i crear-ne dos de nous: limfotoxina alpha (LT-α) i beta (LT-β).

Els lligands de mort són glicoproteïnes transmembrana de tipus II, és a dir, presenten un domini carboxil-terminal a l'espai extracel·lular, una regió transmembrana i una cua citoplasmàtica amb l'extrem amino-terminal [110]. Cada membre de la família del TNF s'uneix, al menys, a un receptor i alguns es poden unir a múltiples receptors. També es pot donar el cas de que varis lligands comparteixin receptor com és el cas de BLYS/BAFF (B-cell activating factor) i APRIL amb els receptors BCMA i TACI (Fig13).

Els lligands formen oligòmers, sovint trímers, que són vitals per reclutar els receptors i iniciar la senyalització. Els processament proteolític per metal·loproteïnases és un fet postraduccional característic dels lligand de mort: la regió carboxil-terminal és processada en una forma soluble i alliberada a l'espai extracel·lular.

Els lligands de la família del TNF exerceixen la seva activitat biològica primerament, però no exclusivament, en el sistema immune modulant la immunitat innata i adaptativa [111]. Alguns dels lligands, com TNF, promouen respostes inflamatòries en resposta a infeccions microbianes. Altres com LT-β, CD40L, LIGHT, RANKL i BLYS/BAFF regulen aspectes específics de la immunitat cel·lular o humoral, incloent la formació d'òrgans limfoides, activació de cèl·lules

dendrítiques i estimulació o supervivència de cèl·lules B i T. Altres lligands com FasL (també anomenat Apo1L o CD95L) i Apo2L/TRAIL regulen l'activació de l'apoptosi induïda per limfòcits perifèrics i també controlen l'apoptosi induïda per les cèl·lules NK (**N**atural **k**iller) i limfòcits citotòxics en cèl·lules infectades per virus o transformades. Alguns membres també tenen papers importants fora del sistema immune en la regulació de teixits i òrgans com la pell i l'os.

3.2.2.2 Introducció als receptors de mort.

La superfamília de receptors del TNF presenta un ampli ventall de receptors. Es tracta de proteïnes integrals de membrana, la majoria de tipus I, és a dir, amb un sol pas transmembrana, amb l'extrem amino-terminal extracel·lular i l'extrem carboxil-terminal intracel·lular.

La majoria dels receptors funcionen com a transductors de senyal transmembrana quan són units als respectius lligands. Alguns dels receptors, però, no senyalitzen i per tant no són capaços de generar una resposta intracel·lular. Enlloc d'això sembla que actuïn com a reclams (*decoy*) que competeixen pels lligands amb els receptors senyalitzadors.

Els receptors senyalitzadors al seu torn poden ser classificats en dos subgrups en base a la seva regió citoplasmàtica: aquells que no presenten dominis de mort, i els coneguts com a receptors de mort (DR, **D**eath **r**eceptors) [110] que contenen un domini de mort citoplasmàtic (DD, **D**eath **d**omain).

Els dominis de mort (DD) faciliten la interacció dels receptors amb proteïnes adaptadores que també contenen els dominis DD. Per tant, un domini DD intacte és vital per la inducció de l'apoptosi.

Fent ús d'assajos doble híbrid en llevats, diversos grups identificaren tres proteïnes que interaccionaven amb els DD d'alguns receptors. Una d'aquestes proteïnes és RIP (**R**eceptor **i**nteracting **p**rotein).

FADD/MORT1 (**F**as-**a**ssociated **d**eath **d**omain containing protein) també és una proteïna adaptadora capaç d'unir-se als receptors a través del DD i a les caspases iniciadores a través del domini DED (**D**eath **e**ffector **d**omain) i iniciar així la senyal apoptòtica.

Finalment, l'adaptador conegut amb el nom de TRADD (*T*NF-*r*eceptor-*a*ssociated *d*eath *d*omain) estimula proteïnes cinases que controlen la transcripció de gens moduladors de la resposta immune. Alternativament, TRADD pot iniciar una senyal apoptòtica a través de FADD.

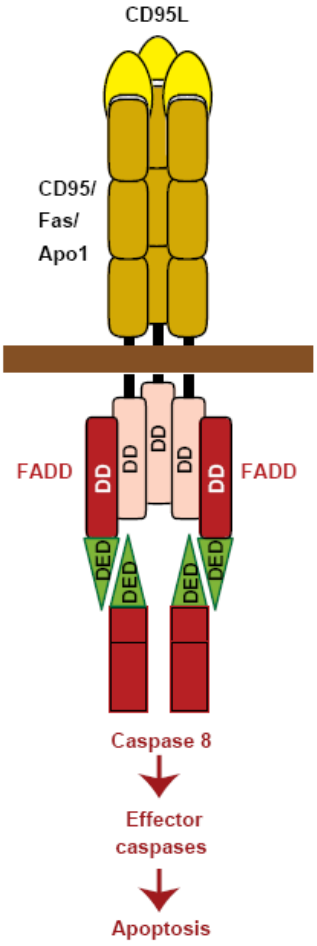


Figura 14: Esquema representatiu de les interaccions entre els dominis DD presents en receptor i proteïna FADD i ensamblatge de les caspases iniciadores a través del domini DED. *(Ashkenazi, A and Dixit, VM. Science, 1998. 281:1305-8)*[110].

Com ja s'ha esmentat anteriorment, també es dóna l'existència de receptors que presenten un domini DD intracel·lular truncat o, en molts casos, inexistent però continuen tenint afinitat pels respectius lligands. Aquesta característica els fa competidors extraordinaris dels receptors senyalitzadors. Un exemple clar d'aquesta classe de receptors és el receptor de l'osteoprotegerina (OPG), un receptor *decoy* soluble per RANKL/OPGL [112] i també per Apo2L/TRAIL [113]. Els DcR1 i DcR2 també són receptors no senyalitzadors competidors per TRAIL [110]. DcR1 i DcR2 estan ancorats a membrana però a diferència del DcR1, el DcR2 presenta un domini

DD intracel·lular truncat incapaç de generar una resposta apoptòtica. Finalment, el receptor DcR3 és, com OPG, un *decoy* soluble per Fas [114] i LIGHT [114,115].

3.2.2.3 L'apoptosi per via extrínseca. Aspectes generals.

Entenem per apoptosi per via extrínseca aquella que està iniciada per receptors de mort. Els receptors, un cop units als seus lligands i en determinades condicions, seran capaços d'activar les caspases iniciadores més apicals com la caspasa-8 o la caspasa-10 a través de FADD. Aquest ensamblatge proteic a membrana es coneix com a DISC (**D**eath-**i**nducing **s**ignaling **c**omplex) [116] (Fig15).

Les caspases iniciadores, un cop actives, activaran les caspases efectores que procediran a la desestructuració cel·lular.

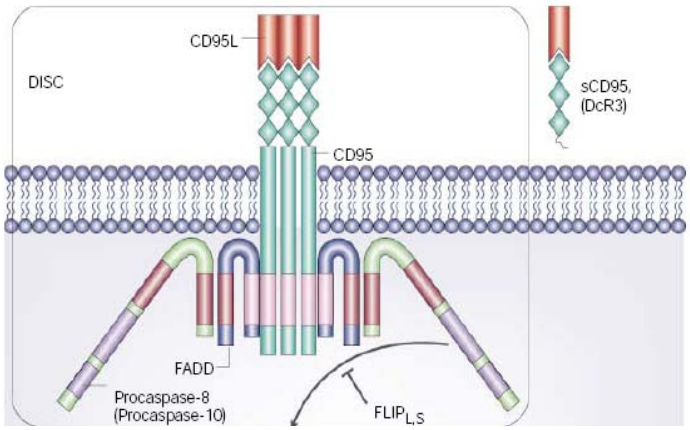


Figura 15: Formació del DISC. La unió dels lligands de mort, en aquest cas FasL, precipita la formació del DISC. La proteïna adaptadora FADD s'uneix a través del seu DD (caixes violeta) a l'extrem carboxil-terminal intracel·lular del receptor i a les caspases iniciadores a través del domini DED (caixes vermella). *(Modificat de: Igney, FH and Krammer, PH. Nature Reviews, 2002. 2:277-88)*[117].

L'apoptosi per via extrínseca i intrínseca semblen ser, doncs, mecanismes independents. Per exemple, ni la sobreexpressió de Bcl-2 [38,118-120] ni la pèrdua de bim, apaf-1 o caspasa-9 [99,100,121] protegeix els limfòcits de l'apoptosi induïda pels lligands de mort. No obstant en altres tipus cel·lulars, les dues vies poden interaccionar ja que la caspasa-8 pot activar la proteïna BH3-*only* Bid que actuarà facilitant la permeabilització de la membrana mitocondrial [56,57,122] (Fig17).

Coetàniament als resultats descrits, Scaffidi i col·laboradors postulen que l'activació individual de la via extrínseca es dóna en cèl·lules amb prous nivells de caspases iniciadores com per activar per si soles les caspases executores i les anomena cèl·lules tipus I. Les cèl·lules tipus II seran aquelles que no puguin activar nivells suficients de caspases iniciadores i que necessitin, per tant, de l'amplificació mitocondrial a través de Bid per executar l'apoptosi [123].

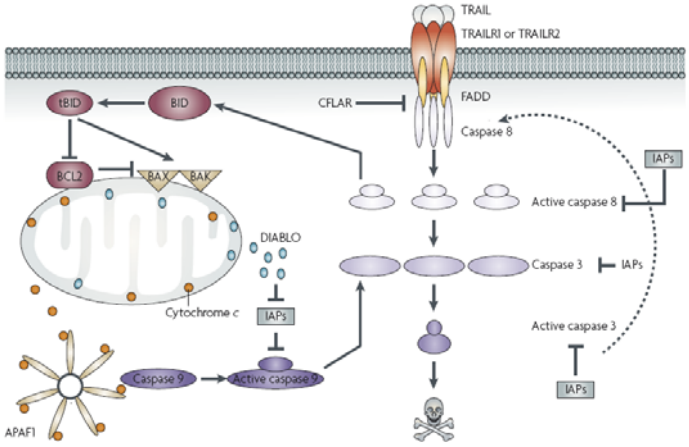


Figura 17: Convergència entre les vies de senyalització per receptors de mort i mitocondrial prenent TRAIL com a model. L'únió de TRAIL al seu receptor desencadenarà la formació del DISC. Un cop format, la caspasa-8 es processarà i s'activarà. Al seu torn, aquesta activarà la caspasa-3 o proteolitzarà Bid. En les cèl.lules tipus I els nivells de caspasa-8 i -10 actius seran prou elevats per activar directament la caspasa-3. Per contra, en les cèl·lules tipus II, les caspases iniciadores processen i activen Bid per iniciar el "loop" d'amplificació mitocondrial que resultarà en l'activació de la caspasa-9. En aquestes condicions hi haurà nivells suficients de caspases iniciadores actives com per activar de forma massiva les caspases executores. *(Johnstone, RW; et al. Nature Reviews, 2008. 8:782-98)*[124].

4. EL PAPER DE L'APOPTOSI EN L'ENDOMETRI NORMAL

Històricament, la menstruació ha estat associada a necrosi isquèmica de l'estrat funcional a causa de la contracció de les artèries irrigants i de ser un fenomen hormono-dependent [125,126]. Estudis posteriors per microscopia electrònica revelaren la presència de cossos apoptòtics en l'epiteli endometrioide humà durant la fase secretora tardana pocs anys després del naixement del terme apoptosi [127].

Evidències acumulades durant anys suggerien que l'apoptosi ajudava a mantenir l'homeòstasi cel·lular durant el cicle a través de l'eliminació de cèl·lules de l'estrat funcional de l'endometri durant les fases secretora i menstrual [127-129]. L'apoptosi va ser detectada en l'epiteli glandular durant la fase secretora i menstrual mentre que rarament era detectada durant la fase proliferativa o als inicis de la fase secretora [128,130,131].

Considerant la naturalesa cíclica de l'apoptosi en l'endometri humà, i tenint en compte que l'endometri inicia un procés ràpid de proliferació, diferenciació i involució sota influència hormonal; sembla probable que estrògens i progesterona podrien regular l'apoptosi en aquest teixit.

Avui dia el mecanisme exacte de l'apoptosi en l'endometri humà no està completament identificat però són diversos els grups que han fet estudis al respecte. En tot cas resulta evident que la regulació és hormonal i que, per tant, és cíclica. S'ha de posar de relleu, però, que l'apoptosi es dóna de forma particular en l'estrat funcional, que és més dinàmic, i de forma menys freqüent en l'estrat basal, que roman més o menys constant durant el cicle.

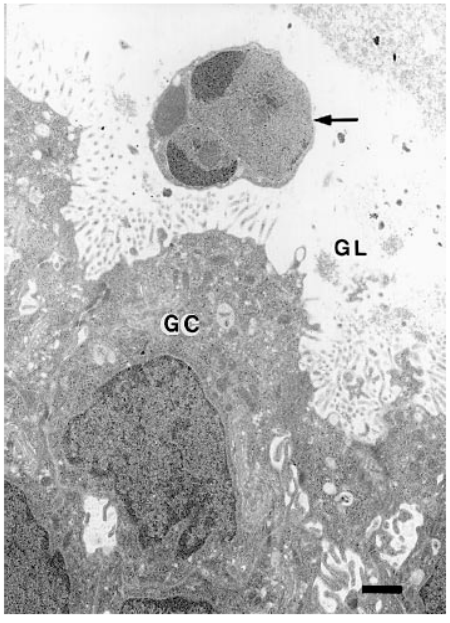


Figura 18: Representació d'un cos apoptòtic (fletxa) en el lumen glandular (GL) durant la fase secretora tardana. El cos es caracteritza per presentar masses de cromatina perifèrica. GC:cèl·lula glandular Barra=1µm
(Yamashita, T. Molecular Human Reproduction, 1999. 5:358-64)[132].

4.1 Bcl-2

Una de les proteïnes més ben estudiada al respecte és Bcl-2. Sembla ser que Bcl-2 té una expressió cíclica en l'endometri glandular i l'estroma que arriba al seu punt àlgid durant la fase proliferativa tardana i decreix durant les fases secretora i menstrual [133,134]. Bax sembla ser també un proteïna que es modula, però a diferència de Bcl-2, els nivells de Bax són modestos durant la fase proliferativa i incrementen dràsticament en la fase secretora quan l'apoptosi és més prevalent [130]. De forma consistent, estudis inmunohistoquímics de Bak demostren que la seva localització és eminentment glandular, especialment durant la fase secretora i pràcticament inexistent en l'endometri proliferatiu [135].

En el seu moment es va postular que aquesta expressió diferencial de proteïnes pro i antiapoptòtiques de forma cíclica havia d'obeir a alguna classe de regulació superior que modulés la natura tant dinàmica de l'endometri humà. Fou en aquesta direcció que diversos científics documentaren la regulació de Bcl-2 i Bax en resposta als esteroides ovàrics [136,137]. Posteriorment, Vaskivuo i col·laboradors demostren que el patró apoptòtic correlaciona inversament amb les concentracions d'estradiol en sèrum durant la fase proliferativa [131].

4.2 FasL

Fas i FasL també s'expressen en l'endometri humà durant el cicle menstrual [132,138,139]. Durant la fase proliferativa aquestes proteïnes són retingudes a l'aparell de Golgi i en vesícules citoplasmàtiques [140].

La inmunotinció per Fas [132,141] i FasL [142] és més intensa durant la fase secretora que en la fase proliferativa.

Amb tot, aquests resultats indiquen que l'apoptosi juga un paper fonamental en la remodelació de l'endometri durant el cicle menstrual.

5. APOPTOSI I CÀNCER

Des dels anys 50, l'estratègia principal per tractar el càncer, fora de la cirurgia, han estat la radioteràpia o la quimioteràpia. Aquests mètodes actuen principalment danyant les cèl·lules a nivell de la replicació del DNA o de la divisió cel·lular i induint la mort cel·lular apoptòtica com una resposta secundaria al dany produït. Tot i que aquests tractaments de vegades s'associen a una aturada en el creixement del tumor o inclús la regressió, rarament són curatius i sovint són secundats per l'aparició de clones de cèl·lules tumorals resistents.

La radioteràpia i la quimioteràpia, a més a més, no distingeixen entre cèl·lules proliferants malignes i no-malignes i poden causar toxicitat en teixits normals com la medul·la òssia o el ronyó. Els últims anys, però, s'han invertit molts esforços en el desenvolupament d'eines biològiques més selectives que puguin vèncer la resistència tumoral alhora que minimitzar els possibles efectes secundaris [104].

El sentit de fer servir l'apoptosi en el tractament contra el càncer es basa en l'observació que aquest és un fenomen que es troba desregulat en les cèl·lules cancerígenes però no en les cèl·lules normals [104,143-146]. Les cèl·lules tumorals haurien adquirit mecanismes de bloqueig de l'apoptosi però tot i així són constantment abocades a iniciar-la com a conseqüència de les alteracions genòmiques que presenten. Per tant, si les vies proapoptòtiques poguessin ser estimulades, aquestes cèl·lules haurien de ser més susceptibles a la mort que les cèl·lules normals [147].

La inducció de l'apoptosi, com ja hem comentat, és un component important de l'activitat antitumoral de les teràpies tradicionals com són la radioteràpia i la quimioteràpia [146,148]. Aquestes, sembla que estimulin l'apoptosi primàriament a través de la via intrínseca o mitocondrial per lo que la incapacitat per executar-la convertiria aquestes cèl·lules canceroses en resistents [146,148,149]. En aquest sentit, mutacions en el gen TP53 (que codifica per la proteïna p53), freqüentment es troben al darrere d'aquesta resistència ja que les cèl·lules presenten desacoblada la connexió entre els sensors de dany a DNA i la maquinària apoptòtica [117,150,151]. Aproximadament es calcula que les mutacions en p53 es donen en un 50% dels tumors.

Donat que l'apoptosi extrínseca o per receptors de mort actua independentment de p53, les teràpies que directament activen aquest mecanisme tenen el potencial

d'induir la mort cel·lular en càncers amb un ampli espectre de resistència enfront les teràpies convencionals [150]. Així doncs, l'estimulació de l'apoptosi ha emergit com una estratègia pel tractament del càncer i s'estan realitzant autèntics esforços per identificar dianes proapoptòtiques amb potencial terapèutic [152-155].

6 TRAIL I ELS SEUS RECEPTORS

TRAIL (TNF-related apoptosis inducing ligand) és una proteïna transmembrana tipus II que inicialment va ser identificada i clonada en base a l'homologia de seqüència del seu domini extracel·lular amb els lligands CD95L (28% d'homologia) i TNF (23% d'homologia) [156]. En la superfície cel·lular, el seu domini carboxil-terminal pot ésser processat per proteases alliberant una molècula circulant d'aproximadament 19 kDa que s'unirà als respectius receptors. Com molts altres membres de la família del TNF, TRAIL forma homotrímers que s'uniran a tres molècules receptores provocant l'activació de la senyal apoptòtica.

S'han identificat diversos receptors pel lligand Apo2L/TRAIL (Fig19): dos receptors de mort, DR4 [157] i DR5/KILLER [158-161] (també anomenats TRAIL-R1 i TRAIL-R2 respectivament) i dos receptors *decoy*, DcR1/TRID [158,159,162] i DcR2 [162,163] (també coneguts com a TRAIL-R3 i TRAIL-R4). Finalment es va demostrar que un tercer receptor *decoy*, OPG, també podia unir TRAIL [113] tot i que inicialment es va caracteritzar com un receptor pel lligand RANKL/OPGL. Tanmateix l'afinitat d'aquest receptor per TRAIL és molt baixa a temperatura fisiològica [164]. La rellevància fisiològica d'OPG com a receptor de TRAIL no és clara però estudis recents indiquen que podria jugar un paper com a factor de supervivència en cèl·lules de càncer de pròstata. L'estudi demostra una correlació negativa entre la capacitat de TRAIL per induir apoptosi i els alts nivells d'OPG en circulació endògenament produïts per les cèl·lules tumorals [165].

En referència als receptors *decoy* DcR1 i DcR2 s'accepta que la seva acció primordial vindria donada de forma indirecta a base de competir amb els pertinents receptors senyalitzadors per la unió a TRAIL. S'ha postulat fins i tot que una disminució en l'expressió d'aquests receptors (observada en algunes cèl·lules tumorals) sensibilitza a la mort induïda per TRAIL [166,167]. Un altre model que podria

complementar aquesta noció postula que els receptor *decoy* DcR2/TRAIL-R4 inhibiria l'apoptosi promovent l'activació de NF-κB [162]. Tanmateix, alguns autors postulen que el model inicial de sensibilització a TRAIL basat en l'expressió dels receptors *decoy* hauria estat massa simplista i han documentat com el DcR2 és capaç d'unir-se al receptor DR4. Els autors demostren que la formació d'aquest heterocomplex seria deficitari en la iniciació d'una senyal de mort prou robusta [124].

Ambdós receptors DR4 i DR5 contenen el domini de mort conservat DD i, per tant, són capaços d'iniciar i senyalitzar apoptosi. Els altres tres receptors actuen com a competidors i són capaços d'inhibir la mort induïda per TRAIL quan són sobreexpressats. Alternativament, DcR1 i DcR2 presenten una alta homologia amb els dominis extracel·lulars de DR4 i DR5. A més, el DcR2 conté una forma truncada, no funcional, del domini DD. D'altra banda el DcR1 no presenta cap regió citosòlica i es troba ancorat a membrana plasmàtica a través d'un motiu glicofosfolípid.

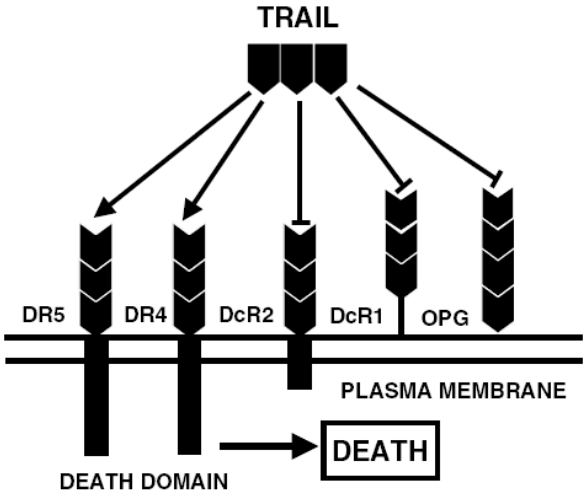


Figura 19: TRAIL i els seus receptors. TRAIL és un lligand homotrimèric capaç d'interaccionar amb els membres de la família dels receptors de TNF. Els receptors DR4 i DR5 contenen el motiu DD, essencial per la senyalització apoptòtica. Els receptors DcR1 i DcR2 competeixen amb DR4 i DR5 per la unió a TRAIL *(Wang, S and El-Deiry, WS.Oncogene, 2003. 22:8628-33)*[84].

S'ha de destacar que en ratolins només un receptor de mort homòleg a l'humà DR5 (mTRAIL-R2/mDR5) i dos receptors *decoy* potencials (mDcTRAIL-R1/mDcR1 i mDcTRAIL-R2/mDcR2) han estat identificats com a receptors

específics del TRAIL murí [168]. Com en humans, mDR5 conté un domini DD intracel·lular capaç d'iniciar la senyal de mort cel·lular, mDcR1 també està ancorat a membrana plasmàtica. Per últim, existeixen dos variants creades per splicing alternatiu de mDcR2: una forma secretada (mDcR2S) i una forma transmembrana (mDcR2L).

Els animals knockout per Apo2L/TRAIL són viables, fèrtils i no presenten defectes hematològics [169,170], suggerint que aquest lligand no té una funció rellevant durant el desenvolupament.

D'altra banda, aquests estudis també indiquen que Apo2L/TRAIL juga un paper important en la vigilància antitumoral per part de les cèl·lules immunes [169]. Experiments en ratolins en els que Apo2L/TRAIL fou bloquejat fent ús d'anticossos neutralitzants impliquen Apo2L/TRAIL específicament en la funció antitumoral de les cèl·lules NK a través de l'interferó-γ [171]. En aquest sentit les cèl·lules NK actives [172,173], monòcits [174] i limfòcits T $CD4^+$ o $CD8^+$ [175-179] expressen Apo2L/TRAIL i usen aquest lligand per induir l'apoptosi en cèl·lules tumorals.

Apo2L/TRAIL també podria ser un mitjancer important de l'apoptosi induïda per interferons tipus I en cèl·lules de carcinoma de ronyó [180] i cèl·lules de mieloma múltiple [181]. La presència d'un element regulador de la transcripció en el gen d'APO2L/TRAIL que respon als interferons dóna suport a aquests resultats [182]. Encara més, la infecció de cèl·lules amb reovirus [183] o herpesvirus [184] incrementa l'expressió d'Apo2L/TRAIL i les cèl·lules NK poden utilitzar l'Apo2L/TRAIL -induït per interferons tipus I que elles mateixes secreten- per matar les cèl·lules infectades [185].

Els limfòcits també expressen Apo2L/TRAIL com una proteïna transmembrana que pot ser alliberada com una forma associada a vesícula [186] o com una forma soluble generada a través de processament enzimàtic a partir del seu extrem carboxil-terminal [187]. Apo2L/TRAIL, per tant, sembla que juga un paper important en la defensa contra la iniciació i metàstasi tumoral.

El descobriment i caracterització d'Apo2L/TRAIL significà en el seu moment tornar a centrar els estudis en els receptors i lligands de mort com una possible eina antitumoral amb aplicacions clíniques. Cal recordar que fins aleshores els dos

lligands de mort majorment coneguts eren el TNF i CD95L/FasL. A més a més es va poder comprovar les seves limitacions quan es van fer els primers assajos in vivo: l'administració sistèmica de FasL resultava en una toxicitat profunda al fetge i la conseqüent mort per apoptosi dels hepatòcits. D'altra banda, l'administració de TNF recombinant causava danys col·laterals severs com caquèxia, hemorràgies, febre i shock en animals de laboratori [188,189], posant de manifest el rol que juga el TNF en la regulació de la immunitat i respostes inflamatòries. En canvi, els assajos amb TRAIL varen ser completament diferents: bàsicament allà on TNF havia mostrat les seves mancances, TRAIL mostrava les seves característiques més valuoses. TRAIL exhibia les mateixes propietats apoptòtiques de forma selectiva que TNF enfront les cèl·lules tumorals in vitro/in vivo però, encara més important i a diferència del TNF, TRAIL era tolerat en els assajos preclínics de toxicitat [166,190].

Probablement la propietat més característica i versàtil de TRAIL és la seva habilitat per matar cèl·lules tumorals sense exercir cap efecte en cèl·lules normals, tot i que el motiu no està esclarit del tot. Nombroses publicacions fonamenten aquesta afirmació: s'ha observat que TRAIL indueix l'apoptosi en un ampli espectre de carcinomes humans incloent còlon, pulmó, mama, pròstata, ronyó, pàncrees i càncer de tiroides així com mieloma múltiple; indicant que aquest lligand efectivament podria arribar a ser útil en el tractament de molts tumors. En aquests sentit s'ha pogut comprovar l'activitat antitumoral derivada de l'administració de TRAIL recombinant en ratolins atímics que presenten xenotransplants de carcinomes humans [191-195].

També s'ha observat que combinacions de TRAIL amb determinades drogues que causen dany a DNA [196,197] o amb radioteràpia [198] tenen efectes de sinergia antitumoral en models murins amb xenotransplants.

Aquesta intrigant capacitat per iniciar l'apoptosi selectivament en cèl·lules tumorals sense afectar les cèl·lules normals ha motivat una extensiva recerca sobre TRAIL i les seves propietats. Tant és així que els assajos clínics pertinents ja es troben actualment en fase I i II [199,200].

Agent/identifier	Mechanism/pharmacology	Development stage
rhApo2L/TRAIL (PRO1762, AMG–951)	rhApo2L/TRAIL targeting DR4 and DR5	Phase II (NHL, NSCLC)
Mapatumumab (HGS-ETR1)	Human mAb targeting DR4	Phase II (NHL, CRC, NSCLC, MM)
Lexatumumab (HGS-ETR2)	Human mAb targeting DR5	Phase I
Apomab	Human mAb targeting DR5	Phase II (NHL, NSCLC)
AMG–655	Human mAb targeting DR5	Phase II (NSCLC)
LBY135	Chimeric mAb targeting DR5	Phase I/II
TRA-8/CS-1008	Murine DR5-targeting antibody (TRA-8) and humanized version CS-1008	Phase I
Ad5-TRAIL	Recombinant adenovirus encoding human Apo2L/TRAIL	Phase I

Taula 1: Estadis de desenvolupament d'agonistes proapoptòtics per als receptors DR4 o DR5. MM, mieloma múltiple. NSCLC, non-small cell lung carcinoma. *(Ashkenazi, A and Herbst, RS. Journal of Clinical Investigation, 2008. 118:1979-90)*[147].

6.1 Vies de senyalització iniciades per TRAIL

A part de les vies de senyalització intracel·lulars implicades en l'apoptosi existeixen vies alternatives iniciades per aquest receptor. Depenent del tipus cel·lular, de la intensitat i durada de l'estímul i de la presència, absència o estat d'activació de les proteïnes intracel·lulars que senyalitzen per sota del receptor, el tractament amb TRAIL o anticossos agonistes poden estimular l'apoptosi o , més rarament, la proliferació cel·lular.

En aquest sentit és presumible que TRAIL pugui induir simultàniament múltiples vies de senyalització que impliquin proteïnes com el factor nuclear kappa B (NFκB; ***N***uclear ***F***actor ***K***appa ***B***), la via de les proteïnes cinases MAPK (***M***itogen ***a***ctivated ***p***rotein ***k***inases) incloent les ERKs (***E***xtracel·lular signal-***r***egulated ***k***inases), les JUN N-terminal cinases (JNK) i la p38. També s'ha documentat l'activació de PI3K (***P***hospho***i***nositide ***3***-***k***inase) i Akt (Fig20).

Tot i que existeix un debat considerable sobre els complexes proteics necessaris per l'activació a través de TRAIL d'aquestes vies alternatives, sembla probable que proteïnes com FADD, TRADD, c-FLIP, les caspases-8 i -10, TRAF2, NEMO (***N***F kappa B ***e***ssential ***mo***dulator) i RIP hi estiguin implicades, possiblement depenent del tipus cel·lular que s'estudiï. L'ensamblantge d'una o més d'una d'aquestes vies de senyalització de forma que tinguin més pes que la pròpia senyal apoptòtica pot causar efectes dramàtics en l'activitat fisiològica o terapèutica de TRAIL o dels anticossos agonistes d'aquest.

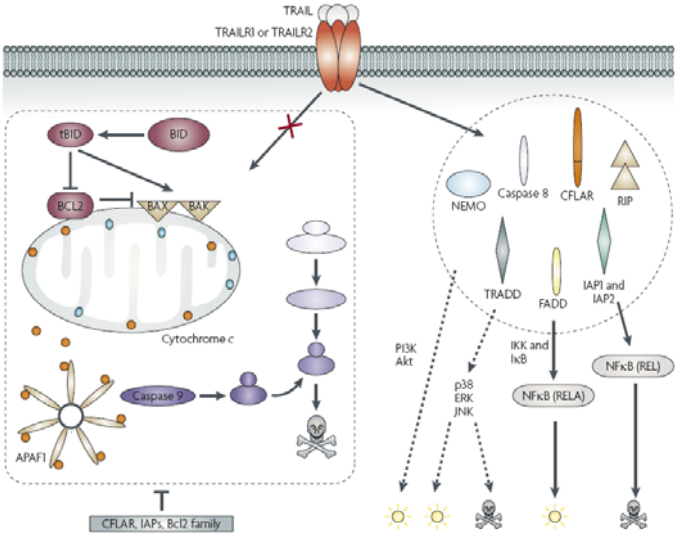


Figura 20: Vies de senyalització adicionals activades per TRAIL. L'unió de TRAIL amb els seus respectius receptors pot resultar en l'activació simultània de vies pro-apoptòtiques i vies alternatives com la PI3K/Akt, NF-kappa B o la via de les MAPK (incloent ERK, p38 i JNK). En general, l'activació d'aquestes vies alternatives en absència d'una resposta apoptòtica funcional tenen un efecte pro-supervivència o proliferatiu. *(Johnstone, RW; et al. Nature Reviews, 2008. 8:782-98[124]).*

6.2 Moduladors de la senyal iniciada per TRAIL i Fas, I: FLIP

FLIP (***F***as-associated death domain-***l***ike inteleukin 1β converting enzime ***i***nhibitory ***p***rotein) és una proteïna que s'ha erigit com a un dels factors més determinants en la regulació de l'apoptosi induïda per TRAIL. Inicialment es van descobrir i caracteritzar els seus respectius homòlegs virals, una família de proteïnes antiapoptòtiques conegudes amb el nom de proteïnes inhibidores de FLICE (***F***as associated death domain-***l***ike ***ICE***) [201]. Les proteïnes v-FLIPs es composen de dos dominis DED que són molt similars en seqüència i estructura als respectius dominis localitzats en la porció amino-terminal de la procaspasa-8 (Fig21).

Durant la senyalització per Fas, les v-FLIPs són reclutades al DISC i interfereixen en l'ensamblatge de les procaspases-8 inhibint la senyalització apoptòtica per Fas [201]. Múltiples grups varen identificar a finals de la dècada dels 90 l'homòleg cel·lular de v-FLIP i va propiciar l'aparició de diverses nomenclatures: Casper, c-FLIP, I-FLICE, CASH, FLAME-1, MRIT, CLARP o Usurpina [202-209].

S'han descrit múltiples variants per splicing de c-FLIP però sembla ser que són dues les formes proteiques les que s'expressen predominantment: la forma llarga (c-$FLIP_L$) i la forma curta (c-$FLIP_S$) [210].

c-$FLIP_L$ conté dominis DED en tàndem i un domini tipus caspasa inactiu ja que li falten els residus aminoacídics necessaris (en especial un residu cisteïna) que confereixen l'activitat catalítica pròpia de les caspases. D'altra banda, c-$FLIP_S$ s'assembla als seus parents vírics i presenta únicament dos dominis DED i una porció carboxil-terminal que difereix de l'observat en c-$FLIP_L$ [206].

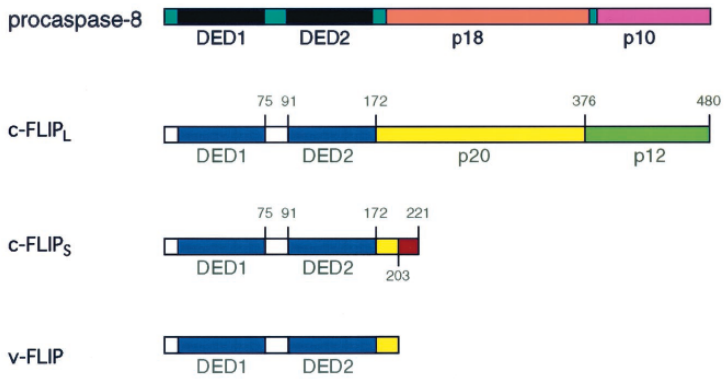


Figura 21: Semblances estructurals entre la procaspasa-8 i les formes llarga i curta de c-FLIP i la forma vírica c-FLIP. *(Krueger, A; et al. Molecular and Cellular Biology, 2001. 21:8247-54)*[211].

El mecanisme d'atenuació de la mort cel·lular per c-FLIP encara no està del tot desxifrat. S'ha suggerit en nombroses ocasions que actuaria com un inhibidor competitiu que bloquejaria el reclutament de la procaspasa-8 al DISC i prevenint així la seva activació [209]. Aquesta idea està complementada per l'observació de que l'homòleg viral v-FLIP-E8 sobreexpressat s'ensambla al DISC desplaçant la procaspasa-8 i inhibint la seva activació [201].

Inicialment, ambdós efectes pro i antiapoptòtics, van ser proposats per c-FLIP [204-208]. No obstant, la inducció de la mort cel·lular es donava únicament en experiments on c-FLIP era sobreexpressat de forma transitòria i la mort podria venir donada per l'ensamblatge i activació de procaspasa-8 no ancorada a membrana [212].

Les dades provinents de ratolins deficients en c-FLIP suporten la funció prosupervivència de c-FLIP [213].

S'ha demostrat que FLIP pot ésser processat en la forma p43 que roman al DISC i en la subunitat p12 que és alliberada (Fig22). En presència de FLIP la procaspasa-8 unida al DISC encara es pot processar [210]. Tanmateix, el processament és incomplet derivant-ne la formació de les subunitats p43 i p41 i el conseqüent alliberament de la forma p10 [211].

Aquest primer processament esdevé de forma autocatalítica però per donar-se, la procaspasa-8 requereix d'un domini caspasa com a homòleg, encara que sigui un domini inactiu com el de FLIP. Aquest raonament es coneix com a model d'activació de la procaspasa-8 induït per proximitat [214]. D'altra banda, per a que la caspasa-8 esdevingui completament activa requereix d'un segon processament. Aquest és trans-catalític, requereix caspasa-8 activa i resultarà en l'alliberació de la forma p18 que dimeritzarà amb la subunitat p10.

c-$FLIP_L$ és capaç d'inhibir la formació de p18 perquè bloqueja el segon processament però no pot impedir el primer perquè ella mateixa el propicia. En canvi, c-$FLIP_S$, al no presentar un domini catalític és capaç per si sol d'inhibir ambdós processaments [211,215].

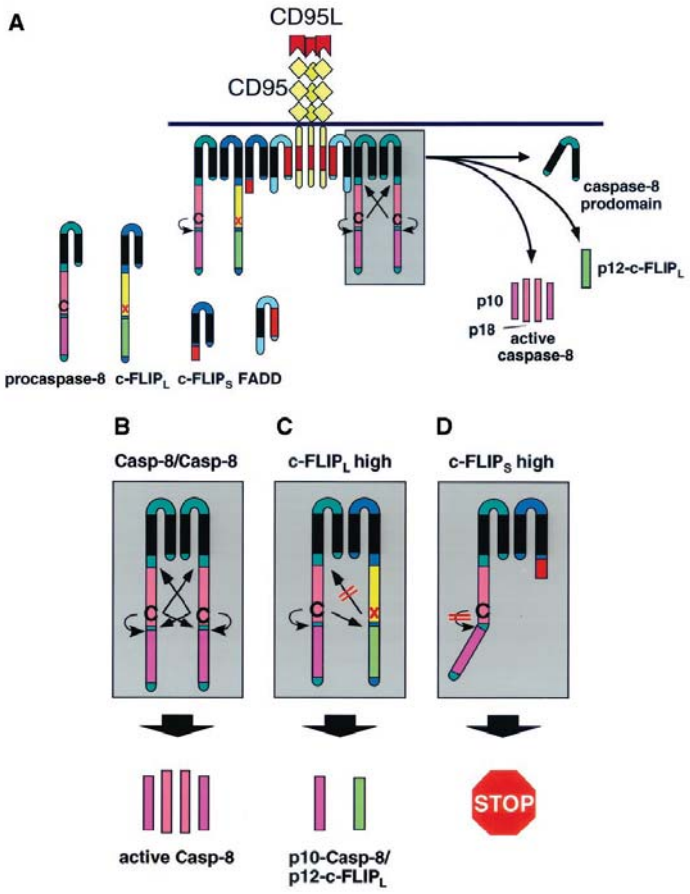


Figura 22: Model establert per la inhibició de FLIP del processament i activació de la caspasa-8. **A**, el lligand CD95L/FasL s'uneix al receptor i indueix el reclutament de FADD que servirà per atraure la procaspasa-8, c-FLIP$_L$ o c-FLIP$_S$. **B**, en el cas que es FLIP estigui absent o en nivells molt baixos es reclutaran les procaspases-8 que s'activaran per processament auto i trans-catalític. **C**, nivells alts de c-FLIP$_L$ propicien la formació d'heterodímers. c-FLIP$_L$ bloquejarà el tall trans-catalític i s'alliberaran les formes p10 i p12 de la caspasa-8 i c.FLIP$_L$ respectivament. **D**, nivells alts de c-FLIP$_S$ impediran qualsevol processament de les procaspases. *(Krueger, A; et al. Molecular and Cellular Biology, 2001. 21:8247-54)*[211].

FLIP no solament actuaria com a inhibidor de la caspasa-8. Diversos autors han documentat que FLIP és capaç d'activar la via de les MAPK o de NF-κB [216,217].

El mecanisme proposat suggereix que els heterodímers formats per les subunitats p43/41 i p43 de la caspasa-8 i FLIP podrien interaccionar amb proteïnes de la família TRAF, RIP o Raf-1 [216,218,219] i iniciar les citades vies (Fig23).

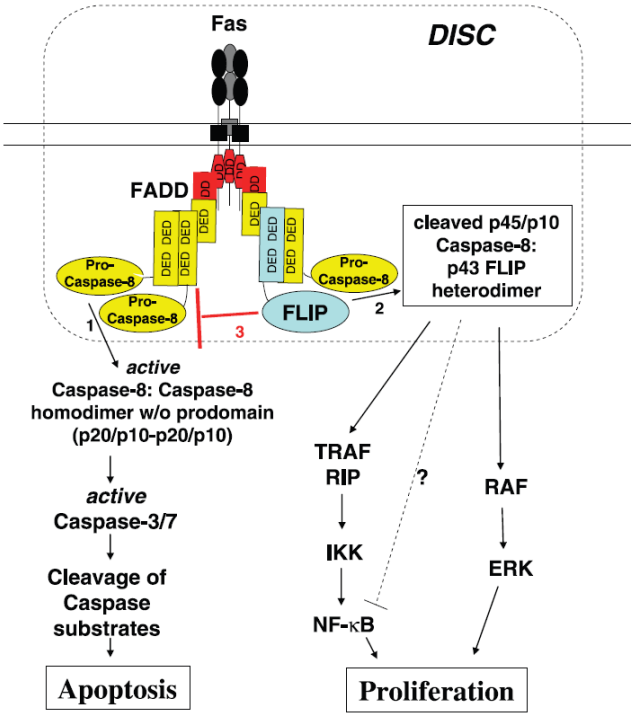


Figura 23: Model esquemàtic de la funcionalitat dual dels components del DISC. El reclutament de dos procaspases-8 al DISC resulta en l'activació d'aquestes i l'inici de la senyal apoptòtica. No obstant, la presència de FLIP no només inhibeix la completa activació de la caspasa-8 sinó que dimeritza amb la subunitat p43 d'aquesta i activa vies de proliferació cel·lular. *(Hyer ML and Reed JC. Clinical Cancer Research, 2006. 12:5929-31)*[220].

Nivells elevats de FLIP s'han pogut observar en nombroses neoplàsies com, per exemple, el melanoma humà o el limfoma de cèl·lules B murí [221,222] i s'ha pogut comprovar in vivo que la sobreexpressió de c-FLIP facilita l'evasió del sistema immune per part dels tumors [223,224].

Finalment, múltiples estudis han mostrat que la disminució de c-FLIP sensibilitza diversos tumors a l'apoptosi induïda per agonistes de Fas i TRAIL [225-228].

6.3 Moduladors de la senyal iniciada per TRAIL i Fas, II: CASEIN CINASA-2

CK2, o casein kinasa 2, és una Ser/Thr cinasa ubiqua i una de les proteïnes més altament conservades en les cèl·lules eucariotes. Aquesta proteïna es presenta sovint en forma de complexes tetramèrics formats per dos subunitats reguladores i dos subunitats catalítiques (Fig24). En diversos organismes, diferents formes isoenzimàtiques s'han identificat per la subunitat catalítica [229-233]. Per exemple en humans, dos subunitats catalítiques denominades CK2α i CK2α' han estat caracteritzades [234] mentre que només s'ha identificat una subunitat reguladora anomenada CK2β. Per contra, múltiples formes de CK2β s'han identificat en altres organismes com *Saccharomyces cerevisiae* [232].

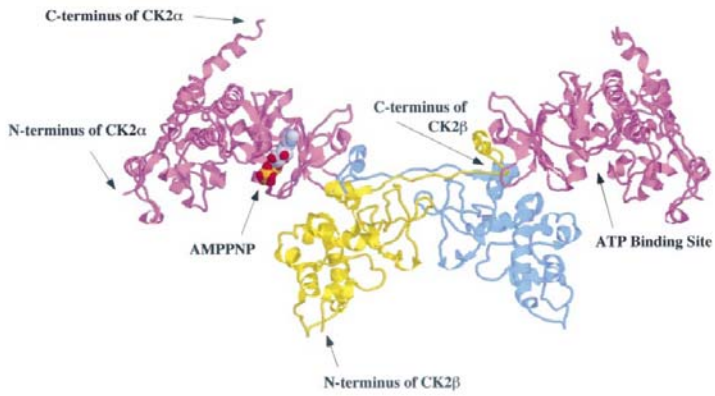


Figura 24: Il·lustració de l'estructura tetramèrica de CK2. Les subunitats catalítiques CK2α s'aprecien de color magenta mentre que les dues reguladores presenten color blau i groc. S'il·lustren els extrems amino i carboxil terminal per una subunitat catalítica i per la reguladora de color groc. L'anàleg d'ATP AMPPNP és present en el lloc d'unió a ATP d'una de les subunitats catalítiques. El significat d'aquesta observació roman irresolta actualment. *(Litchfield, DW. Biochemical Journal, 2003. 369:1-15)*[235].

Diversos estudis indiquen que els dímers de CK2β es troben en el nucli dels complexes tetramèrics de CK2 [236-242]. En mamífers, aquests complexes poden contenir subunitats catalítiques idèntiques (dos CK2α o dos CK2α') o diferents (una subunitat CK2α i una CK2α') (Fig24).

Les subunitats catalítiques comparteixen aproximadament un 90% d'homologia en la seva seqüència en els 330 residus aminoacídics de l'extrem amino-terminal. A més a més, aquestes seqüències es troben altament conservades en els eucariotes superiors. No obstant, difereixen en el seu extrem carboxil-terminal en una regió d'uns 20 aminoàcids que seran clau en la seva regulació diferencial [235].

Tot i que no es coneixen diferències catalítiques entre CK2α i CK2α', hi han evidències de que exhibeixen especialització funcional [243-246]. CK2α és fosforilada per $p34^{cdc2}$ en residus específics (Thr344, Thr360, Ser362, Ser370) localitzats en el seu domini carboxil-terminal únic durant el cicle cel·lular mentre que CK2α' no és fosforilada [229]. A més a més, l'especificitat d'unió a proteïnes com Hsp90, Pin-1, PP2A i CKIP-1 (***CK***2-***i***nteracting ***p***rotein-***1***) amb CK2α però no amb CK2α' suporta la noció de que CK2α i CK2α' tenen funcions cel·lulars independents [247-252].

De forma remarcable, la seqüència aminoacídica de CK2β està inclús més conservada entre espècies que les pertinents a les subunitats catalítiques. De fet, la totalitat de la seva seqüència (215 aminoàcids) és idèntica entre aus i mamífers [231,253,254].

Estructuralment, CK2β conté una seqüència molt semblant a la caixa de destrucció que permet la degradació de la ciclina B via proteasoma durant la mitosi [255-258]. Altres dominis dignes a remarcar són, per exemple, el domini en dits de zinc que permet la interacció entre les subunitats CK2β [239,241,242] i una regió carboxil-terminal *(positive regulatory region)* que permet l'ensamblatge entre les subunitats CK2β amb CK2α/α' [259,260].

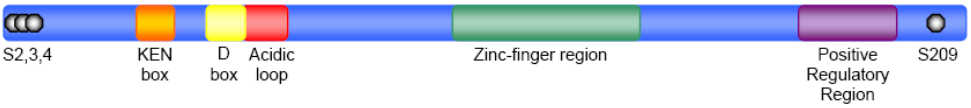


Figura 25: Il·lustració esquemàtica de les principals regions de CK2β. Les caixes KEN i D representen motius de degradació. El motiu acídic regula l'activitat catalítica a través de la a poliamines mentre que la "positive regulatory region" facilita la interacció amb les subunitats catalítiques. *(Bibby, AC and Litchfield, DW. International Journal of Biological Sciences, 2005. 1:67-79)*[261].

La popularitat creixent de CK2 es deu, principalment, a la seva pleiotropia i inusual "modus operandi". CK2 posseeix activitat cinasa de forma constitutiva, característica de bon principi difícil de concebre ja que la majoria de proteïnes cinases es regulen positivament en resposta a determinats estímuls.

D'altra banda, la seva pleiotropia és definitivament una característica destacable si un té present que dos dècades després del seu descobriment CK2 encara era una cinasa per la qual es buscaven substrats fisiològics [262].

Els primers substrats foren descoberts en els inicis dels anys 80 i pels 90 ja se n'havien descrit 50. Al 1994 la xifra ja havia superat els 100 i al 1997 ja s'havia arribat aproximadament als 160 substrats [236].

Actualment el repertori de substrats de la CK2 inclou aproximadament 307 proteïnes i només relativament pocs casos (uns 50) no han estat corroborats amb dades efectuades in vivo.

Més significatiu que el nombre, però, és la natura de les proteïnes fosforil·lades per CK2 i les funcions que efectuen dins la cèl·lula. No menys de 60 representen factors de transcripció, que juntament amb aproximadament 40 proteïnes nuclears implicades en expressió i transcripció gènica i amb més de 80 proteïnes implicades en varies funcions de senyalització representen la majoria de substrats implicats en senyalització cel·lular.

En comparació, enzims metabòlics clàssics com la glicògen sintasa, l'acetil CoA carboxilasa i l'ornitina descarboxilasa representen una minoria dels seus substrats. Més impressionant encara és la llista de proteïnes virals, 38, que són fosforil·lades per CK2, fenomen que es podria explicar donada l'activitat constitutiva de CK2 que seria explotada pels virus com a primera opció per assegurar el seu cicle de vida.

En general, l'activitat de CK2 és elevada en teixits proliferatius: la seva expressió és alta en teixits embrionaris [263,264], fetge en regeneració [265,266] i també en la capa de cèl·lules proliferants situades a la base de les criptes de la mucosa del còlon [267].

L'activitat de CK2 també ha estat observada de forma consistentment elevada en tots els càncer humans examinats [268-270]. La qüestió a fer-se és, doncs, en quant

difereix l'elevació de CK2 en cèl·lules tumorals en comparació amb cèl·lules normals. Sembla ser que en cèl·lules normals estimulades per al creixement l'elevació de CK2 seria transitòria mentre que en el cas de les cèl·lules canceroses l'elevació de la CK2 vindria donada per una desregulació partint dels "nivells normals" en aquest tipus particular de tipus cel·lular.

L'elevada activitat de CK2 ha estat associada amb la transformació maligna de diversos teixits així com també al comportament agressiu dels tumors [271]. La sobreexpressió de CK2 ha estat documentada en nombrosos tipus de carcinomes incloent ronyó [272], glàndul·la mamària [273], pulmó [274] i pròstata [275].

L'expressió condicionada a teixit de CK2α en models animals transgènics ha resultat en la transformació de cèl·lules T i de l'epiteli de la l'anul·la mamària i en la formació de tumors [273,276]. La sobreexpressió en models murins de CK2α en conjunció amb c-Myc [276] o Tal-1 [277] en limfòcits T o la sobreexpressió de CK2α en ratolins p53 $^{-/-}$ [278] resulta en un increment de l'activitat oncogènica i progressió tumoral. Un increment en l'activitat oncogènica també s'ha documentat en fibroblastes 3T3 coexpressant H-Ras i CK2α' [279].

En molts d'aquests estudis, el potencial oncogènic fou apreciable en presència d'una desregulació modesta de la CK2. Això emfatitza la noció de que les cèl·lules no toleren ni un petit canvi sostingut en els nivells de CK2 sense transformar-se en col·laboració amb un estímul oncogènic [268].

Els mecanismes pels quals CK2 regula la proliferació cel·lular no són clars donat que CK2 regula centenars de substrats afectant així múltiples vies de creixement cel·lular [236,269,280]. Tal com succeeix amb HSIX-1, molts altres factors de transcripció poden ésser regulats per CK2, tal com s'ha demostrat amb c-Myc, c-Myb, AP-1, UBF entre d'altres.

La subunitat p65 de NF-κB és un substract directe de CK2 [281]. CK2 també regula NF-κB a través de la fosforilació de l'inhibidor IkB-alpha [282-285]. CK2 també participa en la senyalització de Wnt en cèl·lules mamàries epitelials on la fosforilació de β–catenina per part de la CK2 promou la seva estabilització i translocació a nucli [286] (Fig27).

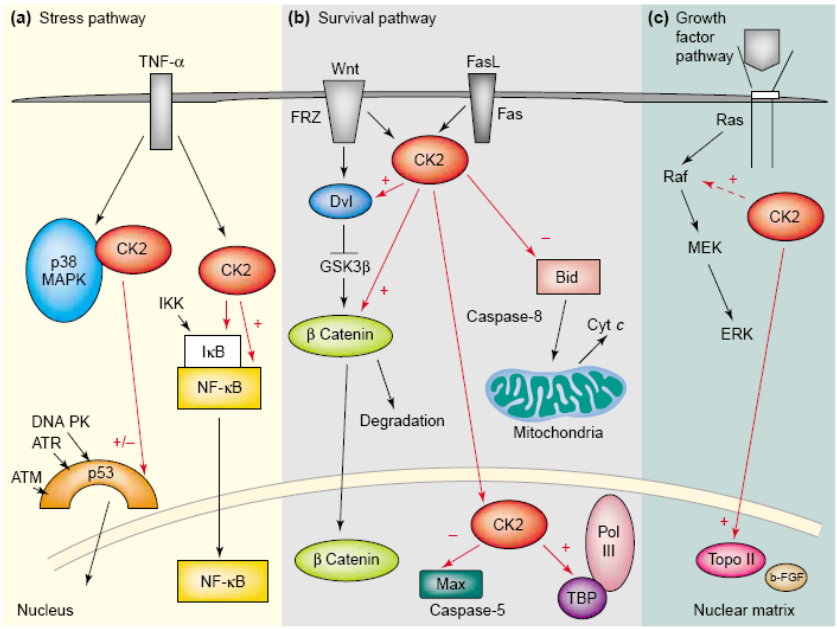


Figura 27: Representació d'algunes de les vies en que CK2 ha estat implicada. **A**, vies d'estrès cel·lular. **B**, vies prosupervivència. **C**, vies dependents de factors de creixement. *(Ahmed, K; et al. TRENDS in Cell Biology, 2002. 12:226-30)*[270].

A part de tenir la capacitat de regular la proliferació cel·lular, la CK2 també juga un paper en prevenir la mort cel·lular per apoptosi. Aparentment, a partir de les dianes de fosforilació de CK2 és possible conformar una seqüència consens de fosforilació. De forma interessant, aquest motiu consens de fosforilació és notablement semblant a la seqüència de tall consens de les caspases. S'ha demostrat que la fosforilació de CK2 en dianes com Bid [287], Max [288], HS1 [289] o la conexina 45.6 [290] resulta en la protecció d'aquestes proteïnes del processament caspasa-dependent. Així doncs, és concebible que la CK2 funcioni com a un sensor de la integritat cel·lular que exerceix una funció general de supervivència o antiapoptòtica a partir de la seva habilitat per fosforilar múltiples proteïnes que estarien destinades altrament al processament per caspases durant l'apoptosi.

La inhibició de la CK2 en cèl·lules canceroses sensibilitza aquestes cèl·lules a l'apoptosi. L'ús de RNAs d'interferència contra CK2α o la sobreexpressió de formes cinasa inactives de CK2α resulten en la sensibilització a la mort induïda per TRAIL en cèl·lules de rhabdomiosarcoma i cèl·lules HT29 de càncer de còlon [291,292].

La sensibilització a l'apoptosi fent ús d'inhibidors de la CK2 també s'ha observat i es caracteritza per un increment en la formació dels complexes DISC en resposta a estímuls com TRAIL, un major processament de Bid per caspasa-8 així com un descens en proteïnes protectives com XIAP o c-IAPs.

L'habilitat de la CK2 de sensibilitzar cèl·lules resistents i no-resistents a l'apoptosi planteja la possibilitat de l'ús d'inhibidors de la CK2 en conjunció amb els tractaments antineoplàsics convencionals per tractar diversos carcinomes.

Diferents tipus de compostos químics competidors d'ATP han estat testats com a inhibidors de la CK2 diferint en la seva eficàcia i especificitat. Alguns d'ells mostren una selectivitat limitada com l'apigenina o la emodina, mentre que d'altres com el TBB (4,5,6,7-tetrabromo-1H-benzotriazole) i IQA (5-oxo-5,6-dihydroindolo(1,2-a)quinazolin-7-yl) àcid acètic presenten una alta especificitat i representen els primers compostos prometedors per estudiar les funcions de la CK2. Derivats del TBB com TBBz (4,5,6-tetrabromobenzimidazole), DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole) i TBCA (àcid tetrabromocinnamic) han estat desenvolupats per incrementar per incrementar la selectivitat cap a CK2.

A comparison of the efficacy of CK2 inhibitors

CK2 inhibitor	IC_{50}=μM	K_i=μM
Apigenin	0.8	0.74
Emodin	2.0	1.85
DRB	13.0	4.50
TBB	0.50	0.40
TBBz (TBI)	0.50	0.70
DMAT	0.14	0.04
TBCA	0.11	0.077
IQA	0.39	0.17
Ellagic acid	0.04	0.02
Inhibitor 7	0.30	0.06

Taula 2: Llistat d'inhibidors de la CK2 amb les eficàcies d'inhibició respectives. *(Duncan, JS and Litchfield, DW. Biochimica et Biophysica Acta, 2008. 1784:33-47)*[293].

6.4 Moduladors de la senyal iniciada per TRAIL i Fas, III: MAPK

La via de senyalització per les MAPK juga un paper crític en la transmissió de nombroses senyals de desenvolupament i creixement i una de les majors vies per les quals Ras transmet les senyals és a través de l'activació seqüencial de les cinases Raf, MEK i MAPK.

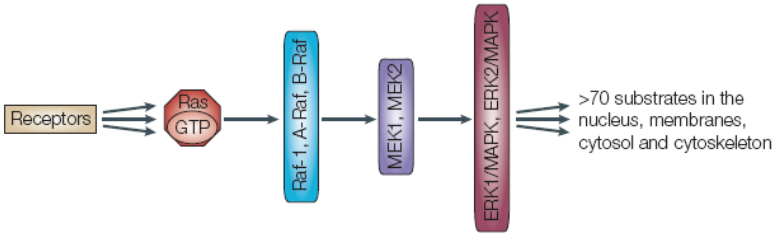


Figura 28: La via ERK/MAPK. Molts receptors de membrana activen les Ras GTPases. Les Ras GTPases inclouen una extensa família de proteïnes residents a membrana que es mouen entre una conformació inactiva unida a GDP i una conformació activa unida a GTP. Els membres més caracteritzats són K-Ras, H-Ras i N-Ras, que estan codificats per proto-oncogens molt potents que es troben mutats en aproximadament el 30% dels tumors humans. Les proteïnes Ras-GTP són capaces de reclutar proteïnes efectores com les proteïnes cinases Raf, la fosfatidilinositol-3-cinasa (PI3K), RalGDS etc. Les proteïnes Raf seran les encarregades d'activar les ERK/MAPK a través de la fosforilació en dos residus de MEK-1, essent b-Raf la més eficient de les tres. Les proteïnes ERK1/2 són considerades les principals efectores d'aquesta via i se'n coneixen més de 70 substrats que inclouen factors de transcripció, proteïnes de citoesquelet, etc. *(Kolch, W. Nature Reviews, 2005. 6:827-37)*[294].

Tal com comentarem més endavant, la via de les MAPK es troba freqüentment alterada en diversos tipus de neoplàsies i aquest "desorde" es tradueix sovint en un increment en la proliferació cel·lular o l'adquisió de resistència enfront diversos tipus d'estrés. En aquest sentit s'ha observat amb anterioritat com la inhibicó d'aquesta via és capaç de sensibilitzar cèl·lules canceroses a la mort induida per lligands de mort com TRAIL[295-299].

Raf (*R*apidly *A*ccelerated *F*ibrosarcoma), és un efector dels receptors amb activitat tirosin-cinasa. Fou descobert als inicis dels anys 80 per dos grups de forma independent com un oncogen retroviral, v-Raf [300] o v-MIL [301] amb activitat cinasa serina/treonina (la proteïna resultant) [302]. Posteriorment, aquesta

proteïna cinasa fou descrita com un component de la via Ras-Raf-MEK-ERK (via MAPK) [303].

Hi han tres isoformes de Raf conegudes en mamífers: A-Raf, B-Raf i C-Raf (o Raf-1). Les proteïnes Raf estan altament conservades al llarg de l'escala filogenètica, tal com succeeix també amb la via de senyalització de les MAPKs en general.

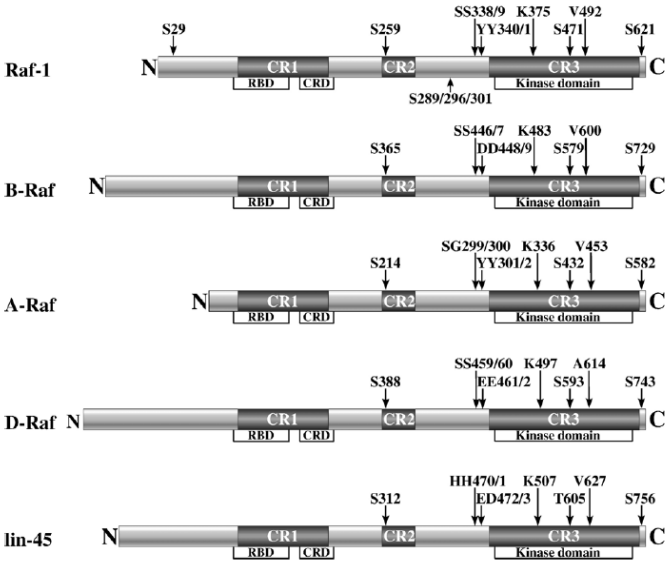
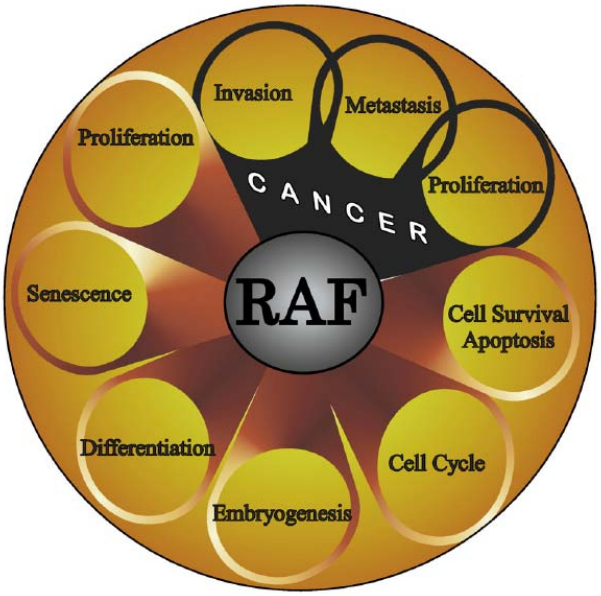


Figura 31: Alineament estructural de les isoformes de Raf. Les isoformes humanes A, B i C-Raf es troben alineades amb la forma D-Raf present en *Drosophila melanogaster* i la forma lin-45 present en *Caenorhabditis elegans*. S'indiquen les regions conservades (CR) 1, 2 i 3, el domini d'unió a Ras (*Ras binding domain*, RBD) i el domini d'estructura en dit de zinc i ric en cisteïnes CRB (**C**isteine **r**ich **d**omain). També s'aprecien els llocs de fosforilació de C-Raf i demés isoformes i la posició del lloc d'unió a ATP (K375, en C-Raf). *(Leicht, DT. Biochimica et Biophysica Acta, 2007. 1773:1196-1212)*[304].

A part del seu rol reconegut en la tumorogènesi, les proteïnes Raf i la via de les MAPK participen en diversos processos fisiològics com són el metabolisme cel·lular, cicle cel·lular, mort cel·lular i funció neurològica.

Figura 32: Funcions fisiològiques conegudes per Raf. *(Leicht, DT. Biochimica et Biophysica Acta, 2007. 1773:1196-1212)*[304].



B-Raf ha estat la segona isoforma de la familia Raf en ésser identificada. L'interés cap a B-Raf s'incrementà quan a partir del 2002 s'identificà B-Raf com un element mutat en numerosos càncer humans [305-307]. A partir d'aquí, mutacions en B-Raf s'han observat en el 70% dels melanomes, 30% dels càncers de tiroides, 15% dels càncers de còlon i en freqüències menors en altres tipus de carcinomes [308,309]. La majoria de les mutacions foren identificades dins el domini cinasa, en una substitució aminoacídica: V600E. Aquesta representa el 90% de les mutacions trobades en B-Raf.

Les mutacions en B-Raf resulten en una activació constitutiva de l'activitat cinasa que correlaciona in vivo en l'activació constitutiva de les seves dianes com són MEK i ERK [306,310,311]. De forma interessant sembla ser que una única alteració en la via MAPK és suficient per l'oncogènesi ja que les dades actuals mostren una molt baixa superposició o solapament de mutacions activadores en Ras i B-Raf en mostres de càncer. Per exemple, en un estudi recent els autors determinen que el càncer colorectal presenta un 36% de mutacions en B-Raf i un 18% de mutacions en Ras sense que cap d'elles coexisteixi en una mateixa mostra [312].

La mutació corresponent a V600E en B-Raf no s'ha detectat en A-Raf i C-Raf en càncers humans. Es postula que això es deu al fet que C-Raf i A-Raf són objecte d'una regulació molt més controlada o estricta que B-Raf, de forma que una mutació simple en B-Raf ja és suficient per un increment substancial de l'activitat cinasa [304,309,313-315]. En efecte, l'activació de C-Raf requereix fosforilacions en les serines 338/339 i en les tirosines 340/341 en la regió amino-terminal del domini cinasa (Fig31), mentre que en B-Raf el residu serina comparable a C-Raf en la posició 445

es troba constitutivament fosforilat i els residus 448/449 estan ocupats pel fosfomimètic àcid aspàrtic, primant doncs B-Raf per l'activació. A-Raf sembla ser regulat per un mecanisme similar a C-Raf.

Tenint en compte la forta prevalència de mutacions presents sobretot en B-Raf, diverses aproximacions s'han realitzat al llarg dels anys per aconseguir inhibir de forma eficient les proteïnes Raf. Les estratègies inclouen la inhibició de l'ectivitat de Raf fent ús de molècules inhibidores, el bloqueig de l'expressió de Raf a través de RNAs d'interferència o bloquejant les interaccions Ras-Raf.

En referència al bloqueig de l'expressió de B-Raf, diversos compostos antisentit i RNAs d'interferència s'han testat. Per exemple, ISIS-5132, que és un oligonucleòtid antisentit de 20 bases per C-Raf, mostrà una eficient inhibició tumoral en models animals i resultà en l'estabilització del carcinoma d'ovari en anteriors fases clíniques [316]. No obstant, les proves realitzades en fase clínica II revelaren que aquest compost no és efectiu quan és administrat com a agent únic. Les proves incloien carcinomes d'ovari [317], carcinoma colorectal [318], càncer de pulmó de cèl.lules petites (SCLC), NSCLC [319] i carcinoma de pròstata [320].

L'èxit en l'ús d'estratègies per inhibir la interacció Ras-Raf han estat limitades i actualment no hi han dades clíniques disponibles [304].

Pel que fa a l'ús de molècules inhibidores, BAY 43-9006 (o Sorafenib) ja es troba en fase clínica III i de moment sembla ser l'inhibidor farmacològic més efectiu i l'estratègia més exitosa.

6.5 Moduladors de la senyal iniciada per TRAIL i Fas, III: NF-kappa B

NF kappa B, un factor dimèric de transcripció nuclear, fou en un inici descobert i estudiat com un dels principals activadors de les funcions immunes i inflamatòries a través de la seva habilitat per induir l'expressió de gens que codifiquen per citoquines, receptors de citoquines i molècules d'adhesió cel·lular [321,322]. Més tard NF kappa B fou associat al control del creixement cel·lular i a l'oncogènesi.

El paper que juga NF kappa B en el càncer sembla complex i probablement englobi múltiples funcions com el control de l'apoptosi, de la progressió del cicle cel·lular i possiblement de la diferenciació cel·lular, angiogènesi i migració cel·lular.

6.5.1 Membres de la família NF-kappa B

Existeixen cinc membres de la família NF-kappa B: p105/p50 (NF-κB1), p100/p52 (NF-κB2), c-Rel, Rel-B i p65 (Rel-A). Aquests es poden classificar en dos subfamílies [323]: 1) Rel-A, c-Rel i Rel-B són sintetitzats en la seva forma madura i contenen un domini de transactivació que interacciona amb l'aparell transcripcional i 2) NF-κB1-p105/p50 i NF-κB2-p100/52 que són sintetitzats en un primer terme com a una forma precursora. Aquestes (p100 i p105) contenen una sèrie de repeticions anquirina en l'extrem carboxil-terminal que són proteolitzades per el proteasoma resultant en la producció de les proteïnes madures (p52 i p50). Ambdues p50 i p52 contenen dominis d'unió al DNA però són defectuoses en dominis de transactivació.

Tots els membres de la família NF-κB estan caracteritzats per presentar dominis d'homologia Rel (RHD) en les seves seqüències que controlen la seva unió al DNA, dimerització i interaccions amb factors inhibitoris com les proteïnes IκB (*I*nhibitor of *κB*) [321,322].

Les proteïnes IκB (IκBα, IκBβ, IκBε i Bcl-3) formen una família de proteïnes que interaccionen amb el domini RHD de les proteïnes NF-κB. IκBα, IκBβ i IκBε interaccionen amb els dímers NF-κB retenint-los a citoplasma. Bcl-3 és un peculiar membre de la família, caracteritzat per contenir un domini de transactivació, que interacciona amb p50 i p52 i que actua com a coactivador transcripcional.

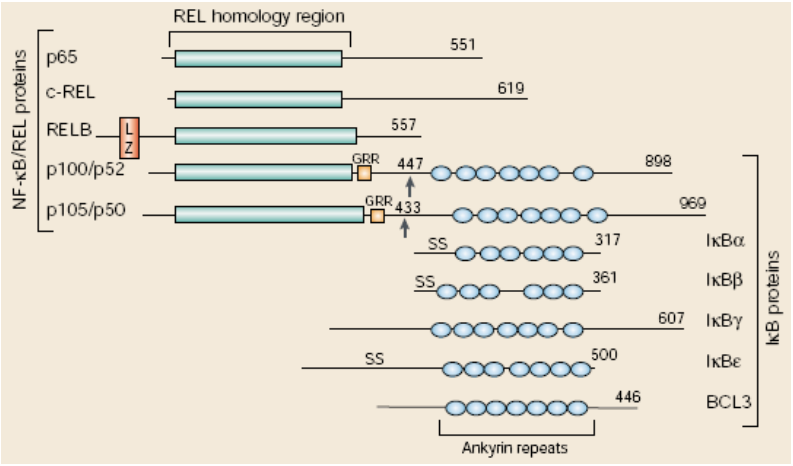


Figura 41: Representació dels membres de la família NF-kappa B i proteïnes inhibidores (IκB). S'aprecien els dominis d'homologia Rel. *(Karin, M; et al. Nature Reviews, 2002. 2:301-10)*[324].

6.5.2 Regulació de NF-kappa B

En la majoria dels tipus cel·lulars, els dímers NF-κB estan retinguts predominantment a citoplasma i són, per tant, transcripcionalment inactius fins que la cèl·lula rep l'estímul pertinent que resultarà en la seva activació i posterior translocació a nucli (Fig 42, 43). Aquesta activació pot ésser deguda en resposta a citoquines proinflamatòries com TRAIL, Fas, el factor de necrosi tumoral (TNF), la interleuquina (IL)-1, el lipopolisacàrid (LPS), etc. (Fig 20, 23, 44).

Determinades vies de senyalització cel·lular com la via Ras/MAPK, PI3K/Akt o la propia casein cinasa-II (CK2) també poden activar la via NF-κB en resposta a diversos estímuls com, per exemple, dany genotòxic (Fig 27).

Diferents mecanismes moleculars d'activació de NF-κB han estat proposats [325,326]. En l'anomenada via canònica o clàssica els dímers NF-κB contenen c-Rel o Rel-A en combinació amb p50. Aquests són retinguts a citoplasma a través de la interacció amb les proteïnes IkBα [326,327]. Aquesta via està típicament activada en resposta a citoquines proinflamatòries i infeccions víriques. Un cop activada la via, la proteïna cinasa IKKβ (*I*nhibitory of **κ**B *k*inase **β**) serà la responsable de fosforilar les proteïnes IkB. Un cop fosforil·lades, aquestes seran ubiquitinitzades i subseqüentment degradades a través del proteasoma. La degradació resultarà en l'alliberació del dímer NF-κB que translocarà a nucli i regularà l'expressió de gens

específics típicament implicats en respostes immunes i inflamatòries i en el control del creixement cel·lular [321,322] (Fig 42).

La via alternativa és activada per membres de la família del TNF com la limfotoxina B o BAFF. En aquest cas els dímers estan formats per Rel-B i p100 [328]. Aquesta via activa principalment la cinasa IKKα, que serà l'encarregada de fosforilar p100. Aquesta, un cop fosforilada serà proteolitzada generant-se la forma madura p52 [327,329]. Finalment els dímers translocaran al nucli on activaran la transcripció de gens diana (Fig 42).

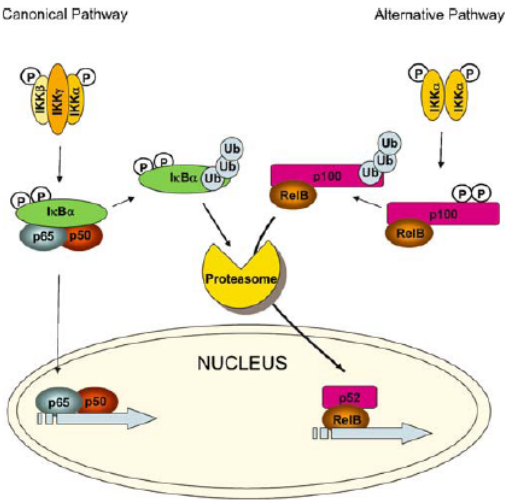


Figura 42: Vies canònica i alternativa d'activació de NF-κB. *(Dolcet, X; et al. Virchows Archives, 2005. 446:475-82)*[330].

Tot i que els dímers que trobem en les vies canònica i alternativa (Rel-A/p50, c-Rel/p50, Rel-B/p52) són els més coneguts, altres tipus d'hetodímers (c-Ral/Rel-A, Rel-A/Rel-B) o homodímers (p50/p50, p52/p52) també es generen.

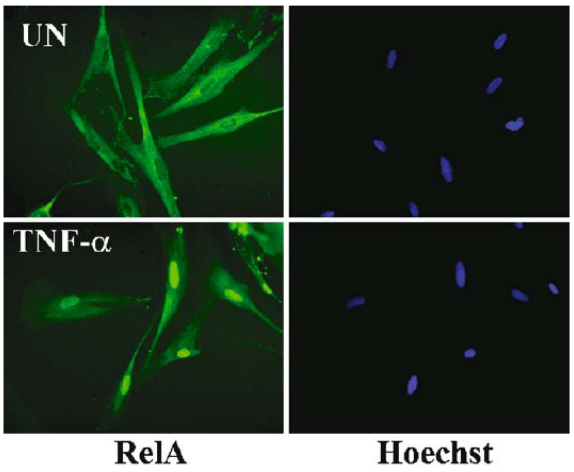


Figura 43: Translocació nuclear de Rel-A (p65) en cèl·lules de l'estroma de l'endometri en resposta a l'estimulació per TNFα. *(Dolcet, X; et al. Virchows Archives, 2005. 446:475-82)*[330].

L'activació de la via NF-κB és doncs una via estrictament regulada en múltiples nivells. En cèl·lules normals, NF-κB és activada només en resposta a estímuls apropiats. Per contra, en cèl·lules tumorals diferents tipus d'alteracions moleculars poden resultar en una regulació defectuosa i una activació constant de la via.

6.5.3 Dianes de NF-kappa B

NF-κB pot activar la transcripció de múltiples gens implicats en el bloqueig de la mort cel·lular programada tant a nivell de la mitocondrial com a nivell de receptors de mort com, per exemple, FLIP [331,332] (Fig44).

Altres proteïnes que participen en la cascada de senyalització de membres de la familia dels receptors de mort com el propi TNFα, TRAIL o Fas com són TRAF2 o TRAF6 també representen possibles dianes de NF-κB. Aquestes proteïnes, al seu torn, faciliten l'activació de vies de supervivència per aquests lligands [333].

NF-κB també indueix l'expressió de proteïnes inhibidores de l'apoptosi (IAPs) [333-335] així com alguns membres de la família antiapoptòtica de Bcl-2 [336,337].

Recentment s'ha documentat que NF-κB també pot bloquejar la transcripció de PTEN, un conegut gen supressor tumoral que es troba freqüentment mutat en el carcinoma d'endometri [338,339]. Per tant, un descens en l'expressió de PTEN pot

causar un increment en l'activitat de Akt i el bloqueig propiciat per aquest últim d'algunes vies proapoptòtiques [340].

NF-κB també pot promoure la progressió del cicle cel·lular a través de la regulació de l'expressió de diversos gens implicats en cicle com les ciclines D1, D2, D3 i la ciclina E [327,341-343], c-myc i c-myb [344-347].

D'altra banda, NF-κB indueix l'expressió de molècules d'adhesió cel·lular (ICAM, E-selectina), i proteïnes implicades en invasió (metal·loproteïnases). Diversos factors importants en fenòmens com l'angiogènesi, incloent el VEGF (*V*ascular *e*ndothelial *g*rowth *f*actor) també són promoguts per NF-κB.

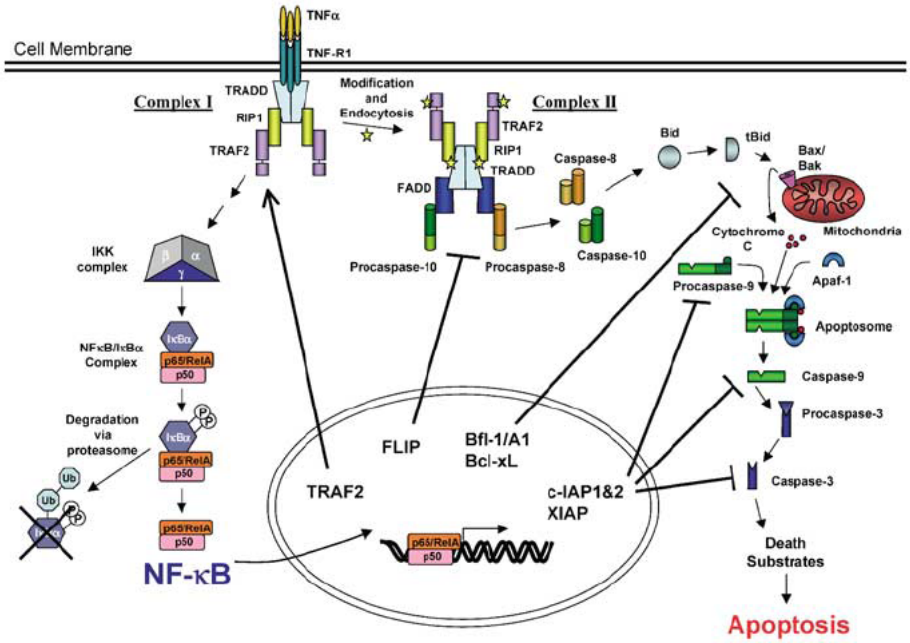


Figura 44: Paradigma d'activació de NF-Kappa B a través de l'estimulació per TNFα. Les dianes transcripcionals de NF-Kappa B actuen en múltiples nivells inhibint l'activació de les caspases apicals (FLIP), inhibint la via mitocondrial i la permeabilització de la seva membrana (Bfl/A1, Bcl-xL) o inhibint la senyalització "per sota" de la mitocondrial (c-IAPs, XIAP). *(Kucharczak, J; et al. Oncogene, 2003. 22:8961-82)*[348].

6.5.4 Implicacions de NF-Kappa B en l'oncogènesi

v-Rel, l'homòleg viral de c-Rel, fou identificat com el gen transformant d'un retrovirus d'aus que era altament oncogènic i que causava tumors agressius en pollastres [323,349].

En referència al càncer humà, els gens codificants per c-Rel, NF-κB2 (p100/52) i Bcl3 estan amplificats i/o reordenats en determinats tumors. Per exemple, el gen Bcl-3 fou localitzat en una translocació cromosomal [t(14,19) (q32;q13.1)] trobada en leucèmies limfàtiques cròniques de cèl·lules B [349,350]. Mutacions en el gens codificant per IκBα s'han detectat en el limfoma de Hodgkin contribuint a l'activitat constitutiva de NF-κB en aquestes cèl·lules [351].

En tumors sòlids, alt nivells de c-Rel s'han observat en el càncer de pulmó de cèl·lules grans (NSCLC) [352] i càncer de mama [353]. Tot i que les alteracions en Rel-A, Rel-B i NF-κB1 (p105/50) són rares en les neoplàsies humanes, aquestes subunitats s'han trobat constitutivament actives en un a àmplia varietat de tumors humans i s'han associat a progressió tumoral [354,355]. A més a més, diversos tumors sòlids com el de mama, còlon, ovari, pàncrees, bufeta, pròstata i melanoma s'han caracteritzat per ésser defectius en IκBα i tenir activitat NF-κB constitutiva [350].

Donada la importància del paper de NF-κB en l'oncogènesi són molts els esforços que s'han invertit en el desenvolupament de compostos per bloquejar i inhibir l'activitat NF-κB. Entre aquests uns dels més exitosos han estat, sens dubte, els inhibidors del proteasoma.

Estudis previs en el nostre laboratori suggereixen que NF-κB pot jugar un paper important en el desenvolupament i progressió del carcinoma d'endometri [356]. Aquests resultats, doncs, donen suport a l'ús d'inhibidors de NF-κB com a eina d'estudi i possible estratègia terapèutica en el tractament del carcinoma d'endometri.

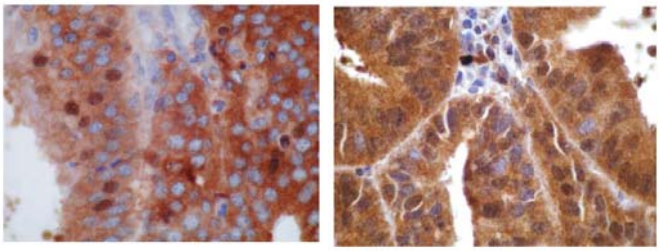


Figura 43: Immunotinció nuclear per p65 (esquerra) i immunotinció nuclear i citoplasmàtica per Rel-B (dreta) en un carcinoma d'endometri endometrioide. *(Pallarés, J; et al. Jounal of Pathology, 2004. 204:569-77)*[356].

7 DIANES MOLECULARS EN EL TRACTAMENT DEL CARCINOMA D'ENDOMETRI

Un dels grans problemes alhora de tractar els carcinomes és la resistència que aquests presenten sovint enfront les teràpies convencionals. Aquest és un dels motius de la constant recerca de nous fàrmacs que presentin, a més a més de capacitat antitumoral, propietats més selectives.

En el disseny de la teràpia "ideal" per al tractament del càncer, tres criteris són els que es troben més amunt en la llista de prioritats: la primera, la teràpia hauria d'ésser efectiva alhora de matar les cèl·lules tumorals i evitar la seva reaparició. En segon lloc, el tractament hauria d'ésser altament selectiu per les cèl·lules canceroses i evitar danys col·laterals. En aquest sentit, els danys secundaris i la pobra tolerància als agents quimioterapèutics convencionals són les principals dificultats clíniques al tractament. Finalment, l'habilitat de fer un seguiment del tractament a través de biomarcadors permet optimitzar temps i recursos [357].

7.1 Sorafenib

Són diverses les estratègies i els agents que actualment es troben en alguna fase clínica per estudiar la seva efectivitat antitumoral i possible administració en humans, des d'estudis amb lligands de mort com TRAIL [200,358], inhibidors de deacetilases d'histones[359], inhibidors del proteasoma [360], triterpenoides [361-363], inhibidors de receptors tirosina cinasa, etc.

En referència als darrers, el inhibidor de cinases en tirosina (TKI, ***T***yrosin ***k***inase ***i***nhibitor) imatinib (Gleevec) ha revolucionat el tractament de pacients amb leucèmia mieloide crònica i ha potenciat la recerca de drogues amb propietats inhibidores de les proteïnes tirosina cinases. Aquests agents es poden classificar en dos grups:

1- anticossos monoclonals dirigits contra receptors tirosina cinases (RTK, ***R***eceptor ***t***yrosin ***c***inases) com el Trastuzumab, un inhibidor del receptor ERBB2 (o HER2).
2- molècules de baix pes molecular inhibidores de proteïnes cinases en residus tirosina (TKIs) com l'imatinib, un inhibidor de la proteïna cinasa ABL. Actualment l'imatinib s'usa en el tractament de la leucèmia mieloide crònica

on les cèl·lules canceroses expressen la proteïna de fusió BCR-ABL codificada en el cromosoma Filadèlfia (Ph).

No obstant, darrerament l'estratègia seguida en el disseny de nous fàrmacs ha variat substancialment. Molts tumors depenen de nova vascularització per al creixement i metàstasi, però també requereixen de l'acció de tirosin cinases per a la proliferació i supervivència de les cèl·lules canceroses. En aquesta línia, els receptors tirosin cinases VEGFR i PDGFR han estat implicats en fenòmens d'angiogènesi tumoral. Teòricament, un agent que inhibís l'angiogènesi i una o més tirosin cinases implicades en proliferació tumoral podria exhibir una activitat antitumoral de més ampli espectre degut a aquest efecte dual. Aquesta és la màxima que es troba al darrere del desenvolupament de fàrmacs com el sunitinib i el sorafenib [364].

7.1.1 Desenvolupament del Sorafenib

Sorafenib, o BAY 43-9006, apareix en escena com a resultat d'una col·laboració entre les empreses Bayer i Onyx iniciada l'any 1994. L'objectiu d'aquesta unió és el de identificar molècules amb propietats inhibidores de l'activitat de Raf-1. L'estudi s'inicia al 1995 amb el testatge d'aproximadament 200.000 compostos.

Durant l'estudi s'identifiquen diverses molècules o compostos candidats que exhibeixen propietats potencialment útils. Les posteriors modificacions imposades en l'estructura química permeten l'evolució des de molècules precursores amb una constant d'inibició modesta cap a molècules més especialitzades fins donar lloc al compost final, el sorafenib, amb una IC_{50}=6 nM (Fig33).

Posteriorment al seu nomenament com a candidat preclínic a entrar en fases de desenvolupament, el sorafenib entra al 2000 en les fases clíniques per a evaluar la seva possible aplicació en humans. Un cop finalitzades les fases II/III els resultats estableixen l'administració oral del sorafenib com a un nou mètode segur i efectiu pel tractament del carcinoma de ronyó metastàsic. Al desembre de l'any 2005 la FDA (**F**ood and **D**rug **A**dministration) aprova la seva comercialització.

Amb tot, el cicle de descobriment i desenvolupament del sorafenib des de l'inici fins la seva aprovació final triga 11 anys.

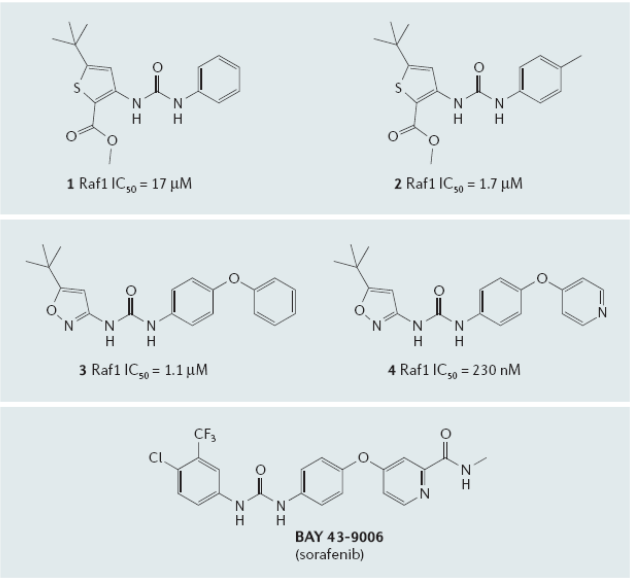


Figura 33: Resum de l'optimització química del sorafenib. *(Wilhelm SW; et al. Nature Reviews, 2007. 5:835-44)*[365].

Actualment s'està evaluant la seva possible aplicació en altres tipus de neoplàsies com el melanoma, el carcinoma hepatocel·lular o el càncer de pulmó de cèl·lules grans (NSCLC)[365].

7.1.2 Mecanismes d'acció del sorafenib

El sorafenib fou identificat inicialment com un inhibidor de Raf-1 però estudis posteriors revelen que el sorafenib és un inhibidor d'ampli espectre que exhibeix la seva activitat inhibitòria en altres homòlegs a Raf-1 com B-Raf i la seva forma mutada B-Raf^{V600E}. Les dades indiquen que el Sorafenib és capaç d'unir-se al domini cinasa i estabilitzar la conformació inactiva d'aquest.

D'altra banda, el Sorafenib també presenta activitat inhibitòria per altres proteïnes cinases com són els receptors de VEGF i PDGF (***V***ascular ***e***ndothelial ***g***rowth ***f***actor i ***P***latelet-***d***erived ***g***rowth ***f***actor respectivament), c-Kit o Ret. Així doncs, alguns dels efectes antitumorals del Sorafenib poden ésser atribuïts a la inhibició de l'angiogènesi i vascularització tumoral.

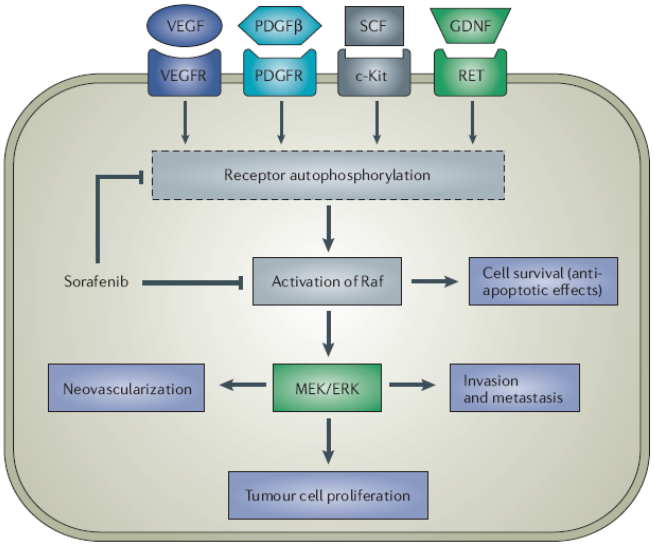


Figura 34: Dianes cel·lulars del sorafenib. El sorafenib bloqueja la senyalització iniciada pels receptors amb activitat tirosina-cinasa VEGF, PDGF, c-Kit i RET i inhibeix l'activitat cinasa de Raf. Aquests efectes anti-angiogènics i anti-proliferatius prevenen el creixement tumoral. *(Wilhelm SW; et al. Nature Reviews, 2007. 5:835-44)*[365].

Evidencies recents sugereixen que el sorafenib és capaç de potenciar els efectes de TRAIL en diferents models de carcinoma. Els mecanismes pels quals el sorafenib sensibilitzaria al tractament per TRAIL cèl·lules altrament resistens inclouen la regulació negativa de Mcl-1, FLIP o c-IAP2[366-368].

Encara més, el paper que juga l'activitat cinasa de Raf i les seves dianes inmediates MEK1/2 i ERK1/2 en aquest fenòmen no està del tot dilucidat i es continuen buscant nous mecanismes d'acció que expliquin en profunditat les propietats antitumorals del sorafenib (Fig35).

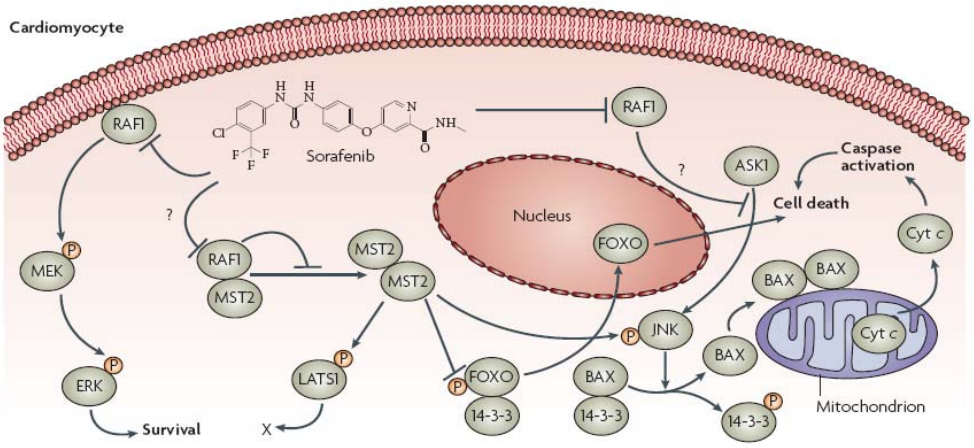


Figura 35: Possibles vies de senyalització afectades per l'acció del Sorafenib. La inhibició de C-Raf (i B-Raf, no mostrat) disrupciona la senyalització a través de ERK, que es creu juga un paper en la supervivència cel·lular en el cor, especialment en condicions d'estrès. Altres vies proapoptòtiques inhibides per C-Raf de manera cinasa-independent com són la via de ASK1 (**A**poptosis **s**ignal-regulated **k**inase 1) o la via MST2 (**M**ammalian **s**terile **t**wenty kinase-**2**) encara no s'ha reconegut com a mecanisme d'acció del Sorafenib. *(Force, T; et al. Nature Reviews, 2007. 7:332-44)*[364].

7.2 El proteasoma

La degradació de les proteïnes no és un procés simple i moltes vies reguladores controlen el què, quan, on i com les proteïnes es degraden.

Les cèl·lules utilitzen diversos mecanismes per regular l'estabilitat i degradació de les proteïnes. Tot i que possiblement existeixin mecanismes addicionals per la degradació proteica encara per identificar, podríem afirmar que existeixen dos rutes principals de degradació: la via lisosomal i la proteasomal (Fig34). De forma simplista, els lisosomes es podrien descriure com a els orgànuls cel·lulars responsables de la degradació de proteïnes extracel·lulars i transmembrana, mentre que els proteasomes s'encarreguen de la degradació de les proteïnes intracel·lulars [369].

Els lisosomes són vesícules citosòliques unides a membrana que inclouen proteases i altres enzims hidrolítics. La compartimentalització d'aquests enzims prevén la degradació proteica de forma incontrolada. Fins que els proteasomes foren descoberts [370,371] i la seva funció estudiada, els lisosomes van ésser considerats com

els principals "aparells" a través dels quals les proteïnes les proteïnes eren degradades en el citoplasma. Ara es coneix que els proteasomes tenen una funció cabdal en la degradació proteica i que una degradació ordenada és crucial per al correcte manteniment de les funcions cel·lulars.

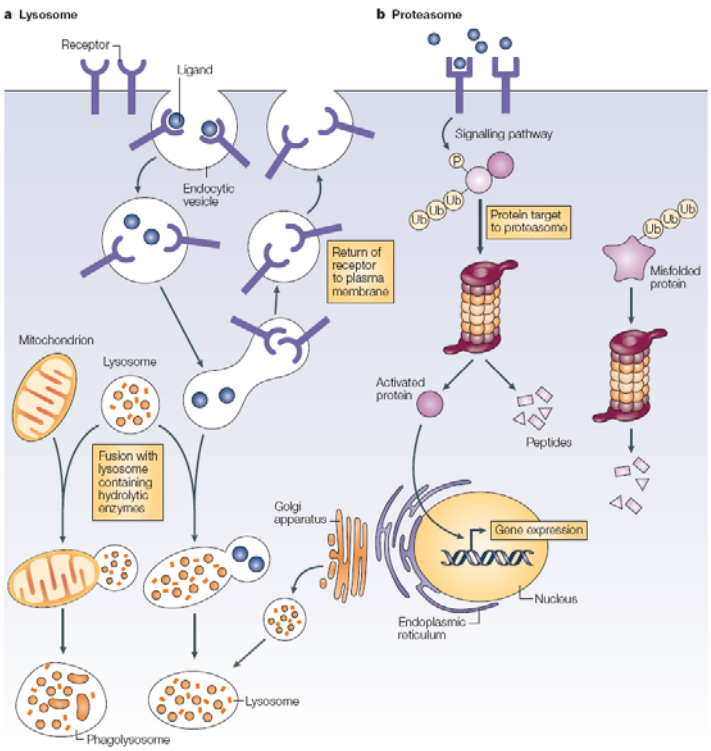


Figura 34: Mecanismes de degradació proteica en eucariotes: **A**, via lisosomal. Els lisosomes degraden proteïnes extracel·lulars i transmembrana captades per endocitosi i també participen en l'autofagocitosi fusionant-se amb orgànuls i formant els fagolisosomes. **B**, via proteasomal. Via implicada en la degradació de proteïnes intracel·lulars que prèviament han sofert modificacions per fosforilació, ubiquitinització o simplement són proteïnes mal plegades. *(Adams, J. Nature Reviews, 2004. 4:349-60)*[360].

Proteasoma 26S, el proteasoma funcionalment actiu, és un complex multiproteic d'aproximadament 2,4 MDa amb activitat proteolítica ATP-dependent que es troba en el citoplasma i nucli de les cèl·lules eucariotes.

Està format per el complex cilíndric 20S que conforma el nucli catalític i dos subunitats reguladores 19S que es localitzen en ambdós extrems del complex 20S [372,373] (Fig35).

El proteasoma identifica proteïnes que han estat marcades per a la degradació a través de la unió a residus ubiquitina, tot i que la ubiquitinització no és un prerequisit per a la degradació proteasoma-dependent per a totes les proteïnes.

El descobriment dels proteasomes ancestrals en l'arqueobacteria *Thermoplasma acidophilum* [374] fou crucial per a la dissecció estructural de la partícula 20S. Aquest està format per dos tipus de subunitats: α i β. Es postulà en el seu moment que aquestes dues subunitats són els ancestres de les diferents subunitats α i β trobades gradualment en els proteasomes eucariotes [375,376].

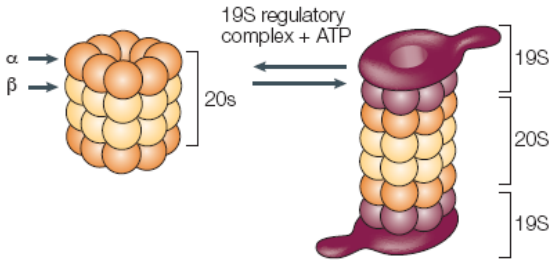


Figura 35: Representació tridimensional del proteasoma. Aquest està compost per la partícula 20S formada per les subunitats α i β i pel complex regulador 19S. La formació del complex definitiu 26S requereix ATP. *(Adams, J. Nature Reviews, 2004. 4:349-60)*[360].

El proteasoma 20S és un complex de 28 subunitats organitzades en 4 anells un a sobre de l'altre formant una estructura cilíndrica. Els dos anells exteriors estan formats per set subunitats α cadascun i els dos interiors per 7 subunitats β respectivament. Els anells interiors seran els que continguin els llocs enzimàticament actius del proteasoma [360,377,378] (Fig36).

Només les subunitats β presenten activitat catalítica. En bactèries, les subunitats α són idèntiques entre elles al igual que succeeix entre les subunitats β [379,380]. A més a més, cada subunitat β és activa [379-381] i es creu que les subunitats α han evolucionat a partir de les subunitats β [382]. En aquest sentit s'ha postulat que en etapes primerenques en l'evolució dels eucariotes ambdues subunitats α i β es van diversificar donant lloc a set subunitats α i β diferents codificades per 14 gens diferents [382,383].

De forma interessant, durant aquest procés el nombre de subunitats β amb activitat catalítica s'ha reduït a tres (sis en el còmput global). Aquestes tres subunitats (β1, β2 i β5) exerceixen l'activitat enzimàtica del proteasoma en eucariotes, que pot ésser classificada en: activitat quimiotripsina (β5), activitat tripsina (β2) i activitat post-glutamil peptidil hidrolasa (β1) [384-386]. La funció de les restants quatre subunitats β no es coneix.

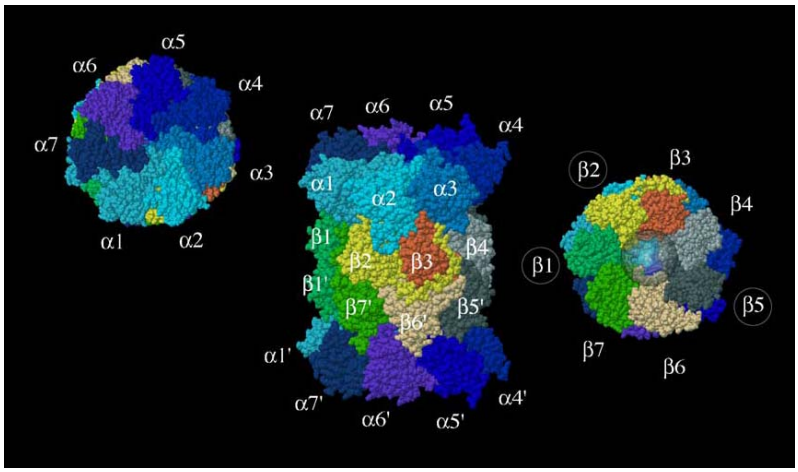


Figura 36: Estructura del complex central 20S. La partícula 20S està formada per quatre anells que contenen cadascun set subunitats. Els dos anells interiors, formats íntegrament per subunitats β, són els que desenvoluparan l'activitat catalítica. *(Wolf, DH and Hilt, W. Biochimica et Biophysica Acta, 2004. 1695:19-31)*[378].

Basat en estudis en llevats, es coneix que la longitud de la majoria dels pèptids detectables generats per la degradació proteasomal es mou entre els quatre i els catorze aminoàcids [387].

L'arquitectura α_7 β_7 β_7 α_7 resulta en la formació de tres cambres en la partícula 20S: la cambra catalítica d'aproximadament 84nm^3 formada pels dos anells interiors i dues antecambres formades per una anell exterior i un interior de 59nm^3 [379,388]. S'accedeix als llocs actius en la cambra catalítica a través de dos porus axials de 2nm formats per les subunitats α. La dimensió d'aquest porus assegura que només els polipèptids en conformació lineal puguin entrar en el centre catalític del proteasoma.

En mamífers existeixen tres gens addicionals induïbles per interferó-γ que codifiquen per subunitats β amb activitat catalítica alternatives: β1/LMP2,

β2/MECL-1 i β5/LMP7. La incorporació d'aquestes subunitats enlloc de les constitutives β1, β2 i β5 dóna lloc a la formació d'un subtipus de proteasoma conegut com a immunoproteasoma. Aquest és el responsable de la generació de pèptids antigènics presentats pel complex major d'histocompatibilitat de classe I [389,390].

Per funcionar in vivo, el proteasoma 20S requereix l'associació d'unitats reguladores que determinin parcialment l'especificitat de la funció del proteasoma. Una d'aquestes partícules reguladores és el complex 19S [385].

La partícula reguladora 19S (o PA700) és un complex d'uns 700kDa format per 20 subunitats polipeptídiques que s'uneixen als dos extrems del proteasoma 20S per formar el proteasoma 26S. La seva funció consisteix en reconèixer els substrats poliubiquitinitzats, la alliberació de la cadena d'ubiquitina, el desplegament del substract, l'obertura del canal que comunica amb la cambra catalítica i la translocació del polipèptid linealitzat als centres catalítics [377].

El complex 19S es divideix en dos parts: la "base", que conté sis ATPases i diverses subunitats més i la "tapa"formada per proteïnes menys caracteritzades localitzades en posició distal [391]. La base per si sola és capaç de degradar pèptids i proteïnes no ubiquitinitzades, però la tapa és requerida per degradar proteïnes ubiquitinitzades proveint d'aquesta manera un nivell superior d'especificitat per la degradació proteica.

La hidròlisi d'ATP és necessària per la formació del complex 26S i pel desplegament i linealització de grans proteïnes per facilitar la seva entrada en el nucli catalític del proteasoma [385,392].

7.2.1 Els inhibidors del proteasoma

Molts dels substrats del proteasoma són coneguts elements que participen en vies de senyalització que sovint trobem desregulades en les neoplàsies. Un gran nombre d'evidències indica que el proteasoma afecta la progressió del cicle cel·lular, vies apoptòtiques i proliferació (Fig37).

El fet que el proteasoma sigui crucial per la execució de moltes funcions cel·lulars indica que seria difícil utilitzar-lo com a diana quimioterapèutica (Fig37). No obstant, els estudis pertinents suggereixen que molts tipus de cèl·lules malignes proliferants són més sensibles al bloqueig de l'activitat del proteasoma que les

cèl·lules no canceroses [393,394]. Per exemple, com a mínim es requereix una concentració 340 vegades superior de PSI (N-carbobenzoxy-$_L$-isoleucyl-$_L$-γ-*t*-butyl-*t*-glutamyl-*t*-alanyl-*t*-leucinal) per induir apoptosi en cèl·lules quiescents en comparació a les cèl·lules endotelials proliferants [395].

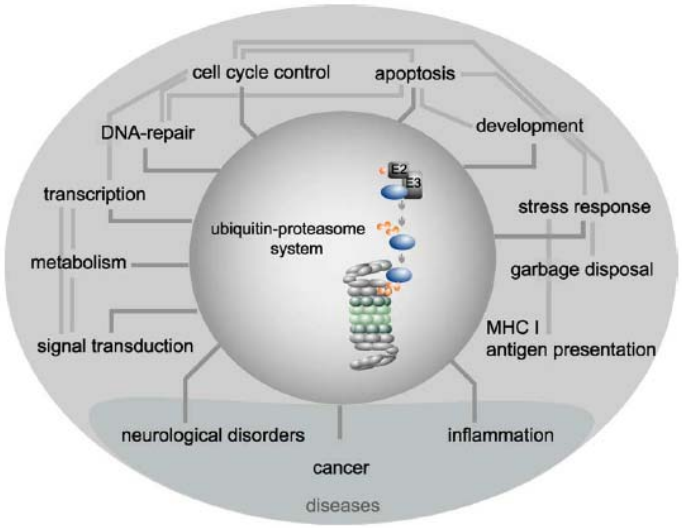


Figura 37: Funcions cel·lulars del sistema ubiquitina-proteasoma. La degradació proteica regulada pel proteasoma juga un paper essencial en múltiples vies cel·lulars, per tant, qualsevol fallida en l'activitat catalítica d'aquest pot resultar en greus conseqüències per la viabilitat cel·lular. *(Wolf, DH and Hilt, W. Biochimica et Biophysica Acta, 2004. 1695:19-31)*[378].

Donat que el proteasoma pot jugar un paper fonamental en la progressió de les cèl·lules tumorals, el disseny d'inhibidors específics de la seva activitat com a eina antitumoral ha estat objecte d'intensos estudis.

Els inhibidors del proteasoma poden ser naturals o sintètics i existeixen en total cinc classes d'inhibidors: els pèptids aldehids, pèptids vinil-sulfones, pèptids boronats, pèptids epoxicetones i β-lactones. Tanmateix, en la actualitat, només un compost ha assolit totes les fases clíniques per les seves propietats antineoplàsiques (bortezomib/PS-341) [396] i ja s'administra en pacients amb mieloma múltiple refractari (Taula3).

Agents	Origin	Phase	Comments	References
Aclacinomycin A	Natural	Preclinical	Laboratory use	87
Benzamide (CVT-634)	Synthetic	Preclinical	Laboratory use	88,89
Bortezomib (PS-341)	Synthetic	Clinical, for lymphoma	Approved by the FDA in 2003 for the treatment of relapsed and refractory multiple myeloma	97
Calpain inhibitors I and II	Synthetic	Preclinical	Laboratory use	90,91
Eponemycin	Natural	Preclinical	Laboratory use	92
Epoxomycin	Natural	Preclinical	Laboratory use	93
Lactacystin	Natural metabolite of *Streptomyces*	Preclinical	Specific, but weak, inhibitor that blocks proteasome activity by targeting the catalytic β-subunit; also inhibits cathepsin A; no effect on serine or cysteine proteases	86
MG132	Synthetic	Preclinical	Targets calpains and cathepsins	95–97
NLVS	Synthetic	Preclinical	Vinyl sulphonate tripeptides that also inhibit cathepsins	94
MLN-519 (a lactacystin derivative)	Synthetic	Clinical, Phase I	Safety and tolerability recently evaluated in a placebo-controlled Phase I study in 39 healthy volunteers	98
Ritonavir	Synthetic	FDA-approved for HIV	HIV1 protease inhibitor that also selectively inhibits the proteasome	99

Taula 3: Llistat d'inhibidors del proteasoma. La principal diana d'aquests compostos és l'activitat quimiotríptica [397]. La llista està en continua expansió i nous compostos amb major eficàcia es van desenvolupant al llarg dels anys. FDA=Food and Drug Administration. *(Adams, J. Nature Reviews, 2004. 4:349-60)*[360].

Tot i l'èxit del descobriment del bortezomib com a eina antitumoral s'han observat efectes secundaris a la seva administració en pacients amb mieloma múltiple avançat com són episodis lleus de diarrea, vòmits, astènia i d'altres més greus com trombocitopènia, neutropènia o neuropatia [360,396]. És per aquest motiu que nous compostos es desenvolupen de forma preclínica. Entre aquests nous compostos destaquen la salinosporamida A (NPI-0052) i el carfilzomib (PR-171).

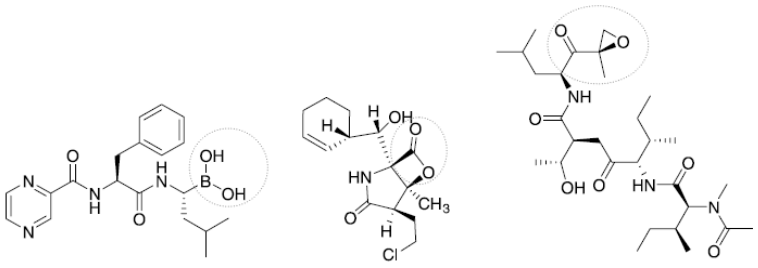


Figura 38: D'esquerra a dreta, estructura química de PS-341 (bortezomib), NPI-0052 (salinosporamida A) i PR-171 (carfilzomib). (*Joazeiro, CAP; et al. Cancer Research, 2006. 66:7840-42)*[398].

La salinosporamida A, a diferència del bortezomib, s'administra oralment. La salinosporamida A és un producte natural derivatiu de la lactacistina que de forma irreversible inhibeix les tres activitats catalítiques del proteasoma essent, però, la quimiotríptica la més rellevant [399,400]. L'altre compost anomenat carfilzomib o PR-171 és un inhibidor sintètic derivat de l'epoxomicina, l'inhibidor del proteasoma més selectiu conegut actualment [398] i inhibeix de forma irreversible principalment l'activitat quimiotríptica del proteasoma [401-403].

De forma interessant, ambdós inhibidors NPI-0052 i PR-171 són capaços d'induir apoptosi en cèl·lules de mieloma múltiple resistents al bortezomib.

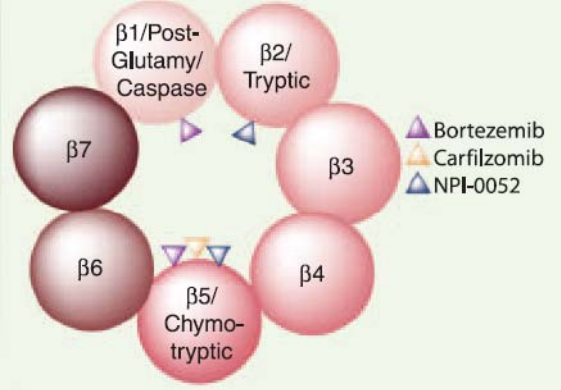


Figura 39: Activitat inhibitòria dels inhibidors del proteasoma en relació a les subunitats catalítiques β1, β2 i β5. El bortezomib inhibeix reversiblement l'activitat quimiotríptica de β5 i amb menys afinitat les altres activitats. NPI-0052 actua de forma similar al bortezomib però de forma irreversible. Finalment, el carfilzomib actua principalment inhibint de forma irreversible l'activitat quimiotríptica de la subunitat β5. *(Orlowski, RZ and Kuhn, DJ. Clinical Cancer Research, 2008. 14:1649-57)*[404].

7.2.2 Acció antitumoral dels inhibidors del proteasoma

Els mecanismes exactes per l'alta susceptibilitat a la inhibició del proteasoma per part de les cèl·lules malignes en comparació a les cèl·lules normals encara no ha estat del tot determinada. Tanmateix sí que s'han caracteritzat alguns dels mecanismes efectors dels inhibidors del proteasoma. Un exemple és el seu efecte en el cicle cel·lular. Sembla ser que els inhibidors del proteasoma són capaços d'aturar el cicle cel·lular en fase G1/S o G2/M [405,406] i que aquest efecte vindria donat per un

progressiu acumulament de proteïnes repressores del cicle cel·lular com WAF1 (o p21, CIP1) i KIP1 (o p27) [407] (Fig40).

D'altra banda, la inhibició del proteasoma també pot donar lloc a l'acumulament de la proteïna p53. Aquest acumulament es podria traduir en un arrest del cicle cel·lular o en una resposta apoptòtica. L'ús dels inhibidors del proteasoma, doncs, podria ser una bona aproximació per tractar carcinomes que presenten sobreexpressió de MDM2 (i que per tant inactiven p53 sense necessitat de presentar mutacions per p53) [408,409].

Finalment un dels mecanismes més ben caracteritzats d'acció dels inhibidors del proteasoma és el bloqueig de la via NF kappa B a través de l'estabilització del repressor IkBα. D'aquesta manera s'aturarà la transcripció de gens implicats en proliferació i supervivència (Fig40).

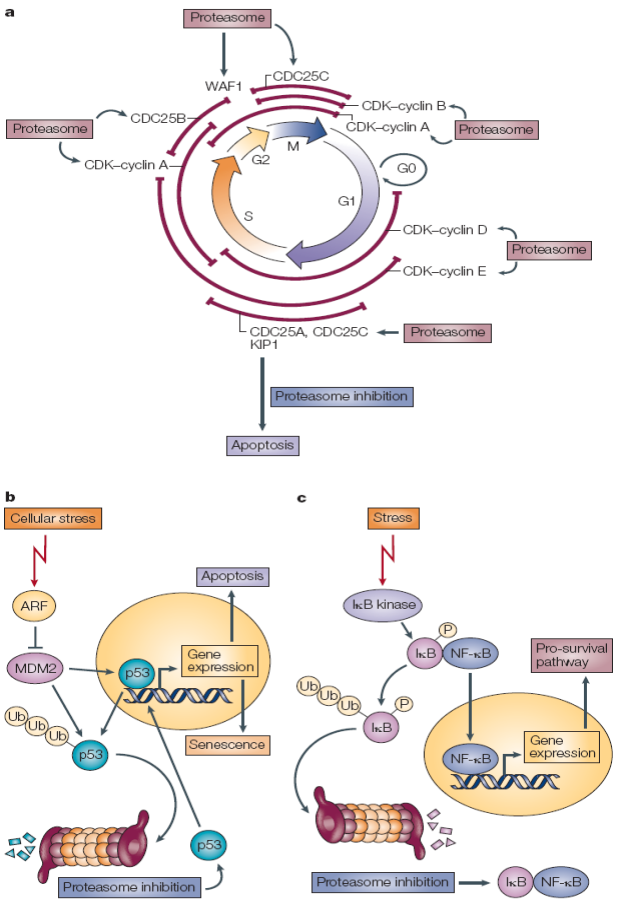


Figura 40: Alguns dels mecanismes d'acció atribuïts als inhibidors del proteasoma. **A**, els inhibidors del proteasoma poden actuar via el bloqueig de la progressió del cicle cel·lular en les fases G1/S i G2/M a través de l'acumulació de proteïnes inhibidores dels complexes ciclina-cdk com són p21 (WAF1) i p27 (KIP1). **B**, la inhibició del proteasoma pot resultar en l'acumulació de p53 a citoplasma i nucli. p53 pot activar la transcripció de gens implicats en l'apoptosi o en l'aturada del cicle cel·lular. **C**, els inhibidors del proteasoma poden causar una aturada en la via de senyalització per NF kappa B a través de l'estabilització en citoplasma del repressor IkBα. *(Adams, J. Nature Reviews, 2004. 4:349-60)*[360].

D'altra banda, i de forma més recent, s'estan explorant noves vies per les quals els inhibidors del proteasoma podrien exercir els seus mecanismes d'acció. Alguns d'aquests mecanismes d'acció tenen a veure amb estrés a reticle endoplasmàtic [410-413] com també amb increments en l'expressió de proteïnes de la subfamília de Bcl-2 BH3-*only* com Bik [414,415] o Noxa [416-418] o increments en l'expressió de receptors de mort com el receptor de TRAIL DR5 [419,420].

També s'ha pogut comprovar que els inhibidors del proteasoma són capaços d'induir canvis en el potencial de membrana de la mitocondrial i també de generar espècies reactives d'oxigen (ROS, ***r***eactive ***o***xygen ***s***pecies) [413,421]. Els ROS són bioproductes que es generen de forma corrent com a conseqüència dels processos oxidatius que tenen lloc dins la cèl·lula. Tanmateix, encara no es coneix amb exactitud com la inhibició del proteasoma resulta en la producció de ROS i la desestabilització de la mitocondrial.

En aquest sentit s'han realitzat diverses aproximacions per caracteritzar el possible impacte de la producció de ROS en la viabilitat cel·lular a través de l'ús d'antioxidants com el Tiron, NAC (***N***-***a***cetyl***c***isteina), BHA (***B***utilat ***h***ydroxyanisol), àcid ascòrbic (Vitamina C), etc. No obstant, les dades obtingudes han resultat a vegades confuses donat que en funció del model a estudiar els inhibidors del proteasoma no són capaços de generar espècies reactives d'oxigen [422,423]o el compost antioxidant ha resultat ésser inapropiat.

Hipòtesi i Objectius

L'apoptosi és un fenomen que se'ns presenta freqüentment desregulat en els tumors. Aquest fet fa que molts tumors siguin resistents a les teràpies antitumorals convencionals i això ha propiciat la recerca de noves dianes moleculars per a desenvolupar nous compostos amb propietats anticancerígenes capaços de superar aquesta resistència. Per tot això la nostra hipòtesi és la següent: "Les alteracions en els mecanismes d'apoptosi en el càncer d'endometri juguen un paper cabdal en el desenvolupament i progressió d'aquest. El seu estudi ens permetrà identificar tumors d'agressivitat diversa".

Amb tot, l'objectiu general d'aquest treball es centra principalment en l'estudi dels efectes que diferents agents antitumorals exerceixen sobre les cèl·lules canceroses d'endometri. El treball realitzat intenta aplicar en l'àmbit del càncer d'endometri possibles estratègies terapèutiques que s'han testat en altres tipus de neoplàsies i avaluar els seus efectes en termes de senyalització i viabilitat cel·lular. Per assolir aquesta fita ens hem marcat els següents objectius:

1. Anàlisi dels efectes que els lligands de mort de la família del TNF, concretament TRAIL i Fas, exerceixen sobre les cèl·lules de carcinoma d'endometri en cultiu. Caracteritzar els mecanismes de senyalització cel·lular encarregats de modular aquesta resposta.

2. Caracterització dels efectes de l'inhibidor de proteïnes cinases Sorafenib (BAY43-009) sobre cultius de carcinoma d'endometri.

3. Estudi dels efectes dels inhibidors del proteasoma com a possible eina terapèutica en el càncer endometrioide.

Resultats

Objectiu 1

1. FLIP ES TROBA FREQÜENTMENT EXPRESSAT EN EL CARCINOMA D'ENDOMETRI I JUGA UN PAPER EN LA RESISTÈNCIA A L'APOPTOSI INDUÏDA PER TRAIL.

1.1 FLIP es troba freqüentment expressat en el càncer d'endometri i presenta correlació amb l'estadiatge.

1.2 Les línies cel·lulars de carcinoma d'endometri en cultiu són resistents a l'apoptosi induïda per TRAIL.

1.3 L'actinomicina D disminueix l'expressió de FLIP i sensibilitza les cèl·lules de càncer d'endometri a l'apoptosi induïda per TRAIL.

1.4 La sobreexpressió de $FLIP_L$ reverteix la mort induïda per TRAIL.

1.5 La inhibició de l'expressió endògena de FLIP bloqueja la resistència a la mort per TRAIL.

(Dolcet X, ***Llobet D****, Pallarés J, Comella JX, Matias-Guiu X. Flip is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. Laboratory Investigation. 85:885-94. 2005.)*

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FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis

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The FLICE-inhibitory protein (FLIP) plays a key role in the regulation of apoptosis triggered by death ligands. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in some types of tumor but not in others. To assess the possible role of FLIP in apoptosis resistance in endometrial carcinoma, we performed an immunohistochemical study on a tissue microarray composed of 95 endometrial carcinomas. We found positive signals in 43% of the cases, as well as a significant difference in FLIP expression between stage I and II tumors. Moreover, we observed that endometrial carcinoma cell lines Ishikawa and KLE did not undergo apoptosis after TRAIL treatment. Cotreatment of these cells with the inhibitor of transcription actinomycin D resulted in a dramatic decrease in cell viability and induced activation of caspase-8. These events coincided with downregulation of FLIP mRNA and protein. Inhibitors of caspase-8 or overexpression of FLIP completely blocked apoptosis induced by actinomycin D plus TRAIL cotreatment. More importantly, downregulation of endogenous FLIP expression by specific siRNAs sensitized endometrial carcinoma cells to TRAIL-induced apoptosis in the absence of actinomycin D. Taken together, our results suggest for the first time a critical role for FLIP in the regulation apoptosis triggered by TRAIL in endometrial carcinoma cells.
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Keywords: endometrial carcinoma; FLIP; TRAIL; tissue microarray, molecular pathology; apoptosis

Apoptosis is an important process that regulates homeostasis in multicellular tissues. Alterations in proteins that regulate apoptosis may result in impaired cell death and the development and progression of cancer.[1] The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the proapoptotic cytokines of the tumor necrosis factor (TNF) superfamily. Several lines of evidence have shown that TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells[2,3] indicating that it may become a promising anticancer agent.[4] TRAIL can bind four different receptors DR4/TRAIL-R1,[5,6] DR5/TRAIL-R2,[7,8] DcR1/TRAIL-R3,[9] and DcR2/TRAIL-R4.[10] DR4 and DR5 are functional receptors that transduce apoptosis after ligation with TRAIL. DcR1 and DcR2, known as decoy receptors, lack the intracellular domains required to induce apoptosis. Recently, it has been shown that osteoprotegerin (OPG) can also bind TRAIL with less affinity.[11]

Engagement of receptors DR4 or Dr5 results in the formation of a death-inducing signalling complex (DISC). The intracellular death domain (DD) of these receptors recruits Fas associated DD-containing protein (FADD), which in turn binds procaspase-8. After recruitment to the DISC, procaspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signalling.[12–14] Active caspase-8 activates the executioner caspase-3, which in turn cleaves the cellular substrates.

The FLICE-inhibitory protein (FLIP) was initially described as a homologue of viral v-FLIP, which is a key regulator of death-receptor signalling.[15,16] Two isoforms of FLIP are generated by alternative splicing: the long form, FLIP-L; and the short form, FLIP-S.[16] Both proteins share a high degree of homology with caspase-8, and contain two Death

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Effector Domains (DED). FLIP-L also contains a caspase-like domain but lacks proteolytic activity. Therefore, high levels of expression of FLIP result in inhibition of caspase-8 activation and apoptosis triggered by death receptors. Recent evidence indicates that FLIP is constitutively or highly expressed in some tumors such as prostate cancer,[17] Hodgkin's lymphoma,[18] gastric cancer,[19] bladder carcinoma,[20] and malignant mesothelial cell lines.[21]

Although TRAIL induces apoptosis in a wide variety of neoplastic cells, certain tumors are resistant to TRAIL killing. The potential use of TRAIL in cancer therapy increases interest in the understanding of the molecular mechanisms by which tumor cells are resistant to TRAIL.

In this article, we provide evidence for the first time that suggests that FLIP may be important in apoptosis resistance in endometrial carcinoma. First, we show that FLIP is frequently expressed in endometrial carcinoma and correlates with stage. Moreover, we show that Ishikawa and KLE cell lines are resistant to TRAIL-induced apoptosis. Inhibition of transcription by actinomycin D results in cell viability decrease and induction of apoptosis. The sensitization to TRAIL by actinomycin D correlated with a significant reduction on FLIP expression and forced expression of FLIP overcame TRAIL plus actinomycin D-induced apoptosis. Moreover, down-regulation of endogenous FLIP by siRNA was sufficient to sensitize endometrial carcinoma cells to TRAIL-induced apoptosis.

Materials and methods

Tissue Microarray Construction and Immunohistochemistry

A tissue microarray (TMA) was constructed from paraffin-embedded blocks of 95 endometrial carcinomas (EC), obtained from the Department of Pathology of Hospital Santa Creu i Sant Pau, Barcelona, Spain. The specimens were collected between 1996 and 2001. The tumors were classified by following the World Health Organization (WHO) criteria. They were surgically staged and graded according to the International Federation of Gynecology and Obstetrics (FIGO) staging and grading systems. The specimens included: 25 endometrioid carcinomas (EEC) grade I, 34 EEC grade II, 19 EEC grade III, 10 serous carcinomas (SC), 4 clear cell carcinomas (CC) and the epithelial components of three malignant mixed Müllerian tumors (MMMT). Totally about 65 cases were stage I, four were stage II, 14 were stage III, and one was stage IV. The study was approved by the local Ethical Committee.

A tissue array device (Beecher Instruments, MD, USA) was used. All ECs were histologically reviewed and representative tumor areas were marked in the corresponding paraffin blocks. Two selected cylinders (0.6 mm in largest diameter) from two different tumor areas were included for each case. Normal control tissues from the same specimens were also included. Thus, two different TMAs blocks were constructed; each of them contained 186 cylinders.

TMA blocks were sectioned at a thickness of 3 μm, dried for 16 h at 56°C. They were dewaxed in xylene, rehydrated through a graded ethanol series, and washed with phosphate-buffered saline. Antigen retrieval was achieved by heat treatment in a pressure-cooker for 3 min in 10 mM citrate buffer (pH 6.5). Endogenous peroxidase was blocked. A rabbit polyclonal antibody, against aminoacids 1–202 at the amino terminus of FLIP was used (H-202, Santa Cruz, Santa Cruz, CA, USA; dilution 1:10). After incubation, the reaction was visualized with the EnVision Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as substrate. Sections were counterstained with hematoxylin. Appropriate external and internal positive and negative controls were used. Antigen preservation was verified with vimentin and Ki-67 immunostaining.

Immunohistochemistry was evaluated by two pathologists who followed uniform pre-established criteria. Immunoreactivity was graded semiquantitatively by considering the percentage and intensity of the staining.

A histological score was obtained from each sample, which ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The score was obtained by applying the following formula Hscore $= 1X$ (% light staining) $+ 2X$ (% moderate staining) $+ 3X$ (% strong staining). Since each TMA included two different tumor cylinders from each case, immunohistochemical evaluation was performed after examining both samples. The apoptotic index was calculated by the percentage of apoptotic bodies in H&E-stained sections, after evaluating a minimal number of 1000 cells.

The reproducibility of TMA immunostaining was confirmed by comparison of TMA results with those obtained in sections from the corresponding paraffin blocks of 37 randomly selected cases. The overall concordance was 89.2%. The Kappa index of agreement between the two methods ranged from 0.68 to 0.83.

FLIP immunostaining was correlated with histological type and grade, stage, and the apoptotic index. The Fisher's exact test was used to assess association between categorical variables. The Wilcoxon rank sum and the Kruskal–Wallis tests were used to assess association between continuous and categorical variables. The Spearman rank correlation test was used to assess association between continuous and/or ordinal variables.

Reagents, Cell lines, Culture Conditions and Transfection

Recombinant TRIAL was obtained from Peprotech EC Ltd. (London, UK), 3-(4,5-dimethylthiazol-2-yl)-

2,5- diphenyl tetrazolium bromide assay (MTT), and actinomycin D and monoclonal antibody to Tubulin were from Sigma (St Louis, MO, USA). z-IETD-fmk was from Calbiochem (La Jolla, CA, USA). Monoclonal antibody to FLIP was purchased from Alexis Corp (Lausen, Switzerland). Antibodies to active caspase-3 and caspase-8 were obtained from Cell-Signal (Beverly, MA). siRNA to FLIP was from SantaCruz Biotechnology, Inc. (SantaCruz, CA, USA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were provided by Amersham-Pharmacia (Uppsala, Sweden).

The Ishikawa 3-H-12 (IK) and the KLE cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbeco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) suplemented with 10% Foetal Bovine Serum (Invitrogen Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma, St Louis, MO, USA), 1 mM sodium pyruvate (Sigma, St Louis, MO, USA), 2 mM L-glutamine (Sigma, St Louis, MO, USA) and 1% of penicillin/streptomicin (Sigma, St Louis, MO, USA) at 37°C with saturating humidity and 5% CO_2. Transfections were performed with Lipofectamine 2000 reagent (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturers instructions.

Western Blot Analysis

Endometrial carcinoma cell lines were washed with cold PBS and lysed with lysis buffer (2% SDS, 125 mM Tris-Hcl pH 6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad, Richmon, CA, USA). Equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Nonspecific binding was blocked by incubation with TBST (20 mM Tris-Hcl pH 7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of nonfat milk. Membranes were incubated with the following antibodies: anti-FLIP NF6, anti-caspase-8, anti-active-caspase-3 or anti-tubulin. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

Caspase-8 Activity

Cells were treated with the indicated conditions for 12 h and harvested with lysis buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% 3-[(3 cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 10% saccharose, 5 mM dithiothreitol. Cell lysates were centrifuged at 10 000 *g*, and protein extracts were quantified with the Protein assay Kit (Bio-Rad, Richmon, CA, USA). Equal amounts of protein were loaded on M96 multiwell plates and the colorimetric substrate *N*-acetyl-Ile-Glu-Thr-Asp*p*-nitroanilide (Ac-IETD-*p*NA) (Biomol, Plymouth, PA, USA) was added. Samples were incubated at 37°C, and the absorbance at 405 nm was read in a microplate reader. Results were expressed as a fold induction over basal.

Cell Viability Assays and Assessment of Apoptosis

Cell viability was determined by MTT assay. Endometrial carcinoma cells were plated on M96 well plates at 15×10^3 cells per well. After the indicated treatments, cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent, lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmon, CA, USA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

RNA Extraction and RT-PCR

Total RNA was extracted using the TRIZOL reagent (Invitrogen Inc., Carlsbad, CA, USA) as indicated by the manufacturer. Of total RNA, 1 μg was subjected to reverse transcription for 1 h at 42°C with the Multiscrive reverse transcription kit (Applied biosystems Inc., Foster City, CA, USA). cDNA was amplified by PCR using FLIP-specific set of primers (forward: 5′-GGACCTTGTGGTTGAGTTGG-3′, reverse: 5′-TCTTCACTGGTTCTTGTTGAGC-3′) for each gene plus the actin primers. Cycling conditions were as follows: 45 s at 94°C, 45 s at 55°C and 45 s at 72°C for 31 cycles. PCR products were resolved in an 8% acrylamide-bisacrylamide gel.

Results

FLIP is Frequently Expressed in Endometrial Carcinoma, and Shows a Correlation with Stage

In all, 10 cylinders were missed in either the construction of the tissue microarray, section or staining procedures. Six cases were excluded because of unreliable staining of vimentin and Ki-67. Thus, FLIP immunostaining was evaluated, in at least one cylinder, in 88 cases. Cytoplasmic staining was observed in 34 cases (43%), and the Hscore varied from 0 to 170 (mean = 33.98) (Figure 1). There was a statistically significant difference in FLIP expression between Stage I and II tumors ($P = 0.016$). The mean Hscore was 25.74 for stage I tumors, and 62.7 for stage II tumors. However, FLIP staining did not show statistically significant correlation with histological type ($P = 0.723$), grade ($P = 0.914$) or apoptotic index ($P = 0.177$) (Table 1).

Actinomycin D Downregulates FLIP and Sensitizes Endometrial Carcinoma Cells to TRAIL-Induced Apoptosis

TRAIL is able to induce apoptosis in a wide variety of tumor cells. However, several types of tumors

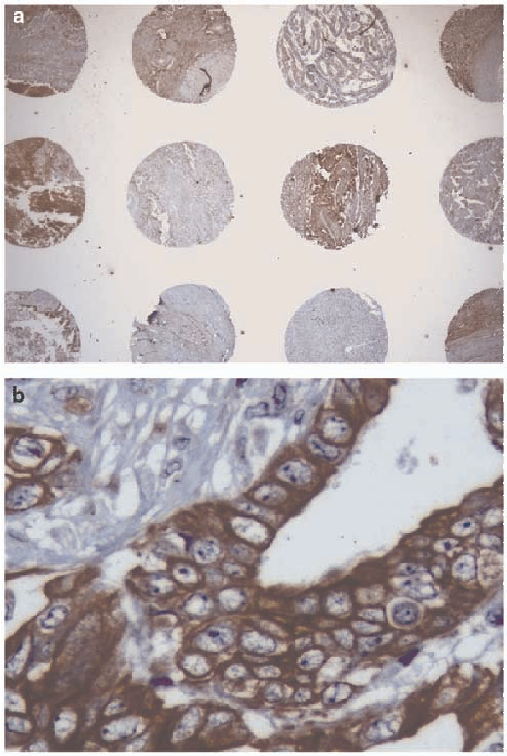

Figure 1 FLIP is frequently expressed in endometrial carcinoma. A tissue microarray constructed from 95 endometrial carcinoma was stained with an anti-FLIP antibody. A significant number of cylinders showed intense staining for FLIP (**a**) with a cytoplasmic pattern (**b**).

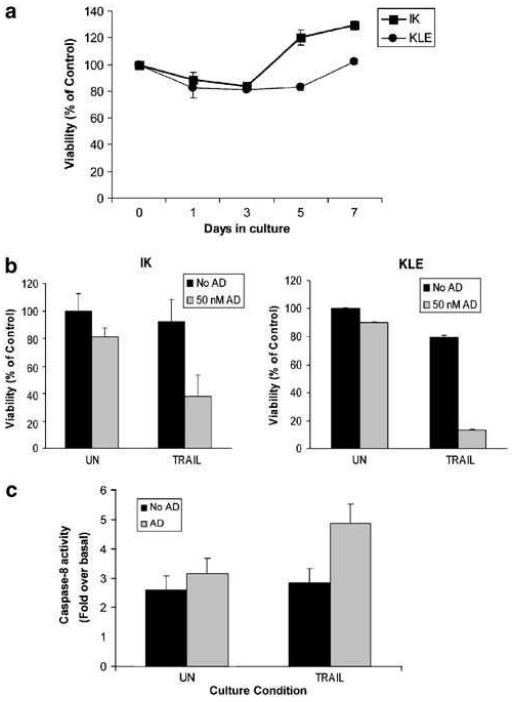

Figure 2 AD sensitizes IK and KLE to TRAIL-induced apoptosis. IK and KLE cell lines were incubated with 50 ng/ml of TRAIL and percentage viability was assessed by MTT viability assay after 1, 3, 5 or 7 days (**a**). IK and KLE cells were untreated (UN) or treated with 50 ng/ml of TRAIL with or without 50 nM actinomycin D (AD) for 24 h (IK) or 48 h (KLE). Cell viability was assessed by MTT and expressed as percentage of the control (**b**). IK cells were untreated (UN) or treated with 50 ng/ml of TRAIL with or without 50 nM actinomycin D (AD) for 12 h and caspase-8 activity was measured (**c**).

Table 1 Summary of the statistical analysis

Protein	*Histological type (EEC/NEEC)*	*Tumor grade*	*Pathological stage*	*Apoptotic index*
Flip	$P=0.723$	$P=0.914$	$P=0.016$	$P=0.177$

display TRAIL resistance. First, we explored the ability of endometrial carcinoma cell lines to undergo apoptosis after TRAIL treatment. IK and KLE cells were treated for several days with 50 ng/ml of TRAIL. Exposure to TRAIL even for long periods of time did not significantly reduce the viability of either cell line. Only a 5–10% reduction in cell viability was observed during the first 24–72 h in culture (Figure 2a).

A classical approach to sensitize resistant cells to TRAIL-triggered apoptosis is the inhibition of transcription or translation.[11,22] Such inhibition may block the expression of antiapoptotic molecules. Cotreatment of TRAIL with actinomycin D (an inhibitor of transcription) resulted in a significant reduction of cell viability of IK and KLE cells, together with an increase of caspase-8 activity (Figure 2b, c). Actinomycin D alone caused marginal changes on either cell viability or caspase-8 activity. Altogether these results indicate that actinomycin D sensitizes endometrial carcinoma cell lines to apoptotic cell death triggered by activation of death receptors. One of the key regulators of TRAIL apoptosis is FLIP. High levels of FLIP block caspase-8 binding to the DISC, uncoupling ligand binding from caspase activation, and ultimately inhibiting cell death. Therefore, regulation of FLIP levels may be a determinant in the response to TRAIL. Treatment of IK and KLE cells with actinomycin D plus TRAIL resulted in a dramatic downregulation of FLIP mRNA and protein (Figure 3) suggesting that high levels of FLIP may

be involved in TRAIL resistance to apoptosis in endometrial carcinoma.

Overexpression of FLIP-L Overcomes TRAIL-Induced Apoptosis

To ascertain whether high levels of FLIP rescue endometrial carcinoma cell lines from TRAIL plus actinomycin D-induced apoptosis, we transiently transfected IK cells with a plasmid coding for FLIP-L. After 24 h of transfection to allow FLIP-L expression, cells were treated with TRAIL or TRAIL plus actinomycin D and apoptotic nuclei were visualized by Hoechst staining. As shown in Figure 4, ectopic expression of FLIP-L resulted in an increase of viable cells in the culture and consequent reduction of nuclei displaying apoptotic morphology, suggesting that FLIP-L overcomes cell death triggered by TRAIL plus actinomycin D. These results indicate that expression of FLIP is sufficient to promote TRAIL resistance in endometrial carcinoma cells. To further investigate the involvement of caspase-8 in transducing TRAIL plus actinomycin D cell killing, we carried out a viability experiment with IK and KLE cells treated with the specific caspase-8 inhibitor z-IETD-fmk. Incubation with such an inhibitor completely prevented the cell death induced by TRAIL plus actinomycin D.

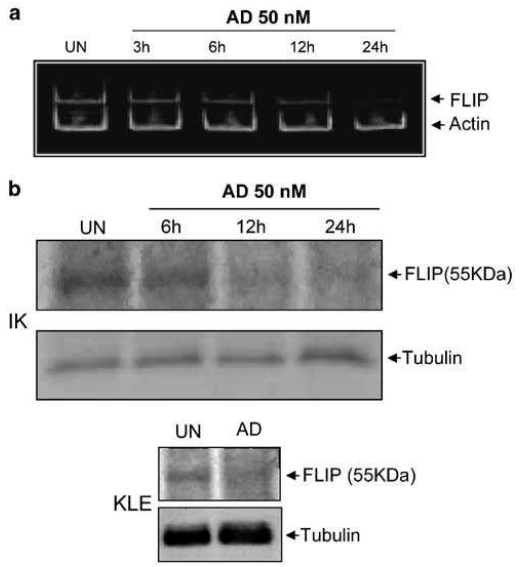

Figure 3 AD causes downregulation of FLIP mRNA and protein. IK cells were treated for the indicated times with 50 nM of actinomycin D and mRNA was subjected to RT-PCR using specific primers for FLIP and Actin (**a**). IK cells were treated with 50 nM AD or left untreated (UN) for the indicated times. Cell lysates were subjected to western blot with anti-FLIP antibody (upper panel) and Tubulin antibody (lower panel). KLE cells were treated with 50 nM AD or left untreated (UN) for 24 h and cell lysates were analyzed by Western blot with anti-FLIP (upper panel) or antitubulin (lower panel) antibodies (**b**).

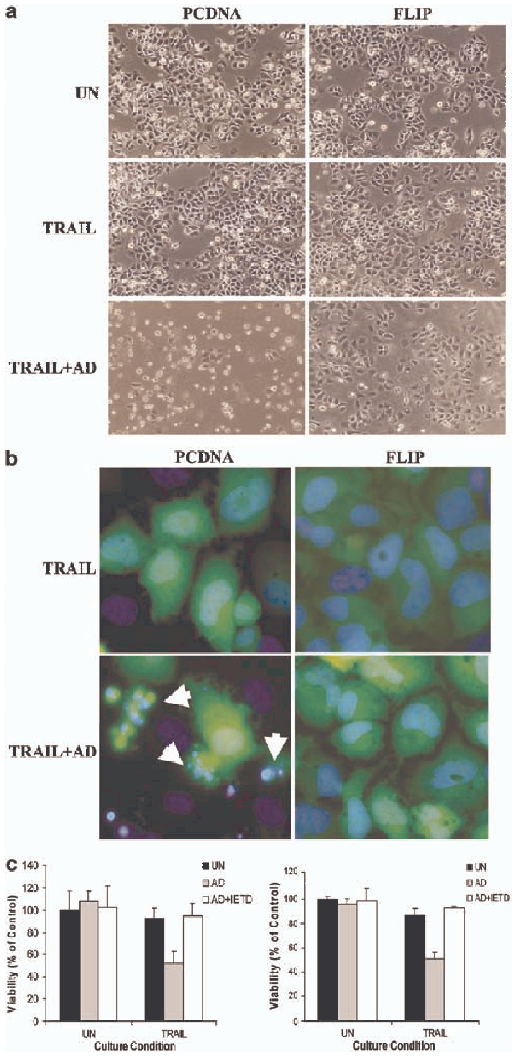

Figure 4 Caspase-8 inhibitors and overexpression of FLIP overcomes TRAIL induced apoptosis. IK were transiently cotransfected with the yellow fluorescent protein plus FLIP cDNA (FLIP) or the empty vector (PCDNA3). After 24 h cells were left untreated (UN), treated with 50 ng/ml of (TRAIL) or TRAIL plus actinomycin D (TRAIL + AD) and incubated for additional 24 h. Representative fields of IK cells transfected with PCDNA3 or FLIP, and incubated for additional 24 h with TRAIL alone or TRAIL plus AD and visualized under contrast phase (**a**) or stained with Hoechst after 24 h of treatment (**b**). IK and KLE cells were incubated with Actinomycin D with or without 100 μM z-IETD-fmk and left untreated or incubated with 50 ng/ml of TRAIL. Cell viability was assessed by MTT assay .

Downregulation of Endogenous FLIP Expression Restores TRAIL-Induced Apoptosis

To confirm the role of endogenous FLIP in the prevention of TRAIL-triggered apoptosis, IK and KLE cells were transfected with FLIP siRNA and incubated for 24 h to allow the specific inhibition of FLIP expression without effects on a homologous protein such as caspase-8 (Figure 5a). Afterwards, we examined the siRNA-mediated downregulation of endogenous FLIP on TRAIL incubation. IK and KLE cells were transfected and, after 24 h, exposed to TRAIL, and incubated 24 additional hours. Apoptosis was assessed by quantification of apoptotic nuclei visualized by Hoechst staining. TRAIL incubation induced a marked increase of apoptotic figures in IK and KLE cultures transfected with FLIP siRNA, when compared to the control siRNA transfected cells (Figurea 5b, c). Furthermore, IK and KLE cells transfected with FLIP siRNA displayed an increased cleavage of caspase-8 and caspase-3 activation, indicating that the intracellular levels of FLIP inhibited caspase activation and thus, cell death (Figure 6). FLIP siRNA transfected KLE cells displayed an increase of apoptotic cell death and caspase activation even in the absence of TRAIL. These results provide clear evidence that the expression of FLIP in these cell lines is sufficient to cause resistance to TRAIL-induced apoptosis.

Discussion

Apoptosis is a key process in the regulation of cellular homeostasis. Alterations of apoptosis plays an important role in development and progression of cancer. Deregulation of proteins involved in apoptosis control may result in cell populations that escape of apoptotic cell death.[1] The lack of response to such stimuli can produce a survival advantage, and the expansion of a population of neoplastic cells. Moreover, defects in the apoptotic pathway can make cancer cells resistant to therapy.

Several proapoptotic members of the TNF superfamily trigger the 'extrinsic' apoptotic pathway.[23] Among these members, TRAIL has raised interest as a potential anti-cancer agent, because of its ability to trigger apoptosis in cancer cells without affecting normal cells.[2,3] However, an increasing number of tumor types show mechanisms of TRAIL resistance. In these tumors, cell death is blocked as a result of molecular alterations of proteins regulating extrinsic apoptotic pathway. In the present study, we demonstrated that FLIP is frequently expressed in endometrial carcinoma cells and suggested, for the first time, that FLIP may play a significant role in resistance of endometrial cancer cells to TRAIL-induced apoptosis.

The results of the present study showed that FLIP expression is frequent in endometrial carcinomas. Interestingly, FLIP expression did not correlate with histological type or grade, suggesting that it may be involved in the process of apoptosis-resistance of both type I (endometrioid) and type II (nonendometrioid) endometrial carcinomas. These two types of endometrial carcinomas do not show only different morphological features, but exhibit significant clinical and molecular differences.[24]

The possible effects of FLIP and TRAIL in the development of apoptotic cell death in endometrial carcinoma cells have not been addressed so far. We first assessed the viability of two endometrial carcinoma cell lines after exposition to TRAIL. Treatment of both IK and KLE with TRAIL failed to cause a significant reduction on viability. Only a marginal reduction of 5–10% was observed on the first 24–72 h in culture. However, incubation of these cell lines with actinomycin D (an inhibitor of transcription) plus TRAIL resulted in a marked decrease of cell viability caused by apoptotic death. Treatment with inhibitors of transcription or translation, such as actinomycin D or cycloheximide, has been shown to be effective in sensitization of different tumor cells to members of the TNF superfamily. These inhibitors may cause the reduction of the expression of some intracellular proteins that inhibit different steps of the apoptotic cascade. The results obtained with actinomycin D treatment indicate that these endometrial carcinoma cell lines may express molecules that are able to block TRAIL-induced apoptosis. Consistently, the cell death caused by actinomycin treatment is accompanied by an increase of caspase-8. These results enabled us to study intracellular proteins involved in the regulation of apoptotic signalling by TRAIL. One of such regulators is FLIP, which competes with caspase-8 for the binding to FADD. Therefore, the expression of FLIP could be important as a mechanism to suppress apoptosis.

An RT-PCR and Western blot analysis on a time course exposition to actinomycin D showed a marked decrease in the levels of both FLIP mRNA and protein suggesting that FLIP could be one of the mechanisms of this resistance. These results are concordant with previous studies that found decreased levels of FLIP after treatment with inhibitors of transcription.[25,26] To investigate whether the decreased FLIP expression was responsible for TRAIL resistance, we transfected endometrial carcinoma cells with a plasmid coding for FLIP. The transfection assays resulted in a prevention of TRAIL-induced apoptosis, which indicated that actinomycin D regulates the activation of the extrinsic apoptotic pathway. Furthermore, the incubation of the endometrial carcinoma cells with an inhibitor of caspase-8 (z-IETD-fmk) resulted in a complete inhibition of apoptosis. Altogether, these

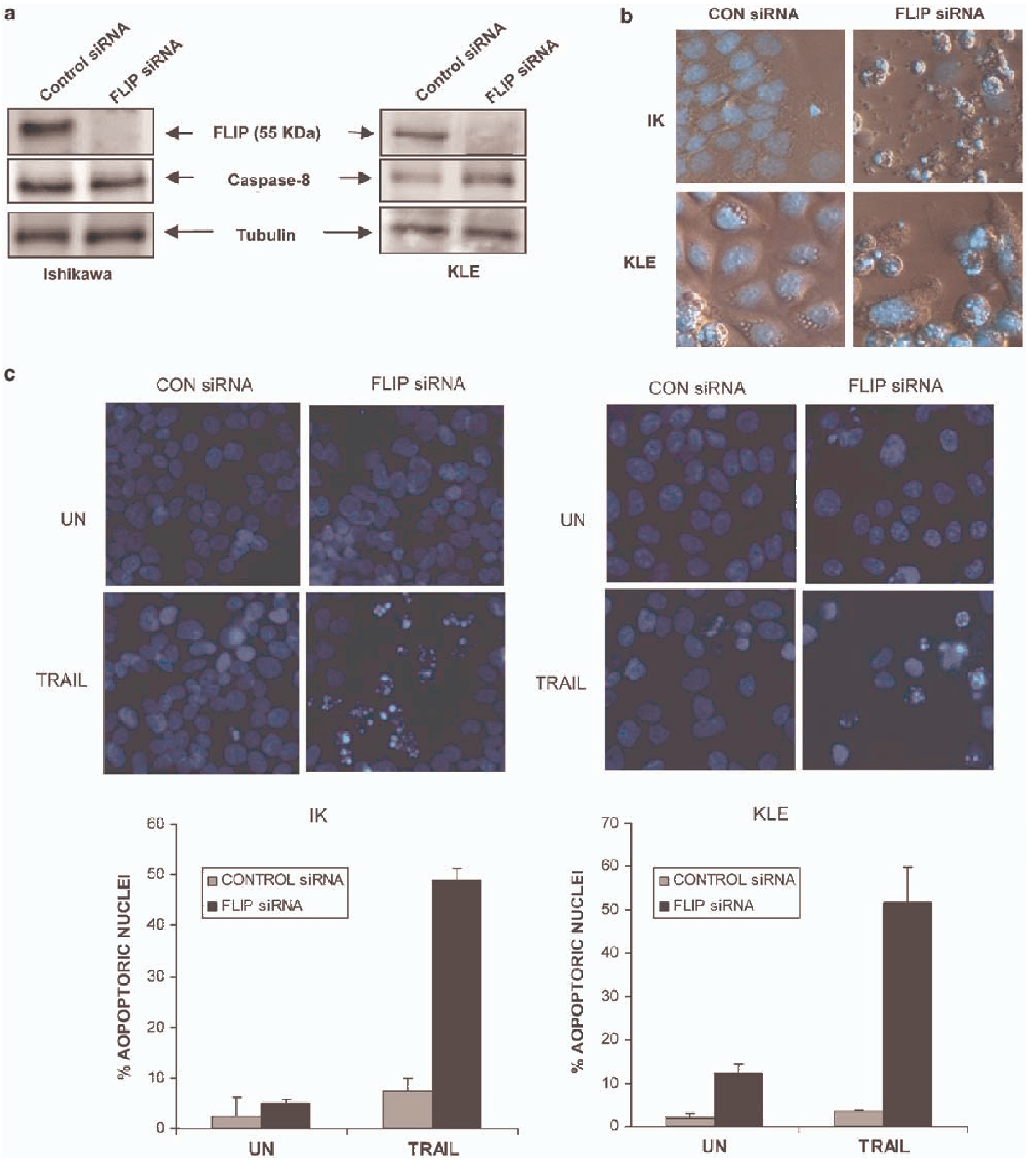

Figure 5 Downregulation of FLIP by specific siRNA sensitizes IK and KLE cells to TRAIL-induced apoptosis. IK and KLE cells were transfected with either FLIP siRNA or control siRNA. After 24 h protein cell lysates were subjected to Western blot with antibodies against caspase-8, FLIP or tubulin as a control of specificity and protein loading. FLIP siRNA reduces the expression of endogenous FLIP (**a**). Micrographs showing Hoechst staining and Hoffman optical image of FLIP siRNA transfected IK and KLE after 24 h of treatment with 50 ng/ml of TRAIL (**b**). IK and KLE cells were transfected with either control or FLIP siRNA and treated or not with 50 ng/ml of TRAIL. Micrographs show representative fields IK and KLE cells stained with Hoechst dye to evidence the apoptotic nuclear morphology. The graphs represent a quantification of nuclei displaying nuclear apoptotic morphology in IK and KLE cells (**c**).

data provide evidence that FLIP is, at least, one of the proteins that are involved in apoptosis resistance to TRAIL.

Direct evidence of the role of endogenous FLIP in TRAIL apoptosis resistance on endometrial carcinoma cells is provided by treatment with specific

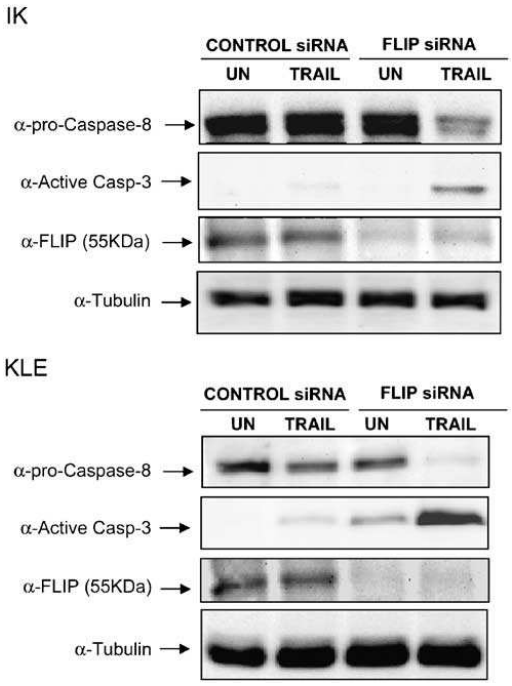

Figure 6 Downregulation of FLIP by specific siRNA activates caspase-8 and caspase-3. IK and KLE cells transfected with either control siRNA or FLIP siRNA, after 24 h cultures were treated with 50 ng/ml of TRAIL and incubated for further 24 h. Protein cell lysates were subjected to Western blot with antibodies against active caspase-3, procaspase-8, FLIP or tubulin. FLIP siRNA transfected cells show an increase of active caspase-3 and decrease of procaspase-8 levels.

siRNA targeting FLIP. Transfection of both IK and KLE cells with FLIP siRNA resulted in a marked decrease in cell viability after TRAIL exposition. This was accompanied by processing of both caspase-8 and caspase-3 suggesting activation of the extrinsic pathway. The fact that endogenous FLIP inhibition induced apoptosis indicates that FLIP is necessary and sufficient to cause resistance to TRAIL-induced apoptosis. KLE cells showed an increase of apoptotic nuclei as well as caspase-3 and caspase-8 processing after 24 h of transfection with FLIP siRNA, but without exogenous supplied TRAIL to the medium. This result suggests that KLE cells may produce an endogenous factor that may promote self-induced cell death in an autocrine manner.

Although several studies have demonstrated an anti-apoptotic role for FLIP by interfering caspase-8 recruitment to the DISC, some recent evidences suggest that the amount of FLIP that is present in the DISC may be responsible for either suppressing or inducing apoptosis.[27] Thus, FLIP may cause activation of both caspase-8 and caspase-10.[28,29] Our findings demonstrated that in endometrial carcinoma cell lines, the endogenous levels of expression of FLIP may inhibit caspase activation. The results also showed that downregulation of FLIP enables caspase cleavage and apoptosis.

There is evidence suggesting a possible role of FLIP in the resistance to apoptosis in cancer. FLIP is a human cellular homologue of viral FLICE-inhibitory proteins, which are expressed by γ-herpes viruses, and were shown to be capable of blocking CD95-mediated apoptosis through association with the receptor in the DISC.[16] In cells that are latently infected with human herpes virus 8, v-FLIP is expressed at low levels but its expression is increased in Kaposi sarcomas. Human melanomas and a murine B-cell lymphoma cell line have been shown to express high levels of FLIP, which interferes with apoptosis induction by death receptors. On the other hand, there is high constitutive expression of FLIP in Reed-Sternberg cells of Hodgkin's lymphomas; and some evidences suggest that it prevents FAS-induced apoptosis in these cells.[30,18] There are also evidences that FLIP may inhibit the extrinsic apoptotic pathway in carcinomas of the prostate,[17] the stomach,[19] and the urinary bladder,[20] and melanoma.[31] On the other hand, FLIP has also been shown to be expressed in ovarian cancer,[32] a gynecologic type of tumor very similar to endometrial carcinoma. Interestingly, in some types of tumor, such as in carcinomas of the urinary bladder, FLIP expression was shown to correlate with advanced stage.[20] Similarly, in the present series, FLIP expression also showed a statistically significant difference between stage I and II endometrial carcinomas, which may suggest that resistance to TRAIL-mediated apoptosis could be related to tumor progression.

Finally, it is worth mentioning that FLIP is an important target of the nuclear factor-Kappa B (NF-κB) signalling pathway.[33] NF-κB encompasses different members of a family of transcription factors, involved in the regulation of genes encoding cytokines, cytokine receptors, and cell adhesion molecules, that drive immune and inflammatory responses. However, NF-κB has been recently found related to carcinogenesis, by regulating genes involved in apoptosis, the cell cycle, differentiation, and cell migration. In a previous study, we demonstrated that NF-κB family members are frequently expressed in endometrial carcinomas.[34] Although assessment of FLIP expression was not the main goal of that study, we tried to correlate FLIP and NF-κB expression, but we did not find any statistical association between them. However, it is important to emphasize that increased expression of FLIP may result from activation of some signalling pathways, other than NF-κB.

In summary, we provide evidence that FLIP is expressed in endometrial carcinoma, and that it may

be responsible for resistance to TRAIL-induced apoptosis in this type of tumor.

Acknowledgements

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References

1 Kaufmann SH, Vaux DL. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. Oncogene 2003;22:7414–7430.
2 Ashkenazi A, Pai RC, Fong S, *et al.* Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–162.
3 Walczak H, Miller RE, Ariail K, *et al.* Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157–163.
4 Srivastava RK. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. Neoplasia 2001;3: 535–546.
5 Pan G, O'Rourke K, Chinnaiyan AM, *et al.* The receptor for the cytotoxic ligand TRAIL. Science 1997;276: 111–113.
6 MacFarlane M, Ahmad M, Srinivasula SM, *et al.* Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J Biol Chem 1997;272:25417–25420.
7 Walczak H, Degli-Esposti MA, Johnson RS, *et al.* TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997;16:5386–5397.
8 Sheridan JP, Marsters SA, Pitti RM, *et al.* Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997;277:818–821.
9 Pan G, Ni J, Wei YF, *et al.* An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 1997;277:815–818.
10 Pan G, Ni J, Yu G, *et al.* TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. FEBS Lett 1998;424:41–45.
11 LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003; 10:66–75.
12 Bodmer JL, Holler N, Reynard S, *et al.* TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol 2000;2:241–243.
13 Sprick MR, Weigand MA, Rieser E, *et al.* FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 2000;12:599–609.
14 Kischkel FC, Lawrence DA, Chuntharapai A, *et al.* Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 2000;12:611–620.
15 Thome M, Schneider P, Hofmann K, *et al.* Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 1997;386:517–521.
16 Irmler M, Thome M, Hahne M, *et al.* Inhibition of death receptor signals by cellular FLIP. Nature 1997; 388:190–195.
17 Zhang X, Jin TG, Yang H, *et al.* Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. Cancer Res 2004;64:7086–7091.
18 Dutton A, O'Neil JD, Milner AE, *et al.* Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. Proc Natl Acad Sci USA 2004;101: 6611–6616.
19 Lee SH, Kim HS, Kim SY, *et al.* Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. APMIS 2003;111:309–314.
20 Korkolopoulou P, Goudopoulou A, Voutsinas G, *et al.* c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. Urology 2004;63: 1198–1204.
21 Rippo MR, Moretti S, Vescovi S, *et al.* FLIP over-expression inhibits death receptor-induced apoptosis in malignant mesothelial cells. Oncogene 2004;23: 7753–7760.
22 Olsson A, Diaz T, Aguilar-Santelises M, *et al.* Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitoryprotein in B chronic lymphocytic leukemia by actinomycin D. Leukemia 2001;15: 1868–1877.
23 Muppidi JR, Tschopp J, Siegel RM. Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity 2004; 21:461–465.
24 Matias-Guiu X, Catasus L, Bussaglia E, *et al.* Molecular pathology of endometrial hyperplasia and carcinoma. Hum Pathol 2001;32:569–577.
25 Griffith TS, Chin WA, Jackson GC, *et al.* Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol 1998;161:2833–2840.
26 Okano H, Shiraki K, Inoue H, *et al.* Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Invest 2003;83:1033–1043.
27 Chang DW, Xing Z, Pan Y, *et al.* c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J 2002;21: 3704–3714.
28 Micheau O, Thome M, Schneider P, *et al.* The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem 2002; 277:45162–45171.
29 Boatright KM, Deis C, Denault JB, *et al.* Activation of caspases-8 and -10 by FLIP(L). Biochem J 2004;382: 651–657.
30 Thomas RK, Kallenborn A, Wickenhauser C, *et al.* Constitutive expression of c-FLIP in Hodgkin and Reed-Sternberg cells. Am J Pathol 2002;160:1521–1528.

31 Zeise E, Weichenthal M, Schwarz T, *et al*. Resistance of human melanoma cells against the death ligand TRAIL is reversed by ultraviolet-B radiation via downregulation of FLIP. J Invest Dermatol 2004;123:746–754.
32 Abedini MR, Qiu Q, Yan X, *et al*. Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells *in vitro*. Oncogene 2004;23:6997–7004.
33 Micheau O, Lens S, Gaide O, *et al*. NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol 2001;21:5299–5305.
34 Pallares J, Martinez-Guitarte JL, Dolcet X, *et al*. Abnormalities in the NF-kappaB family and related proteins in endometrial carcinoma. J Pathol 2004;204:569–577.

2. CK2 CONTROLA LA SENSIBILITAT A TRAIL I FAS A TRAVÉS DELS NIVELLS DE FLIP EN LES CÈL·LULES DE CARCINOMA D'ENDOMETRI.

2.1 CK2 regula l'apoptosi induïda per TRAIL i Fas en cèl·lules de carcinoma d'endometri.

2.2 La inhibició de CK2 disminueix els nivells de FLIP.

2.3 La sobreexpressió de FLIP supera la sensibilització a través de CK2 de la mort per TRAIL i Fas.

2.4 El bloqueig de l'expressió de FADD o caspasa-8 restaura la resistència a TRAIL i Fas.

2.5 El DRB i l'apigenina sensibilitzen els cultius primaris de carcinoma d'endometri a l'apoptosi induïda per TRAIL.

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ORIGINAL ARTICLE

CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has emerged as a promising antineoplastic agent because of its ability to selectively kill tumoral cells. However, some cancer cells are resistant to TRAIL-induced apoptosis. We have previously demonstrated that in endometrial carcinoma cells such resistance is caused by elevated FLICE-inhibitory protein (FLIP) levels. The present study focuses on the mechanisms by which FLIP could be modulated to sensitize endometrial carcinoma cells to TRAIL-induced apoptosis. We find that inhibition of casein kinase (CK2) sensitizes endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis. CK2 inhibition correlates with a reduction of FLIP protein, suggesting that CK2 regulates resistance to TRAIL and Fas by controlling FLIP levels. FLIP downregulation correlates with a reduction of mRNA and is prevented by addition of the MG-132, suggesting that CK2 inhibition results in a proteasome-mediated degradation of FLIP. Consistently, forced expression of FLIP restores resistance to TRAIL and Fas. Moreover, knockdown of either FADD or caspase-8 abrogates apoptosis triggered by inhibition of CK2, indicating that CK2 sensitization requires formation of functional DISC. Finally, because of the possible role of both TRAIL and CK2 in cancer therapy, we demonstrate that CK2 inhibition sensitizes primary endometrial carcinoma explants to TRAIL apoptosis. In conclusion, we demonstrate that CK2 regulates endometrial carcinoma cell sensitivity to TRAIL and Fas by regulating FLIP levels.
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Keywords: endometrial carcinoma; apoptosis; death receptor; casein kinase 2; FLIP

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Introduction

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas Ligand (FasL) belong to the pro-apoptotic cytokines of the tumor necrosis factor (TNF) superfamily. TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells (Ashkenazi *et al*., 1999; Walczak *et al*., 1999) indicating that it may become a promising anticancer agent (Srivastava, 2001; Takeda *et al*., 2007). However, some neoplastic cells are resistant to TRAIL and recent evidences show that both cytokines can even induce proliferation of normal or neoplastic cells. TRAIL can bind four different receptors DR4/TRAIL-R1 (MacFarlane *et al*., 1997; Pan *et al*., 1997b), DR5/TRAIL-R2 (Sheridan *et al*., 1997; Walczak *et al*., 1997), DcR1/TRAIL-R3 (Pan *et al*., 1997a) and DcR2/TRAIL-R4 (Pan *et al*., 1998). DR4 and DR5 are functional receptors that induce apoptosis upon ligation with TRAIL. DcR1 and DcR2, known as decoy receptors, lack the intracellular domains required to induce apoptosis (LeBlanc and Ashkenazi, 2003).

Both FasL and TRAIL trigger similar intracellular signaling pathways. Engagement of Fas or TRAIL receptors leads to the formation of a death-inducing signaling complex (DISC). The intracellular death domain (DD) of these receptors recruits Fas Associated DD-containing protein (FADD) which in turn binds procaspase-8. After recruitment to the DISC, procaspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signaling (Bodmer *et al*., 2000; Kischkel *et al*., 2000; Sprick *et al*., 2000). One of the critical regulators of apoptosis triggered by FasL and TRAIL is the FLICE-inhibitory protein (FLIP) (Thome *et al*., 1997; Irmler *et al*., 2000). High levels of FLIP are found in many tumoral tissues including endometrial carcinoma. We have recently demonstrated that FLIP plays a key role in the regulation of sensitivity of endometrial carcinoma cells (ECCs) to TRAIL-induced apoptosis. In this previous work, we show that inhibition of FLIP expression is enough to sensitize endometrial cancer cells to TRAIL-induced apoptosis (Dolcet *et al*., 2005). FLIP shares a high degree of homology with caspase-8, and contain two death effector domains (DED) and a defective caspase-like

domain that lacks proteolytic activity. Thus, high levels of FLIP compete with caspase-8 and displace its binding to FADD, which results in inhibition of apoptosis.

Because of the importance of FLIP in sensitization of ECC to TRAIL-induced apoptosis, we focused in the search for possible mechanisms by which FLIP expression could be modulated. Increasing evidences in the literature suggest that the protein casein kinase (CK2) may play an important role in the regulation of TRAIL-induced apoptosis in many cell types. CK2 is a ubiquitous, highly conserved tetrameric serine threonine/kinase integrated by two regulatory subunits called CK2β and two catalytic subunits which can be two of either the subunits CK2α or CK2α′ (Padmanabha *et al*., 1990; Kikkawa *et al*., 1992; Allende and Allende, 1995). It is well established that increased CK2 activity is associated with cell growth and proliferation and many types of tumors display aberrant or increased CK2 activity (Tawfic *et al*., 2001; Ahmed *et al*., 2002; Litchfield, 2003). Importantly, transgenic mice expressing CK2α in lymphocytes or mammary gland display an increased incidence of lymphomas and breast carcinomas (Seldin and Leder, 1995; Landesman-Bollag *et al*., 2001). Recent evidences point to CK2 as an important regulator of apoptosis by both intrinsic/mitochondrial and extrinsic/death receptor apoptotic pathways. Inhibition of CK2 results in sensitization of resistant prostate (Wang *et al*., 2005a, b, 2006), esophageal (Shin *et al*., 2005), colon (Ravi and Bedi, 2002; Izeradjene *et al*., 2005) and rhabdomyosarcoma cancer cells (Izeradjene *et al*., 2004) to apoptosis induced by TRAIL treatment. Such evidences have pointed CK2 as a possible target for cancer therapy (Ahmad *et al*., 2005; Wang *et al*., 2005b).

Here, we provide evidence that CK2 inhibition sensitizes ECC to TRAIL and agonistic Fas antibodies (aFas) by regulating FLIP protein levels. Importantly, CK2 inhibition correlates with reduction of endogenous FLIP levels. Such reduction is caused by both transcriptional downregulation of FLIP expression and increased FLIP protein proteasomal degradation Consistently, in ECC treated with CK2 inhibitors or CK2β short hairpin RNAs (shRNAs), overexpression of FLIP completely abolishes caspase activation and restores resistance to both ligands. Accordingly, downregulation of FADD or caspase-8 by specific shRNA also blocks apoptosis triggered by TRAIL or aFas. Finally, we show that inhibition of CK2 sensitizes primary endometrial carcinoma explants to TRAIL-induced apoptosis, suggesting that CK2 might be an important target for cancer therapy.

Results

CK2 regulates TRAIL- and Fas-induced apoptosis in endometrial carcinoma cells

First, we explored the viability of ECC after TRAIL or aFas treatment by an 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT) cytotoxicity assay. The Ishikawa 3-H-12 cell line (IK) cell line displayed no reduction of viability after 24 or 48 h treatment with doses up to 100 ng ml^{-1} of either aFas or TRAIL. To ascertain whether CK2 could regulate cytotoxicity induced by such death ligands, we treated IK with three pharmacological inhibitors of CK2, namely 5,6-dichloro-ribifuranosylbenzimidazole (DRB), apigenin and emodin in presence or absence of increasing doses of aFas or TRAIL. Both aFas and TRAIL together with any of all three CK2 inhibitors significantly reduced cell viability of IK after 24 or 48 h (Figure 1). In contrast, the RL-95 cell line showed a significant reduction of cell viability upon aFas or TRAIL stimulation after 24 h treatment which was further increased at 48 h (Figure 1). Despite the sensitivity of RL-95 cells, addition of CK2 inhibitors caused further decrease on viability induced by both ligands. These results suggest that CK2 regulates sensitivity to both TRAIL and aFas.

To ascertain whether the decrease in cell viability was specifically caused by apoptotic cell death, we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining and we assessed caspase processing and activation by western blot to initiator caspases-8, -9 and -2 and the effector caspase-3. Treatment of IK cells with DRB, apigenin or emodin plus either aFas or TRAIL caused a marked increase in the number of nuclei showing apoptotic morphology (Figure 2a). As we show in Figure 2b, treatment of IK cells with either TRAIL or aFas plus CK2 inhibitors resulted in processing of the initiator caspases-8, -9 and -2, and the effector caspase-3.

Next, we used lentiviral delivery of shRNA to achieve a more specific inhibition of CK2. To avoid problems associated with possible redundancy of CK2α and CK2α′ catalytic subunits, we decided to target the regulatory subunit CK2β for shRNA design. One of the shRNAs designed significantly reduced the expression of the CK2β subunits and was chosen for subsequent experiments (data not shown). IK cells were infected with lentiviruses carrying shRNA to CK2β. After 3–4 days to allow gene silencing, cells were treated with 50 ng ml^{-1} of TRAIL. Apoptotic cell death was also assessed by Hoechst staining and caspase activation. Knockdown of CK2β by itself caused a slightly increase in the number of apoptotic cells (10–12%), which was dramatically increased after addition of TRAIL (Figure 2c). In line with the results obtained with pharmacological CK2 inhibitors, IK cells in which CK2β was silenced, addition of TRAIL or aFas resulted in processing of the initiator caspase-8, -9 and -2, and the effector caspase-3 (Figure 2d). It is worth to mention that downregulation of CK2β caused a remarkable increase in the amount caspase-8 fragment p43/p41 and residual activation of caspases. This activation is compatible with the slight increase in the number of apoptotic cells observed by Hoechst staining. Taken together, these results strongly support a key role in regulation of sensitivity to apoptosis triggered by aFas and TRAIL in ECC.

Inhibition of CK2 depletes endogenous FLIP

Having demonstrated that CK2 is critical in the regulation of cell death induced by either TRAIL or

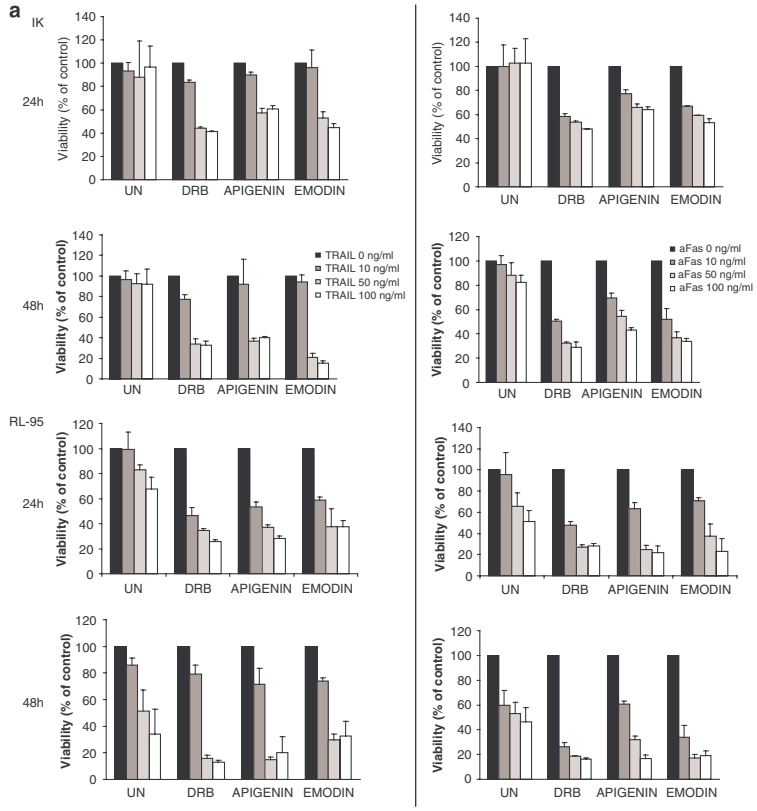

Figure 1 CK2 inhibitors plus tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or agonistic Fas antibodies (aFas) reduce cell viability of ECC. The ECC lines IK and RL-95 were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM or emodin 150 μM and then left untreated (UN) or treated with 10, 50 or 100 ng ml^{-1} of either TRAIL or agonistic antibody (aFas). Cell viability was measured 24 or 48 h later. Results are presented as percentage of survival of TRAIL or aFas conditions over each control condition.

aFas on ECC, we focused our interest in investigating the mechanism of such regulation. FLIP is a key regulator of both TRAIL- and FasL-induced of apoptosis in many cell types, and we have recently demonstrated that FLIP is necessary and sufficient to prevent TRAIL-induced apoptosis of ECC (Dolcet *et al*., 2005). In this scenario, we postulated that FLIP protein levels might be regulated by CK2. To test this hypothesis, we analysed FLIP expression under different experimental conditions. First, we pretreated IK cells with DRB for 6–8 h and then treated IK cells with TRAIL for the indicated times (Figure 3a). FLIP levels decreased after treatment with DRB alone, but addition of TRAIL further decreased FLIP levels as soon as 3 h after its addition. Such reduction was also observed when IK and RL-95 cells were treated with DRB plus either TRAIL (Figure 3b). It is worth to mention that in RL-95 cells, treatment with TRAIL without DRB, reduced the levels of FLIP, which correlated with the sensitivity of these cells to TRAIL apoptosis after 24 h treatment (Figure 3b). Similar results were obtained with IK treated with apigenin or DRB plus either TRAIL or aFas. Treatment of IK cells with DRB or apigenin reduced FLIP levels which were even increased after addition of either TRAIL or aFas (Figure 3c). In agreement with the data obtained with CK2 inhibitors, silencing of CK2β by shRNA reduced the levels of FLIP, which were further diminished after addition of TRAIL (Figure 3d). All these data support the hypothesis that CK2 sensitizes ECCs to TRAIL-induced apoptosis by diminishing FLIP protein levels.

Next, we investigated the mechanisms by which CK2 regulates FLIP levels. The levels of endogenous FLIP protein can be controlled transcriptionally but recent evidences also suggest that endogenous FLIP protein levels may be regulated by the ubiquitin proteasome system. To ascertain whether FLIP levels are transcriptionally regulated, we performed real-time PCR on mRNA extracted from IK cells treated with apigenin or CK2β shRNA. Both apigenin and shRNA cause a significant decrease of the

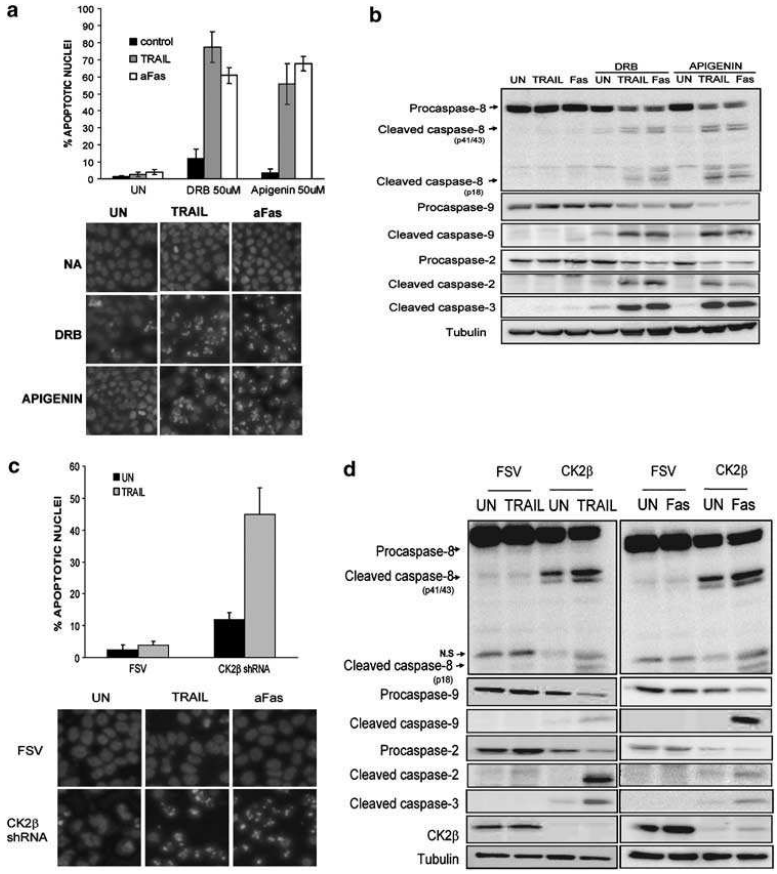

Figure 2 Inhibition of casein kinase (CK2) sensitize ECC to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas)-induced apoptosis. (**a**) Treatment of ECC with pharmacological inhibitors of CK2 plus either TRAIL or aFas increase the number of apoptotic nuclei. Top, IK cells were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Bottom, representative micrographs of IK cells treated as indicated. (**b**). Pharmacological inhibitors of CK2 plus 50 ng ml^{-1} of either TRAIL or aFas activate caspases-8, -9, -2 and -3. IK cells were pretreated for 2 h with DRB 50 μM, apigenin 50 μM and stimulated with 50 ng ml^{-1} TRAIL or aFas or left untreated (UN). After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) Treatment of ECC with short hairpin RNAs (shRNA) to CK2β plus either TRAIL or aFas increases the number of apoptotic nuclei. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} TRAIL. Top, nuclei displaying apoptotic morphology were visualized by Hoechst staining and counted 24 h later. Results are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Bottom, representative micrographs of IK cells treated as indicated. (**d**) shRNA to CK2β plus 50 ng ml^{-1} of either TRAIL or aFas activate caspases-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} of TRAIL or aFas. After 8 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA. NS (nonspecific bands).

mRNA levels compared to the untreated cells (Figure 3e). As a control of FLIP downregulation, we analysed FLIP mRNA from IK cells infected with FLIP shRNA.

FLIP levels are also regulated by ubiquitin–proteasome-mediated degradation (Kim *et al*., 2002; Poukkula *et al*., 2005; Chang *et al*., 2006). To determine whether proteasomal degradation was also involved in downregulation of FLIP protein, we treated IK cells with apigenin in presence or absence of the proteasome inhibitor MG-132. As we show in Figure 3f, addition of MG-132 completely inhibits the reduction in FLIP protein caused by either DRB or apigenin. These results suggest that inhibition of CK2 triggers FLIP degradation through the proteasome.

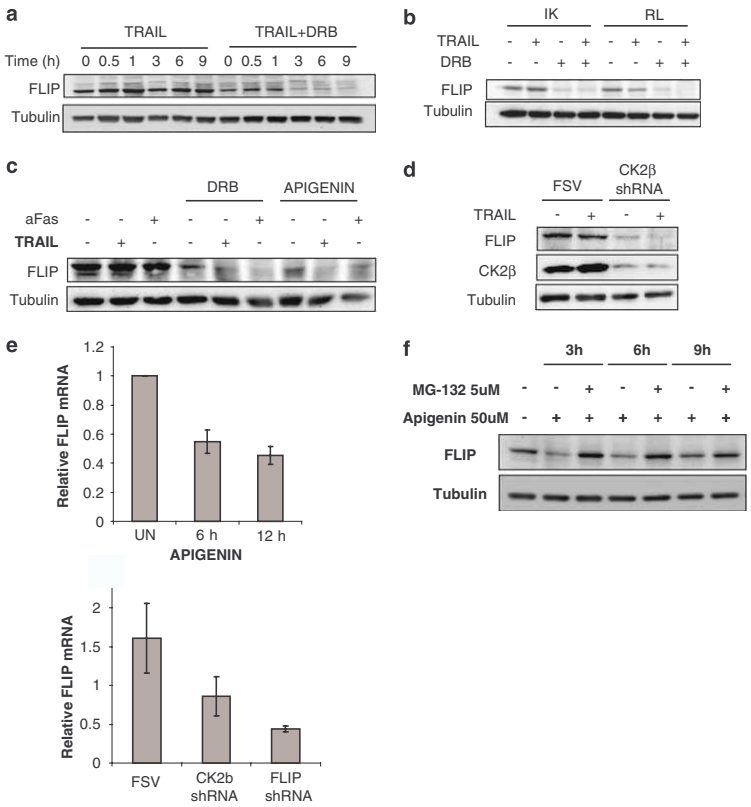

Figure 3 Inhibition of casein kinase (CK2) reduces FLICE-inhibitory protein (FLIP) levels. (**a**) FLIP protein levels are reduced by 5,6-dichloro-ribifuranosylbenzimidazole (DRB) and further decreased after addition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Ishikawa 3-H-12 cell line (IK) cells were pretreated for 3 h with DRB 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL. Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts. (**b**) IK and RL-95 cells were pretreated for 3 h with DRB 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL. Cells lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts. (**c**) IK cells were pretreated for 3 h with DRB 50 μM or apigenin 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL or 50 ng ml^{-1} agonistic Fas antibodies (aFas). Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts (**d**) IK cells were infected with lentiviruses carrying short hairpin RNAs (shRNA) to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} of TRAIL. After 8 h, cells were lysed and protein extracts were analysed by western blot with antibodies to FLIP. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA. (**e**) Apigenin and CK2β shRNA downregulate FLIP mRNA levels. IK cells were treated with apigenin for 6 or 12 h (top graph) or transfected with lentiviruses carrying CK2β or FLIP shRNAs. mRNA was extracted and subjected to reverse transcription. mRNA relative levels were analysed by real-time PCR. Results are expressed as relative mRNA levels compared to untreated (UN) cells (**f**) Proteasome inhibition block degradation of FLIP. IK cells were pretreated in presence or absence of 5 μM MG-132 for 30 min and then apigenin was added to the medium for the indicated periods of time (3, 6, 9 h). Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reproved with tubulin to ensure equal protein amounts.

Altogether, our results suggest that CK2 regulate both the transcription of FLIP mRNA and FLIP protein degradation through the proteasome.

Overexpression of FLIP overcomes CK2 sensitization to TRAIL and aFas

To ascertain whether high levels of FLIP blocked TRAIL- and aFas-induced apoptosis in conditions where CK2 was inhibited, we infected IK cells with a plasmid coding for a Flag-tagged mouse FLIP. After 3–4 days to allow FLIP expression, cells were treated with aFas or TRAIL in presence or absence of the CK2 inhibitors DRB or apigenin. Apoptotic nuclei were then visualized by Hoechst staining and caspase processing by western blotting. As shown in Figure 4a, overexpression of FLIP resulted in a dramatic reduction of apoptotic nuclei caused by CK2 inhibitors plus either TRAIL or

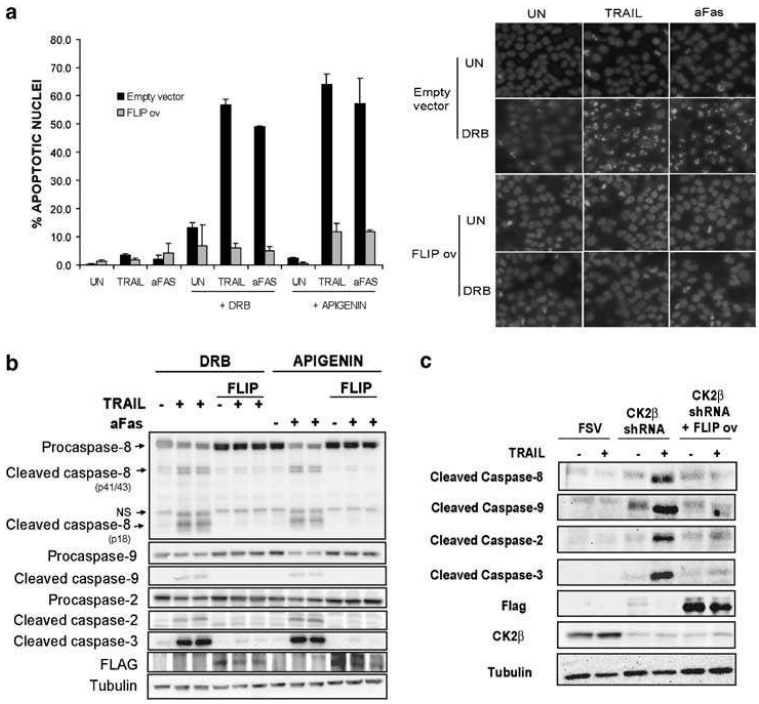

Figure 4 FLICE-inhibitory protein (FLIP) overexpression overrides sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas) caused by casein kinase (CK2) inhibition. (**a**) FLIP overexpression reduces the number of apoptotic nuclei caused by CK2 inhibitors plus TRAIL or aFas treatment. IK cells were infected with lentiviruses carrying a FLIP expression plasmid. After 3 days, cells were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Left: results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Right: representative micrographs of IK infected with the empty vector or plasmid encoding FLIP (FLIP ov) and cells treated as indicated. (**b**) FLIP overexpression inhibits activation of caspase-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying a FLIP expression plasmid. After 3 days, cells were pretreated for 2 h with DRB 50 μM, apigenin 50 μM and then stimulated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) FLIP overexpression suppresses caspase activation caused by downregulation of CK2β. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit, lentiviruses carrying FLIP overexpression plasmid (FLIP ov) or co-infected with both viruses. After 3 days, cells were treated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA.

aFas. Consistent with this observation, FLIP overexpression inhibited processing of the caspases-8, -9, -2 and 3 caused by TRAIL or aFas in presence of either DRB or apigenin (Figure 4b). Similar results were obtained using shRNA to silence CK2β (Figure 4c).

Downregulation of endogenous FADD or caspase-8 expression restores TRAIL and aFas resistance to apoptosis

The results obtained suggested that CK2 is modulating apoptosis triggered after engagement of TRAIL and Fas receptors at the DISC level, since apoptosis induced by TRAIL or Fas requires the formation of a functional DISC. The adaptor protein FADD directly binds TRAIL or Fas receptor through its DD. FADD in turn binds caspase-8/10 which initiate caspase processing of apoptosis. To determine whether CK2 requires formation of DISC to regulate the sensitivity of ECC to either TRAIL or aFas, we infected IK cells with viruses carrying shRNAs to the two main components of Fas and TRAIL receptor DISC, FADD and caspase-8. After 3 days of infection, cells were pretreated with DRB or apigenin for 2 h and then treated with TRAIL or aFas, and apoptotic morphology and caspase activation were analysed. Both FADD and caspase-8 shRNA markedly reduced the number of apoptotic nuclei observed in condition containing any of the two CK2 inhibitors or CK2β shRNA plus either TRAIL or aFas (Figures 5a and c). Accordingly, caspase-8 and FADD shRNA

reduced activation of caspase-8, 2 and -3. We would like to point out that caspase-8 shRNA, which strongly inhibited caspase-8 expression, completely blocked caspase-2 activation, indicating that the activation of the later caspase-2 requires caspase-8 (Figure 5b).

DRB and apigenin sensitize primary endometrial carcinoma explants to TRAIL-induced apoptosis

Among all the members of TNF superfamily, TRAIL has raised some interest as a potential anti-cancer agent, because of its ability to trigger apoptosis in cancer cells without affecting normal cells. However, an increasing number of tumor types show mechanisms of TRAIL resistance. Such resistance has increased the interest of combinatorial therapies (Takeda *et al*., 2007). Since CK2 seems to be critical in the regulation of TRAIL apoptosis, we decided to test whether pharmacological inhibition of CK2 could be effective to kill primary endometrial carcinoma explants treated with TRAIL. We cultured two different endometrial carcinoma explants obtained from biopsies of patients with endometrial carcinoma. We have previously characterized these explant cultures to be of epithelial origin by means of cytokeratin and β-catenin expression (Dolcet *et al*., 2006). Both DRB and apigenin lead to a dramatic increase of nuclei displaying apoptotic morphology (Figure 6a). Accordingly, DRB or apigenin plus TRAIL treatment activate caspases-8, -9, -2 and -3 (Figure 6b). In agreement with the results observed in ECC lines, both DRB and apigenin dramatically decrease the levels of endogenous FLIP protein in primary endometrial explants.

Discussion

In the present study, we have assessed the mechanisms by which FLIP could be modulated to sensitize ECCs to TRAIL-induced apoptosis. Among the regulators of death receptor-induced apoptosis, recent evidences point to CK2 as an important regulator of apoptosis. Here, we demonstrate that CK2 is an important determinant in sensitivity to TRAIL and aFas. Blockade of CK2 activity by different pharmacological inhibitors or lentiviral-mediated transduction of shRNA targeting the regulatory CK2β subunit, sensitizes resistant ECC to both TRAIL- and aFas-induced apoptosis. Such sensitization correlates with a reduction of endogenous FLIP, which is a key regulator of apoptosis triggered by death ligands in ECC. To determine the contribution of FLIP downregulation to CK2 sensitization, we overexpressed of FLIP in presence of shRNA or CK2 inhibitors. FLIP overexpression overcomes CK2 sensibilization to TRAIL and aFas. Importantly, downregulation of endogenous FADD or caspase-8 expression restores TRAIL and aFas resistance to apoptosis.

First, we assessed the effects of CK2 inhibitors in sensitization of ECC to TRAIL or aFas. All of three inhibitors of CK2 activity used in this study (DRB, apigenin or emodin), sensitized ECC to death ligand-induced apoptosis, suggesting that such sensitization

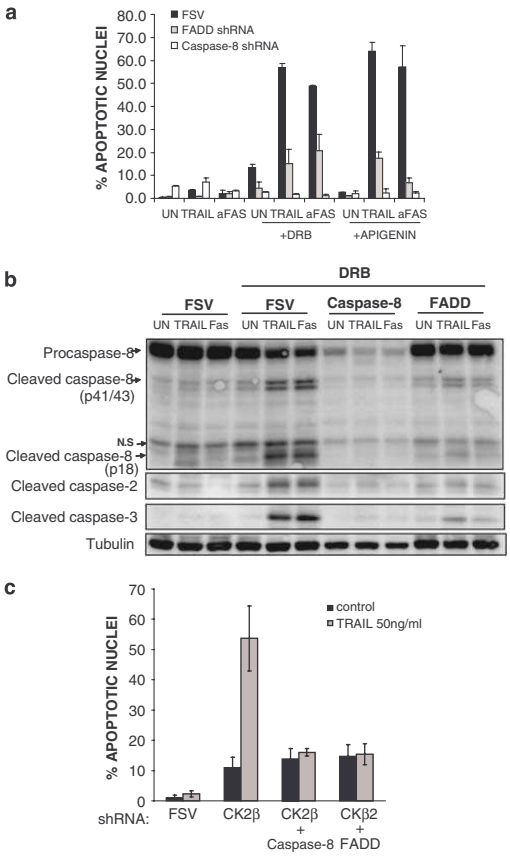

Figure 5 Silencing of Fas associated DD-containing protein (FADD) and caspase-8 restores tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas) resistance. (**a**) IK cells were infected with lentiviruses carrying FADD or caspase-8 short hairpin RNAs (shRNAs). After 3 days, cells were pretreated for 2 h with 5,6-dichloro-ribifurano-sylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei. (**b**) Downregulation of caspase-8 or FADD inhibit activation of caspase-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying shRNA to FADD or caspase-8. After 3 days, cells were pretreated for 2 h with DRB 50 μM, and then stimulated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) Caspase-8 and FADD shRNA reduces apoptotic nuclei caused down-regulation of CK2β. IK cells were co-infected with lentiviruses carrying the empty vector (FSV) or shRNA to CK2β subunit together with shRNA to either caspase-8 or FADD. After 3 days, cells were treated with 50 ng ml^{-1} of TRAIL. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei.

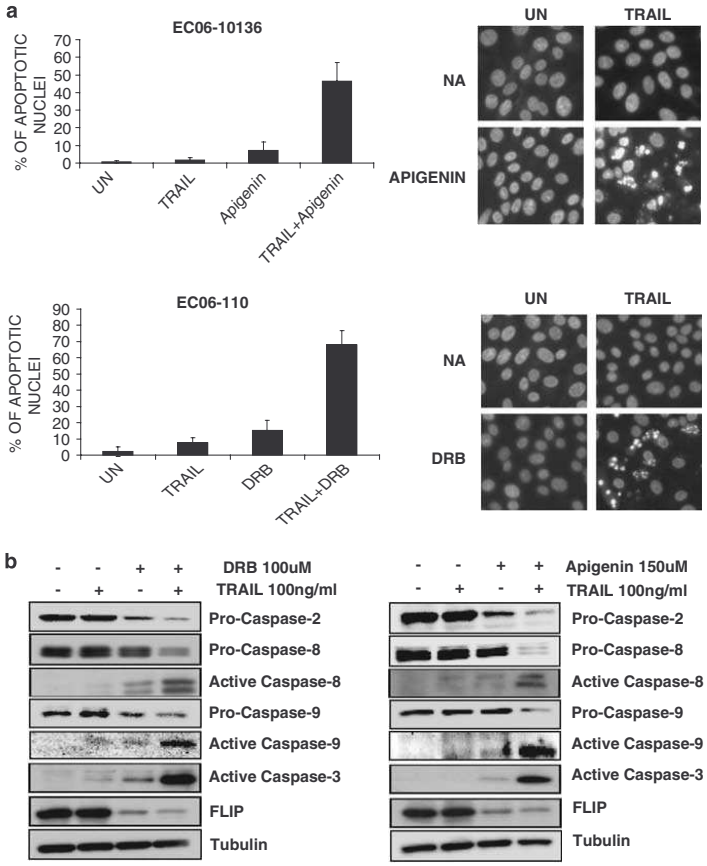

Figure 6 Casein kinase (CK2) inhibitors sensitize primary endometrial explants to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Primary explants were established from two different biopsies (top panel EC06-10136 and bottom panel EC06-110) as described in Materials and methods. Explants were treated with the indicated doses of 5,6-dichloro-ribifuranosylbenzimidazole (DRB) or apigenin or with no additives (NA) and left untreated (UN) of treated with TRAIL. (**a**) Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Left: results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number cells. Right: representative micrographs of primary explants treated as indicated. (**b**) Parallel wells of the two explants were lysed and protein extracts were analysed by western blot with antibodies to caspases and FLICE-inhibitory protein (FLIP). Membranes were reprobed with tubulin to ensure equal protein amounts.

depends on the kinase activity of the CK2. There are some evidences that CK2 catalytic subunits may have compensatory effects in some cell types. To preclude the problem of redundancy between subunits (Tawfic *et al*., 2001; Ahmed *et al*., 2002; Litchfield, 2003), we targeted CK2β regulatory subunit activity. We found that shRNA to CK2β had the same effects that pharmacological inhibition of CK2 kinase activity, suggesting that downregulation of CK2 regulatory subunit is effective to disrupt CK2 activity.

FLIP is a well-established regulator of TRAIL- and FasL-triggered apoptosis in many cell types. FLIP is constitutively or highly expressed in some tumors such as prostate cancer (Zhang *et al*., 2004), Hodgkin's lymphoma (Dutton *et al*., 2004), gastric cancer (Lee *et al*., 2003), bladder carcinoma (Korkolopoulou *et al*., 2004) and malignant mesothelial cell lines (Rippo *et al*., 2004). Increased levels of FLIP inhibit caspase-8 activation and apoptosis. In a previous study, we demonstrated that siRNA to FLIP is enough to sensitize IK cells to TRAIL-induced apoptosis (Dolcet *et al*., 2005), suggesting that FLIP levels are critical in sensitization to TRAIL-induced apoptosis. Although increasing evidence suggest an important role for CK2, there are few and controversial evidences concerning the role of FLIP in regulation of CK2 sensitization to death ligands. Shin *et al*. (2005) showed that overexpression of FLIP does not prevent caspase activation of the FLIP-L negative HCE4 cells after treatment with TRAIL plus DRB. The authors postulated that FLIP might not be

an essential regulator of TRAIL apoptosis in these cells. In contrast to the observations of Shin and collaborators, our results demonstrate that FLIP overexpression completely blocks caspase activation and apoptosis triggered by aFas or TRAIL plus either DRB, apigenin or downregulation of CK2β subunits by specific shRNA. Another recent report also suggested a role for FLIP in regulation of CK2 sensitization to TRAIL (Wang *et al*., 2006). The authors demonstrated in the prostate carcinoma lines PC-3 and ALVA-41 that treatment with suboptimal TRAIL plus the CK2 inhibitor TBB leads to downregulation of FLIP expression and concomitant caspase-8 activation. They also showed that overexpression of CK2α restores FLIP expression and TRAIL resistance. Accordingly, we have found that in ECC both pharmacological inhibition and CK2β knockdown reduce the levels of FLIP.

Next, we investigated the mechanism by which CK2 may regulate FLIP levels. Our data suggest that CK2 regulates FLIP levels by both transcriptional and post-transcriptional mechanisms. It is well known that FLIP can be transcriptionally downregulated by some antineoplastic drugs such as 5-fluorouracil, oxaliplatin and irinotecan in colon carcinoma cells (Galligan *et al*., 2005). Such FLIP mRNA downregulation has been shown to sensitize these cells to TRAIL-induced apoptosis. FLIP levels are also regulated by ubiquitin-proteasome-mediated degradation (Kim *et al*., 2002; Poukkula *et al*., 2005; Chang *et al*., 2006). Moreover, recent findings suggest that some anticancer drugs such as the cyclooxygenase-2 inhibitor celecoxib (Liu *et al*., 2006) or the flavonoids flavopiridol (Son *et al*., 2007) and flavopiridol (Palacios *et al*., 2006) can sensitize cancer cells to TRAIL-induced apoptosis by inducing a proteasome-mediated degradation of FLIP. In agreement with these previous reports, addition of proteasome inhibitors such as MG-132 restores FLIP levels in IK cells treated with CK2 inhibitors. Thus, in our model, CK2 maintains FLIP levels by controlling FLIP expression and degradation.

We showed that inhibition of CK2 plus addition of either TRAIL- or aFas-activated caspase-8 as initiator caspase of the extrinsic pathway; however, it also activated caspase-9 and -2 and the effector caspase-3. These data suggest that sensitization to TRAIL and aFas requires mitochondrial amplification. That is, caspase-8 cleaves the 'BH3 only' protein Bid. Truncated Bid translocates to the mitochondria, which ultimately results in the release of cytochrome c and activation of caspase-9 (Li *et al*., 1998; Luo *et al*., 1998). Previous reports have demonstrated that inhibition of CK2 results in Bid processing (Ravi and Bedi, 2002; Izeradjene *et al*., 2005). Among the caspases activated by CK2 inhibition, the role caspase-2 has been recently studied in death receptor signaling. Caspase-2 is shown to be activated by both stress-induced (Lassus *et al*., 2002) and death receptor apoptosis (Droin *et al*., 2001; Wagner *et al*., 2004; Lavrik *et al*., 2006). However, its role as initiator caspase in death receptor signaling is controversial. A recent publication suggested that caspase-2 prime cancer cells to TRAIL-induced apoptosis by processing caspase-8 (Shin *et al*., 2005). In this work, the authors demonstrated that CK2 directly phosphorylates and inhibits caspase-2. Thus, inhibition of CK2 results in dephosphorylation and processing of procaspase-2 which in turn processes caspase-8. The authors postulated that the inhibition of CK2 is enough to induce a caspase-2 dependent processing of procaspase-8 to the p41/p43 fragments and the addition of TRAIL triggers the second processing of caspase-8 that generates the active form. In contradiction to these data, Lavrik and collaborators demonstrated that in T- and B-cell lines, procaspase-2 is recruited and activated at the DISC after Fas engagement. Importantly, authors showed that caspase-2 failed to initiate apoptosis in caspase-8 deficient cells (Droin *et al*., 2001; Wagner *et al*., 2004; Lavrik *et al*., 2006). We have found that CK2 inhibition generates p41/43 fragment of caspase-8 even in absence of TRAIL and, addition of TRAIL or Fas, gives rise to the p18 active fragments and apoptosis. However caspase-2 active fragment is only activated when we add TRAIL or Fas, suggesting that activation of caspase-2 depends on the addition of the ligand. Moreover, FADD and caspase-8 shRNA or FLIP overexpression block caspase-2 activation and CK2 sensitization to TRAIL and Fas apoptosis, which suggest that in our cell system caspase-2, although activated, might not be an initiator caspase.

Finally, we demonstrated that pharmacological inhibition of CK2 sensitizes primary endometrial carcinoma explants to TRAIL-induced apoptosis. TRAIL has emerged as a promising antineoplastic agent, these data suggest that pharmacological inhibition of CK2 may be an interesting target for combinatorial therapies for endometrial carcinomas.

In summary, we provide evidence that CK2 is an important regulator of TRAIL- and FasL-induced apoptosis. We demonstrate that the mechanism used by CK2 requires downregulation of FLIP, enhancing the importance of this protein in TRAIL and Fas signaling and, ultimately, the cell fate.

Materials and methods

Reagents, plasmids and antibodies

MTT and monoclonal antibody to Tubulin and anti-Flag M2 were from Sigma (St Louis, MO, USA). CK2 inhibitors DRB, apigenin and emodin and monoclonal antibody to caspase-8 was from Calbiochem (La Jolla, CA, USA). Antibody to caspase-9 and cleaved caspase-3 were obtained from Cell Signalling (Beverly, MA, USA). Monoclonal antibody to FLIP (NF6) was purchased from Alexis Corp (Lausen, Switzerland). Anti-CK2β antibody was from SantaCruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody to caspase-2 was from BD Biosciences (San Jose, CA, USA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden).

Lentiviral vector containing Flag-tagged mouse FLIP complementary DNA (cDNA) was a gift from Dr Joan Comella.

Cell lines, culture conditions and transfection

IK was obtained from the American Type Culture Collection (Manassas, VA, USA). RL-95 cell line was a gift from

Dr Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% Fetal Bovine Serum (Invitrogen Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicillin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% CO_2.

When indicated, transfections plasmids constructs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.

Explant culture of endometrial adenocarcinoma

Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (JP). A specific informed consent was obtained from each patient, and the study was approved by the local Ethic Committee. Tissue was collected in DMEM, chopped in 1 mm pieces and incubated with collagenase in DMEM for 1.5 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 10 ml pipette and a 1 ml blue tip and resuspended in 2 ml of fresh DMEM medium. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM medium and tissue was allowed to sediment by gravity for 5 min. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% of penicillin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37 °C with saturating humidity and 5% CO_2.

Lentiviral production and infection

Oligonucleotides to produce plasmid-based shRNA were cloned into the FSV vector using *Age*I-*Bam*HI restriction sites. shRNA target sequence were CK2β, TGGTTTCCC TCACATGCTCT; FADD, CATGGAACTCAGACGC ATCT; caspase-8, GAATCACAGACTTTGGACAA. To produce infective lentiviral particles, 293T cells were co-transfected by calcium phosphate method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA producing vector (FSV) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3–4 days in same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 min at 1000 r.p.m. and filtered through a 0.45 μM filter (Millipore, Bedford, MA, USA). The medium was diluted 1:2–1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24–48 h in presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for 2 additional days to allow endogenous protein knockdown or protein overexpression.

Cell viability assays and assessment of apoptosis

Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated on M96 well plates at 15×10^3 cells per well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg ml^{-1} of MTT reagent and lysed with dimethyl sulfoxide. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, CA, USA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg ml^{-1} to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Western blot analysis

Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and lysed with lysis buffer (2% sodium dodecyl sulfate (SDS), 125 mM Tris–HCl pH 6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Nonspecific binding was blocked by incubation with Tris-buffered saline/Tween (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4 °C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from IK cells treated as indicated using Trizol reagent (Invitrogen). One microgram of total RNA was converted into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems P/N N808-0234) according to the manufacturer's recommendations. A total of 2 μl of the reverse transcription reaction were used as template for a for real-time detection of human FLIP expression using TaqMan Technology on an Applied Biosystems 7000 sequence detection system. Gene expression quantitation was performed in separate tubes (singleplex) for both target gene and endogenous control gene using the primer and probe sequences for human FLIP and β-glucuronidase (GUSB) obtained commercially from Applied Biosystems Assay-on-demand Gene (ABI P/N 4331182: Hs00236002_m1 and ABI P/N 4326320E). The reaction was performed with 10 μl TaqMan Universal PCR Master Mix, No AmpErase UNG 2X (P/N 4324018), 1 μl 20 × Assay-on-demand Gene and 2 μl of cDNA diluted in RNase-free water adjusted to 20 μl volume reaction. The thermal cycler conditions was UNG activation 2 min at 50 °C, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (repeat 40 times) on ABI7000. Triplicate cycle threshold (CT) values were analysed with Quantitative Relative software using de comparative CT (ΔΔCT) method as described by the manufacturer. The amount of target($2^{-\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference gene (GUSB). Results are presented as a relative mRNA amount compared to the untreated samples.

Acknowledgements

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References

Ahmad KA, Wang G, Slaton J, Unger G, Ahmed K. (2005). Targeting CK2 for cancer therapy. *Anticancer Drugs* **16**: 1037–1043.

Ahmed K, Gerber DA, Cochet C. (2002). Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* **12**: 226–230.

Allende JE, Allende CC. (1995). Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J* **9**: 313–323.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA *et al*. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**: 155–162.

Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P *et al*. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2**: 241–243.

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K *et al*. (2006). The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124**: 601–613.

Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA *et al*. (2006). Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* **281**: 22118–22130.

Dolcet X, Llobet D, Pallares J, Rue M, Comella JX, Matias-Guiu X. (2005). FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Lab Invest* **85**: 885–894.

Droin N, Bichat F, Rebe C, Wotawa A, Sordet O, Hammann A *et al*. (2001). Involvement of caspase-2 long isoform in Fas-mediated cell death of human leukemic cells. *Blood* **97**: 1835–1844.

Dutton A, O'Neil JD, Milner AE, Reynolds GM, Starczynski J, Crocker J *et al*. (2004). Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci USA* **101**: 6611–6616.

Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. (2005). Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* **4**: 2026–2036.

Irmler M, Steiner V, Ruegg C, Wajant H, Tschopp J. (2000). Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* **468**: 129–133.

Izeradjene K, Douglas L, Delaney A, Houghton JA. (2004). Influence of casein kinase II in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human rhabdomyosarcoma cells. *Clin Cancer Res* **10**: 6650–6660.

Izeradjene K, Douglas L, Delaney A, Houghton JA. (2005). Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. *Oncogene* **24**: 2050–2058.

Kikkawa U, Mann SK, Firtel RA, Hunter T. (1992). Molecular cloning of casein kinase II alpha subunit from *Dictyostelium discoideum* and its expression in the life cycle. *Mol Cell Biol* **12**: 5711–5723.

Kim Y, Suh N, Sporn M, Reed JC. (2002). An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* **277**: 22320–22329.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12**: 611–620.

Korkolopoulou P, Goudopoulou A, Voutsinas G, Thomas-Tsagli E, Kapralos P, Patsouris E *et al*. (2004). c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. *Urology* **63**: 1198–1204.

Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. (2001). Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**: 3247–3257.

Lassus P, Opitz-Araya X, Lazebnik Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297**: 1352–1354.

Lavrik IN, Golks A, Baumann S, Krammer PH. (2006). Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. *Blood* **108**: 559–565.

LeBlanc HN, Ashkenazi A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10**: 66–75.

Lee SH, Kim HS, Kim SY, Lee YS, Park WS, Kim SH *et al*. (2003). Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. *APMIS* **111**: 309–314.

Li H, Zhu H, Xu CJ, Yuan J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**: 491–501.

Litchfield DW. (2003). Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* **369**: 1–15.

Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. (2006). Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* **66**: 11115–11119.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**: 481–490.

MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. (1997). Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* **272**: 25417–25420.

Padmanabha R, Chen-Wu JL, Hanna DE, Glover CV. (1990). Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**: 4089–4099.

Palacios C, Yerbes R, Lopez-Rivas A. (2006). Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. *Cancer Res* **66**: 8858–8869.

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. (1997a). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**: 815–818.

Pan G, Ni J, Yu G, Wei YF, Dixit VM. (1998). TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* **424**: 41–45.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J *et al*. (1997b). The receptor for the cytotoxic ligand TRAIL. *Science* **276**: 111–113.

Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson MS *et al*. (2005). Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* **280**: 27345–27355.

Ravi R, Bedi A. (2002). Sensitization of tumor cells to Apo2 ligand/TRAIL-induced apoptosis by inhibition of casein kinase II. *Cancer Res* **62**: 4180–4185.

Rippo MR, Moretti S, Vescovi S, Tomasetti M, Orecchia S, Amici G *et al*. (2004). FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. *Oncogene* **23**: 7753–7760.

Seldin DC, Leder P. (1995). Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* **267**: 894–897.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D *et al*. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**: 818–821.

Shin S, Lee Y, Kim W, Ko H, Choi H, Kim K. (2005). Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. *EMBO J* **24**: 3532–3542.

Son YG, Kim EH, Kim JY, Kim SU, Kwon TK, Yoon AR *et al*. (2007). Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* **67**: 8274–8284.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J *et al*. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12**: 599–609.

Srivastava RK. (2001). TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* **3**: 535–546.

Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. (2007). Targeting death-inducing receptors in cancer therapy. *Oncogene* **26**: 3745–3757.

Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K. (2001). Protein kinase CK2 signal in neoplasia. *Histol Histopathol* **16**: 573–582.

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F *et al*. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**: 517–521.

Wagner KW, Engels IH, Deveraux QL. (2004). Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J Biol Chem* **279**: 35047–35052.

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N *et al*. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* **16**: 5386–5397.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M *et al*. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* **5**: 157–163.

Wang G, Ahmad KA, Ahmed K. (2005a). Modulation of death receptor-mediated apoptosis by CK2. *Mol Cell Biochem* **274**: 201–205.

Wang G, Ahmad KA, Ahmed K. (2006). Role of protein kinase CK2 in the regulation of tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis in prostate cancer cells. *Cancer Res* **66**: 2242–2249.

Wang G, Unger G, Ahmad KA, Slaton JW, Ahmed K. (2005b). Downregulation of CK2 induces apoptosis in cancer cells—a potential approach to cancer therapy. *Mol Cell Biochem* **274**: 77–84.

Zhang X, Jin TG, Yang H, DeWolf WC, Khosravi-Far R, Olumi AF. (2004). Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* **64**: 7086–7091.

3. CK2β S'EXPRESSA EN EL CARCINOMA D'ENDOMETRI, I JUGA UN PAPER EN LA RESISTÈNCIA A L'APOPTOSI I PROLIFERACIÓ CEL·LULAR.

3.1 CK2β s'expressa en mostres de carcinoma d'endometri i endometri normal.

3.2 L'expressió de CK2β correlaciona amb el marcador de proliferació cel·lular Ki67.

3.3 L'expressió de CK2β correlaciona amb l'expressió de Akt, PTEN i β-catenina.

3.4 El marcatge per CK2β mostra correlació significativa amb l'expressió de FLIP

3.5 CK2β regula la proliferació i el creixement cel·lular independent d'ancoratge en cèl·lules de carcinoma d'endometri.

3.6 Els efectes citostàtics derivats de l'ús de shRNA contra CK2β no són causats per una resposta apoptòtica en les cèl·lules de carcinoma d'endometri.

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Tumorigenesis and Neoplastic Progression

CK2β Is Expressed in Endometrial Carcinoma and Has a Role in Apoptosis Resistance and Cell Proliferation

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Protein kinase CK2 (CK2) is a serine/threonine kinase that participates in important cellular processes. We have recently demonstrated that CK2 plays a role in resistance to TRAIL/Fas-induced apoptosis in endometrial carcinoma (EC) by regulating FLIP. Here, we assessed the immunohistochemical expression of CK2β in EC and checked its role in cell proliferation and anchorage-independent cell growth. CK2β immunostaining was assessed in two tissue microarrays, one constructed from paraffin-embedded blocks of 95 ECs and another from 70 samples of normal endometrium. CK2β expression was correlated with histological type; grade and stage; cell proliferation (Ki-67) and apoptotic index; immunostaining for cyclin D1, PTEN, AKT, β-catenin, and FLIP. Moreover, the Ishikawa EC cell line was subjected to down-regulation of CK2 by shRNA. CK2β expression was frequent in EC (nuclear, 100%; cytoplasmic, 87.5%). The staining was more intense in EC than in normal endometrium ($P = 0.000$), and statistically correlated with AKT, PTEN, β-catenin, and FLIP. In EC, CK2β expression correlated with cell proliferation. Knock-down of CK2β blocked colony formation of EC in soft agar, and also resulted in decreased expression of cyclin D1 and ERK phosphorylation. The results confirm that CK2β is widely expressed in EC, and suggest a role in cell proliferation and anchorage-independent cell growth. ***(Am J Pathol 2009, 174:287–296; DOI: 10.2353/ajpath.2009.080552)***

Endometrial carcinoma (EC) is one of the most common gynecological malignant tumors in Europe and the United States.[1] EC can be divided into two main clinicopathological variants. Type I ECs are endometrioid EC; they are estrogen-related tumors, frequently well differentiated and developing mostly in pre- and perimenopausal women. Type I ECs are associated more frequently with four main molecular alterations: microsatellite instability and mutations of K-RAS, PTEN, and β-catenin. Type II ECs are nonendometrioid EC (papillary serous and clear cell carcinomas); they tend to occur in older women, and are estrogen-unrelated tumors, frequently aneuploid, associated with p53 mutations, and are clinically more aggressive tumors.[2]

Protein kinase CK2 (previously known as casein kinase II) is a serine/threonine kinase that has been implicated in cell growth, differentiation, proliferation, and apoptosis. CK2 has been shown to be deregulated in several kinds of tumors.[3] CK2 is an enzyme with extensive homology across species that consists of two catalytic subunits (α, α') and the regulatory subunit (β). CK2 exists as $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ configurations. In the β subunit, certain cysteine residues may play a role in anchoring the kinase to nuclear structures. CK2 activity may have a role in cell growth through its signaling to key sites in nuclear matrix and chromatin structures.[4] Several growth stimuli can enhance CK2 nuclear shuttling, so that higher nuclear localization is observed in tumor cells compared with normal cells.[5,6] Moreover, CK2 dysregulation in tumor

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cells may influence the apoptotic activity and to enhance cell survival.[7,8]

We have recently demonstrated a role for CK2β in apoptosis resistance to TRAIL in EC.[9] We first showed that pharmacological inhibition or silencing of CK2β sensitized EC cell lines to TRAIL and Fas-induced apoptosis, by down-regulating FLIP. Moreover, we found that forced expression of FLIP restored resistance to TRAIL and Fas in cells lacking CK2 activity or CK2β expression.

In the present study, we assessed the expression of CK2β in a large series of ECs by using an anti-CK2β antibody in two tissue microarrays that included 95 ECs and 70 samples of normal endometrium (NE). CK2β immunostaining was correlated with histological type, grade and stage, proliferation cell (Ki-67), and apoptotic index. Moreover, CK2β immunoreactivity was correlated with immunostaining for PTEN, AKT, β-catenin, and FLIP, to check the relationship of CK2β with the PI3K and Wnt signaling pathways, as well as to confirm the association between CK2β and FLIP. Finally, the role of CK2 on cell proliferation and anchorage-independent cell growth was assessed in one EC cell line, which was subjected to CK2β knock-down.

Materials and Methods

Tissue Microarray

Two tissue microarrays (TMAs) were constructed. One contained paraffin-embedded blocks of 95 ECs that were classified by following the World Health Organization criteria, and were staged and graded according to the International Federation of Gynecology and Obstetrics. The cases included 25 endometrioid carcinomas grade I, 34 endometrioid carcinomas grade II, 19 endometrioid carcinomas grade III, 10 serous carcinomas, 4 clear cell carcinomas, and 3 mixed Müllerian malignant tumors; 65 cases were stage I, 4 were stage II, 14 were stage III, and 1 was stage IV. Staging information was incomplete in 11 cases. A second TMA was constructed from 70 paraffin-embedded samples of NE in different phases of the menstrual cycle: 20 in proliferative phase, 40 in secretory phase, and 10 menstrual endometria. A tissue arrayer device (Beecher Instruments, Sun Prairie, WI) was used to construct the TMA. Briefly, all of the samples were histologically reviewed and representative areas were marked in the corresponding paraffin blocks. Two selected cylinders (0.6 mm in largest diameter) from two different areas were included in each case. Control normal tissues from the same specimens were also included. The study was approved by the local ethical committee. Specific informed consent was obtained.

Immunohistochemical Study

TMA blocks were sectioned at a thickness of 3 μm, dried for 16 hours at 56°C before being dewaxed in xylene and rehydrated through a graded ethanol series, and washed with phosphate-buffered saline (PBS). Antigen retrieval was achieved by heat treatment in a pressure-cooker for 2 minutes in ethylenediaminetetraacetic acid (pH 8.9). Before staining the sections, endogenous peroxidase was blocked. The antibodies used were: anti-CK2β (monoclonal, 6D5, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 (monoclonal, MIB-1, 1:100 dilution; DAKO, Glostrup, Denmark), cyclin D1 (monoclonal, DCS6, 1:25 dilution; DAKO), antiphosphorylated AKT (polyclonal, 1:25 dilution; Cell Signaling, Beverly, MA), PTEN (monoclonal, 6H2.1, 1:300 dilution; Cascade Bioscience, Winchester, MA), β-catenin (monoclonal, clone 14, 1:300 dilution; Transduction Laboratories, Lexington, KY), anti-FLIP (polyclonal H-202, 1:10 dilution; Santa Cruz Biotechnology). After incubation, the reaction was visualized with the EnVision Detection Kit (DAKO) using diaminobenzidine chromogen as a substrate. Sections were counterstained with hematoxylin. Immunohistochemical results were evaluated by two pathologists, by following uniform pre-established criteria. CK2β, AKT, PTEN, and FLIP immunoexpressions were graded semiquantitatively by considering the percentage and intensity of the staining. A histological score was obtained from each sample, which ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The score was obtained by applying the following formula, Hscore = 1× (% light staining) + 2× (% moderate staining) + 3× (% strong staining). The reliability of the scoring system in TMA evaluation in EC has been demonstrated previously.[10–13] Because each TMA included two different tumor cylinders from each case, immunohistochemical evaluation was done after examining both samples. To assess the cellular proliferation (Ki-67) and the cyclin D1 expression, we used the percentage of positive nuclei in each case, and finally for β-catenin we noted the membranous or nuclear staining. The specificity of the CK2β antibody was confirmed by obtaining a negative staining in cell blocks from Ishikawa cells infected with lentiviruses carrying CK2β shRNA; whereas, Ishikawa cells infected with lentiviruses carrying the empty vector were positive.

Cell Lines and Culture Conditions

The Ishikawa 3-H-12 cell line (IK) was obtained from the American Type Culture Collection (Manassas, VA). IK cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Inc., Carlsbad, CA), 1 mmol/L HEPES (Sigma), 1 mmol/L sodium pyruvate (Sigma), 2 mmol/L L-glutamine (Sigma), and 1% of penicillin/streptomycin (Sigma) at 37°C with saturating humidity and 5% CO_2.

Lentiviral Production and Infection

Oligonucleotides to produce plasmid-based shRNA were cloned into the FSV vector using *Age*I-*Bam*HI restriction sites. shRNA target sequence was: Ck2β, 5′-TGGTTTC-CCTCACATGCTCT-3′. To produce infective lentiviral particles, 293T cells were co-transfected by the calcium phosphate method with the virion-packaging elements

(VSV-G and Δ8.9) and the shRNA-producing vector (FSV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles for 3 to 4 days in same culture medium used for endometrial cell lines. Culture medium was collected, centrifuged for 5 minutes at 1000 rpm, and filtered through a 0.45-μm filter (Millipore, Bedford, MA). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24 to 48 hours in the presence of medium containing lentiviral particles. After this period, medium was replaced with fresh medium and the cells were incubated for an additional 2 to 3 days to allow endogenous protein knock-down.

Western Blot Analysis

The EC cell line was washed with cold phosphate-buffered saline (PBS) and lysed with lysis buffer (2% sodium dodecyl sulfate, 125 mmol/L Tris-HCl, pH 6.8). Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Nonspecific binding was blocked by incubation with TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20) plus 5% of nonfat milk. Membranes were incubated with the primary antibodies overnight at 4°C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK). The antibodies used were: anti-CK2β (monoclonal, 6D5; Santa Cruz Biotechnology), CK2α (monoclonal, 1AD9; Santa Cruz Biotechnology), FLIP (monoclonal, NF6; Alexis Biochemicals, Lausen, Switzerland), anti-phospho ERK-1/2 MAPK (Thr202/Tyr204) (polyclonal, Cell Signaling), Pan-ERK (monoclonal, BD Transduction), α-tubulin (monoclonal, Sigma), and cyclin D1 (monoclonal, DCS-6; Santa Cruz Biotechnology). Antibody to caspase-9 and cleaved caspase-3 were obtained from Cell Signaling.

Colony Formation Assay and Assessment of Apoptosis

Three days after infection FSV or CK2β shRNA-transduced cells were trypsinized and resuspended in 0.3% agar diluted in medium at concentration of 3000 cells/ml. One ml of cell suspension was layered on a 0.6% agar-coated 35 mm culture dish. Dishes were incubated at 37°C with saturating humidity and 5% CO_2 for 15 days. Colonies were visualized by staining with MTT. Colonies were counted with Quantity One software (Bio-Rad). Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

Statistical analysis was performed on a database using SPSS for Windows (version 11.5; SPSS Inc., Chicago, IL). The Hscore results were compared by the Mann-Whitney *U*-test, Student's *t*-test, Kruskal-Wallis test, and analysis of variance test when applicable. Correlations between quantitative variables were established through Pearson and Spearman Rho tests. Statistical significance was set at $P \leq 0.05$.

Relative Quantification Analysis of CK2β Gene Expression

For gene expression analysis, predesigned gene-specific primer pairs and probe were selected for one target gene (CK2β) and one endogenous control (18s rRNA) within a list of predesigned assays (Assays-on-Demand; Applied Biosystems, Foster City, CA). All assays were based on TaqMan hydrolysis probes labeled with FAM (green fluorescent fluorophore 6-carboxyfluorescein). Assays were performed on an ABI Prism 7000 HT sequence detection system (Applied Biosystems). RNA was obtained from fresh-frozen tissue obtained in the Pathology Department, Hospital Universitari Arnau de Vilanova, shortly after surgery. They included three samples of NE in the proliferative phase, three samples of secretory endometrium, and paired normal and tumor tissue from six patients with EC. Corresponding formalin-fixed, paraffin-embedded tumor tissue from these patients had been included in the tissue microarray that was subjected to immunohistochemical analysis of CK2β. Once RNA was extracted, subsequent reverse transcription analyses to cDNA were performed in 20-μl reaction volumes. For the assays each reaction was comprised of 4 μl of cDNA, 10 μl of 2× qPCR Master Mix, 1 μl of 20× primer/probe assay mix (Applied Biosystems). Samples were assayed in triplicate for each gene, and the mean expression was used during subsequent analysis. Relative expression was calculated using the comparative $\Delta\Delta C_T$ method (Bulletin no.2, Applied Biosystems).

5-Bromodeoxyuridine Incorporation

For the determination of DNA, cells were incubated with 3 ng/ml of 5-bromodeoxyuridine (BrdU) (Sigma) for 20 minutes and then fixed with 4% paraformaldehyde. After DNA denaturing with 2 mol/L HCl for 30 minutes and neutralization with 0.1 mol/L $Na_2B_4O_7$ (pH 8.5) for 2 minutes, cells were blocked in PBS solution containing 5% horse serum, 5% fetal bovine serum, 0.2% glycine, and 0.1% Triton X-100 for 1 hour. Subsequently, cells were subjected to indirect immunofluorescence with a mouse anti-BrdU monoclonal antibody (DAKO), and fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR). Nuclei were counterstained with 5 μg/ml Hoechst 33258 and cells were visualized under an epifluorescence microscope (Leica Microsystems).

Propidium Iodide Cell Death Analysis

Analysis of cell death distribution was determined by propidium iodide (PI) staining and flow cytometry. After

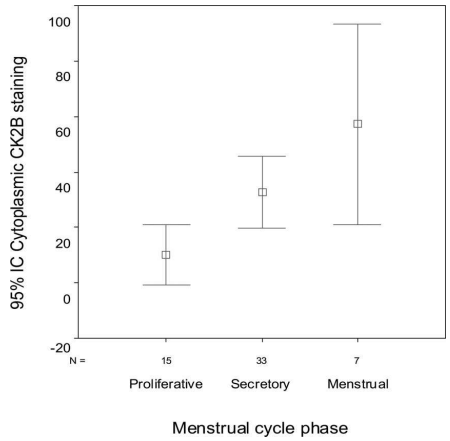

Figure 1. Error bar showing that CK2β cytoplasmic expression was significantly higher in the secretory and menstrual endometrium than in proliferative endometrium (analysis of variance, $P = 0.017$).

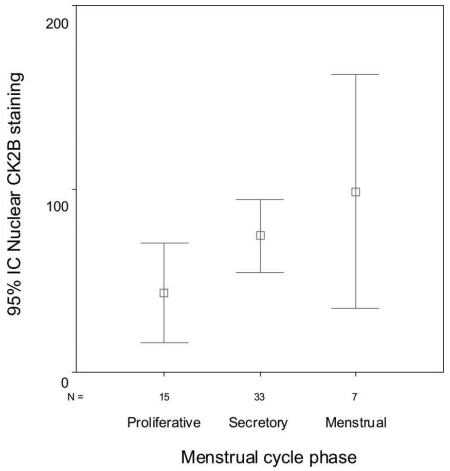

Figure 2. Error bar showing that CK2β nuclear immunoexpression was significantly lower in the proliferative phase of the menstrual cycle, and increases in the secretory and menstrual endometrium (analysis of variance $P = 0.001$).

treatment, $\sim 10^6$ cells were fixed in 70% ethanol for at least 1 hour on ice. Cells were then resuspended in 2 ml of cell cycle buffer (20 μg/ml PI in PBS containing 0.1% Triton X-100 and 50 μg/ml RNase A) for 1 hour at 37°C. PI fluorescence emission was measured using a FACSCalibur (BD Biosciences, San Jose, CA), and cell cycle distribution was analyzed with WinMDI 2.9 software (The Scripps Research Institute, La Jolla, CA).

Results

CK2β Expression in EC and NE Samples

CK2β expression was assessed in NE. There were statistically significant differences in the cytoplasmic (analysis of variance, $P = 0.017$) and nuclear (analysis of variance, $P = 0.001$) expression of CK2β among different phases of the menstrual cycle (Figures 1 and 2). Thus, cytoplasmic and nuclear CK2β immunoexpression was low in the proliferative endometrium (mean Hscores, 10.00 and 43.00, respectively) and increased in the secretory (mean Hscores, 32.73 and 74.24, respectively) and menstrual endometrium (mean Hscores, 57.14 and 98.57, respectively). A positive correlation between cytoplasmic and nuclear CK2β immunostaining was detected in NE (Pearson, $r = 0.371$; $P = 0.003$). Immunohistochemical results were confirmed at the mRNA level by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). All but one of the samples of secretory endometrium had higher levels of CK2β mRNA than samples of proliferative endometrium.

CK2β immunoexpression was evaluated in 88 cases, from the initial series of 95 ECs of the TMA; 6 cases were lost during TMA construction, and 1 case did not have representative tumor sample. All of the EC samples showed nuclear CK2β immunostaining (88 of 88; 100%). Cytoplasmic staining was observed in 77 cases (87.5%), but the staining was heterogeneous. The Hscore ranged from 10 to 300 (mean, 100.34); and only 11 cases had negative cytoplasmic staining (Figure 3). There was a strong positive correlation between the cytoplasmic and the nuclear expression in each case (Pearson, $r = 0.514$; $P = 0.000$). Thus, EC samples with high cytoplasmic CK2β expression showed also strong nuclear CK2 staining. EC had a very significant higher nuclear CK2β immunostaining compared with NE (mean Hscore in NE, 79.44; mean Hscore in EC, 167.39; Student's *t*-test, $P = 0.000$). Also, the tumor cells showed significantly higher CK2β cytoplasmic expression (mean Hscore, 100.34) than the normal cells (mean Hscore, 29.05; $P = 0.000$) (Figure 4, A and B). CK2β expression was also checked in full sections of five ECs, to check if the heterogeneity of the staining was attributable to increased staining in the myoinvasive front. However, no statistically significant differences in CK2β expression were seen between the superficial and the deepest area of invasion (Mann-Whitney *U*-test; $P = 0.401$ for cytoplasmic staining, and $P = 0.599$ for nuclear staining). Differences in CK2β expression between EC and corresponding NE were evaluated by quantitative RT-PCR. In all but one of the cases, the CK2β mRNA levels were higher in EC samples than in the corresponding NE samples from the same patients.

CK2β immunoexpression was slightly higher in the cytoplasm of the endometrioid type of EC (mean Hscore, 107.15) compared with nonendometrioid EC (mean Hscore, 67.00). Also, nonendometrioid EC showed a strong CK2β nuclear staining (mean Hscore, 198.00), but these differences were not statistically significant (Mann-Whitney *U*-test; $P = 0.158$, and $P = 0.287$, respectively). Finally, no significant differences were found in CK2β expression in correlation with the International Federation of Gynecology and Obstetrics grade (Kruskal-Wallis, $P = 0.636$) or pathological stage (Kruskall-Wallis, $P = 0.675$).

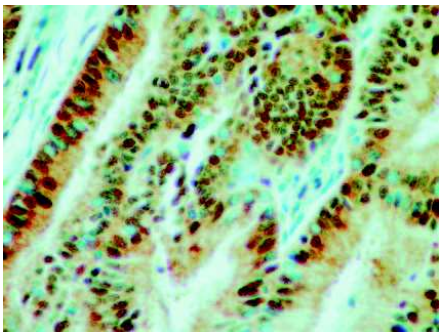

Figure 3. CK2β immunoexpression in ECs. The staining was heterogeneous, cytoplasmic, and nuclear.

CK2β and Cellular Proliferation (Ki-67) and Cyclin D1

CK2β nuclear immunostaining demonstrated a trend of correlation with the proliferation index (Ki-67/MIB-1) in EC (Spearman, $r = 0.210$; $P = 0.070$). Moreover, CK2β cytoplasmic and nuclear expression increased in cases with high nuclear cyclin D1 expression, but these differences were not significant (Kruskal-Wallis; $P = 0.477$ for cytoplasmic, and $P = 0.494$ for nuclear).

CK2β Correlation with AKT, PTEN, and β-Catenin Expression in ECs

We found a positive correlation between CK2β cytoplasmic expression and AKT immunostaining in EC (Spearman, $r = 0.243$; $P = 0.025$) and a nearly significant negative correlation between CK2β and PTEN immunoexpression (Spearman, $r = -0.194$; $P = 0.093$) (Figure 5, A and B). The correlation between β-catenin and CK2β immunostaining was assessed in 82 ECs. CK2β cytoplasmic and nuclear expression increased in ECs with nuclear β-catenin staining (mean cytoplasmic Hscore,112.50; mean nuclear Hscore, 214.58) (Mann-Whitney *U*-test, $P = 0.054$) compared with cases with only β-catenin membranous staining (mean cytoplasmic Hscore, 99.50; mean nuclear Hscore, 158.71).

CK2β, FLIP Expression, and Apoptotic Index in ECs

Correlation between CK2β and FLIP immunoexpression was assessed in 83 ECs. We demonstrated a significant positive correlation between CK2β and FLIP cytoplasmic immunostaining (Pearson, $r = 0.238$; $P = 0.030$) (Figure 6, A and B). However, no correlation was found between CK2β cytoplasmic and nuclear expression and the apoptotic index (Pearson, $P = 0.480$ and $P = 0.282$).

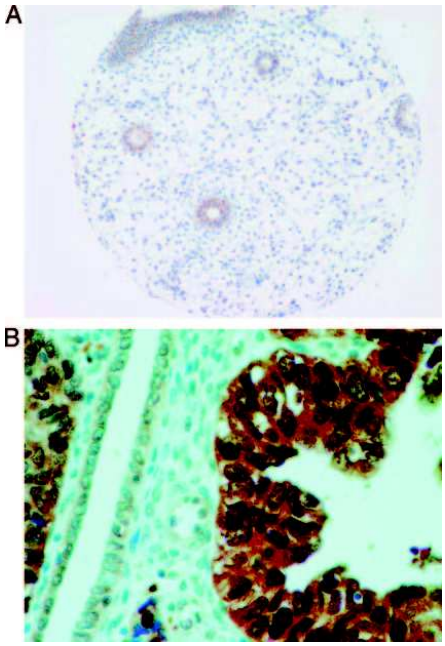

Figure 4. CK2β immunostaining was significantly lower in NE samples (**A**) compared with ECs (**B**).

CK2β Regulates Proliferation and Anchorage-Independent Cell Growth of EC Cells

To ascertain whether CK2 regulates proliferation of EC cells, we infected IK cells with lentiviruses carrying either an empty vector (FSV) or a CK2 shRNAs (FSV-CK2β) and 4 days later cell lysates were analyzed by Western blot for the expression of CK2β. As we show in Figure 7A, CK2β shRNA caused a dramatic down-regulation of CK2β protein levels whereas catalytic α CK2 subunit protein levels remained unaffected. To investigate the role of CK2β in proliferation, we infected IK cells with lentiviruses carrying CK2β shRNA, and we assessed cell proliferation by cell counting throughout time. CK2 shRNA (FSV-CK2β)-transduced cells displayed a dramatic decrease in proliferation rate when compared with the empty vector (FSV)-infected cells (Figure 7A). Moreover, to further confirm the role of CK2β in cell proliferation we performed a BrdU analysis. Results indicated that unlike control infected cells, CK2β-silenced cells displayed a marked reduction in cell proliferation. Bortezomib-treated cells were used as positive control (Figure 7B). These data prompted us to determine any putative proliferation marker susceptible to be modulated on CK2β silencing. Western blot analysis suggested a possible role for cyclin D1 and ERK. Figure 7C shows that CK2β shRNA caused a marked decrease in expression of cyclin D1 and phosphorylation of ERK-1/2 without affecting total ERK-1/2 protein levels. Furthermore, consistently with the results described above, CK2β knock-

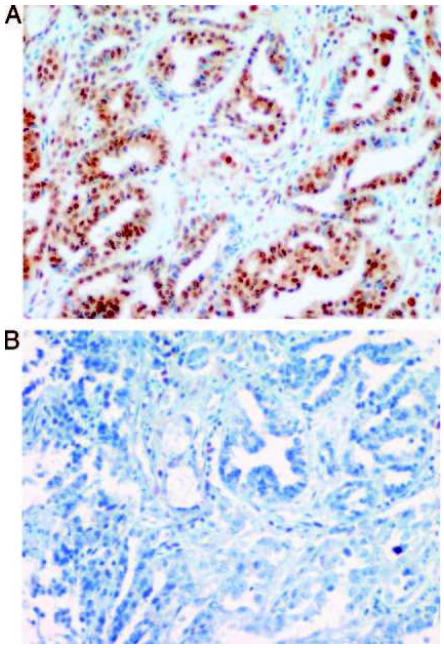

Figure 5. High CK2β immunoexpression in ECs (**A**) was frequently associated with low or negative PTEN expression (**B**).

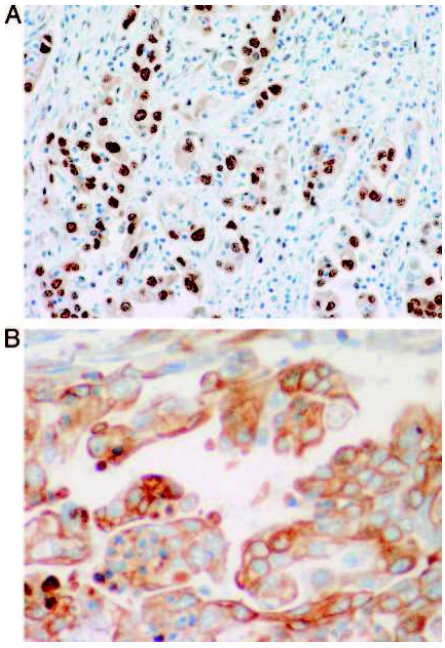

Figure 6. ECs positive for CK2β immunoexpression (**A**) with high FLIP immunostaining (**B**).

down also resulted in a pronounced decrease in FLIP protein levels. Altogether these results indicated that CK2β is involved in endometrial cancer cell proliferation, and that silencing of CK2β by means of shRNA results in a pronounced blockage of cell growth. To investigate the role of CK2 in growing conditions independent of substrate attachment, we infected IK cells with lentivirus carrying CK2β shRNA or the empty vector. After 3 days, cells were trypsinized and resuspended in soft agar as described in the Materials and Methods section. After 15 days, FSV-infected cells displayed cell growth and colony formation. In contrast, IK cells infected with CK2β shRNA failed to develop colonies (Figure 7D). These results suggest that CK2 regulates both cell proliferation and anchorage-independent cell growth.

CK2β shRNA Cytostatic Effects Are Not Caused by an Apoptotic Response of EC Cells

We have previously demonstrated that CK2β is involved in control of TRAIL-induced apoptosis.[9] To discard the role of apoptosis in the CK2β-silencing cytostatic effect, FSV/CK2β shRNA-infected cells and bortezomib-treated cells were subjected to propidium iodide staining and apoptotic index was measured by flow cytometry. $SubG_1$ data indicated that CK2β shRNA did not trigger a pronounced apoptotic response compared with those cells treated with bortezomib (Figure 8A). Moreover, Hoechst staining indicated that the decrease in cell proliferation was not attributable to a massive apoptotic cell death in FSV/CK2β-transduced cells because nuclei displaying apoptotic morphology were not primarily appreciated. Consistent with previous results obtained in our laboratory, CK2β-silenced cells treated with TRAIL resulted in an increase in percentage of apoptotic nuclei. This condition was used as a positive control (Figure 8B). Furthermore, we demonstrated by Western blot that caspases were not activated in response to CK2β shRNA because neither inductor caspase-9 nor effector caspase-3 were processed. In contrast, and in agreement with previous available data, we appreciated remarkable caspase activation on treatment of CK2β-silenced cells with TRAIL. Altogether, these data support the notion that CK2β is involved in progression of cell cycle and proliferation and that its decrease is not caused by an apoptotic response.

Discussion

Protein kinase CK2 is a serine/threonine kinase that is highly conserved and ubiquitously distributed in eukaryotic organisms. It is typically found in tetrameric complexes consisting of two catalytic (α, and/or α') subunits, and two regulatory subunits (β). CK2 has been shown to be deregulated in different types of tumors. Several studies in hematopoietic malignancies and solid tumors, such as carcinomas of the prostate, kidney, colon, liver, and lung have demonstrated elevated CK2 activity, and changes in the intracellular location with an enhanced

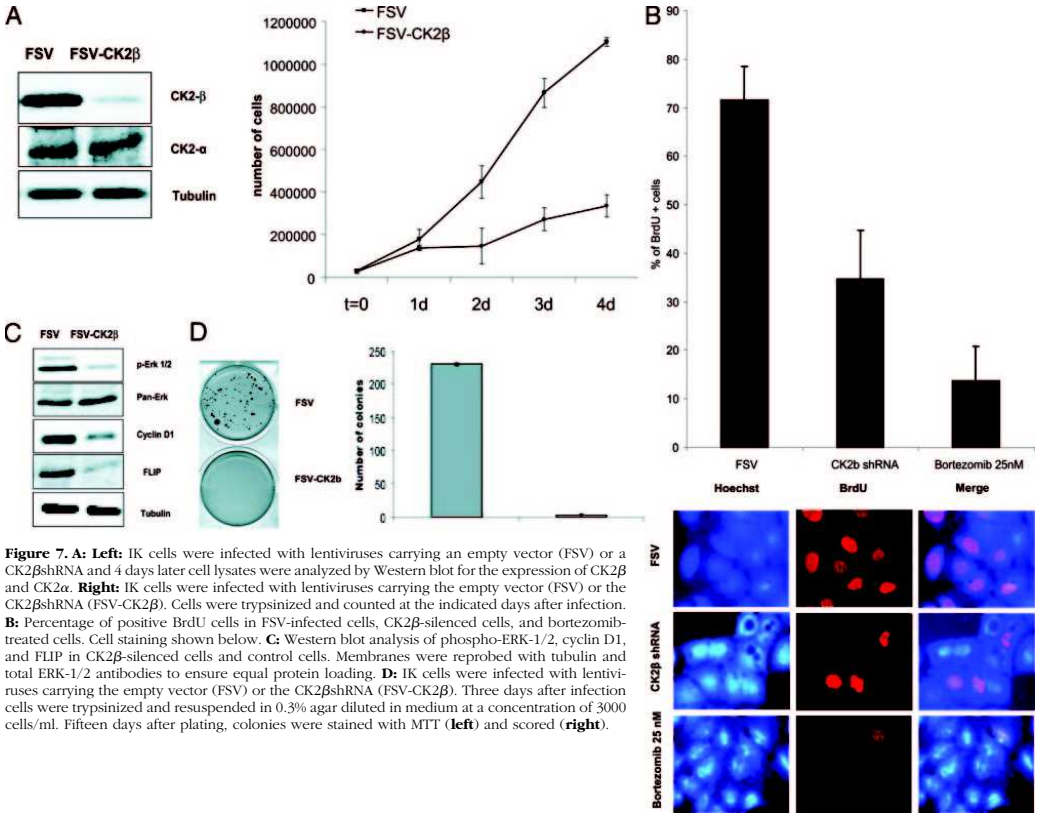

Figure 7. A: Left: IK cells were infected with lentiviruses carrying an empty vector (FSV) or a CK2βshRNA and 4 days later cell lysates were analyzed by Western blot for the expression of CK2β and CK2α. **Right:** IK cells were infected with lentiviruses carrying the empty vector (FSV) or the CK2βshRNA (FSV-CK2β). Cells were trypsinized and counted at the indicated days after infection. **B:** Percentage of positive BrdU cells in FSV-infected cells, CK2β-silenced cells, and bortezomib-treated cells. Cell staining shown below. **C:** Western blot analysis of phospho-ERK-1/2, cyclin D1, and FLIP in CK2β-silenced cells and control cells. Membranes were reprobed with tubulin and total ERK-1/2 antibodies to ensure equal protein loading. **D:** IK cells were infected with lentiviruses carrying the empty vector (FSV) or the CK2βshRNA (FSV-CK2β). Three days after infection cells were trypsinized and resuspended in 0.3% agar diluted in medium at a concentration of 3000 cells/ml. Fifteen days after plating, colonies were stained with MTT (**left**) and scored (**right**).

nuclear translocation.[3] These studies have demonstrated an increase in the CK2 activity or expression by using biochemical analysis of tissue extracts or immunohistochemical techniques. In some types of tumor, such as those of the head and neck, the level of CK2 is correlated with tumor grade, stage, and clinical outcome. CK2 is involved in protein kinase networks controlling cell growth, proliferation, but also as a potent suppressor of apoptosis. Further evidence about the role of CK2 in tumorigenesis is provided by transgenic mice expressing CK2α in lymphocytes or mammary gland. Both animal models display an increased incidence of lymphomas and breast carcinomas.[14,15] More than 300 potential substrates of CK2 (cytoplasmic and nuclear) have been identified, which points to its multiple functional activities. In a recent study we were able to demonstrate for the first time that CK2 played an important role in TRAIL- and Fas-induced apoptosis in EC, by regulating FLIP.[9]

In the present study we have shown, for the first time, that CK2β is frequently expressed in EC. We have observed a significant increase in cytoplasmic and nuclear CK2β immunostaining in EC in comparison with NE in different phases of the menstrual cycle. Results were confirmed at the mRNA level by RT-PCR. The preferential nuclear distribution of CK2 in tumor cells has also been demonstrated in other tumor types, such as in prostatic carcinoma and squamous carcinoma of the head and neck.[16,17] In some types of tumors, CK2 immunoexpression is higher in the infiltrating edge of the tumor and lymphocytes. However, we have not been able to demonstrate such a finding in our series of cases, particularly after having studied full sections from a small selected series of ECs, which allowed us to evaluate the comparative expression of CK2β in the superficial tumor and in areas of deep myometrial invasion.

Interestingly, our results showed a trend of positive correlation between CK2β immunoexpression with the cellular proliferation (Ki-67) in EC, but not in NE. CK2β expression was higher in the secretory and menstrual endometrium than in the proliferative phase, whereas in EC, CK2β expression was associated with increased cell proliferation. As discussed later on, these results obtained in EC samples are in agreement with our results obtained in the Ishikawa cell line. Studies on yeasts and mammalian cells have revealed requirements for CK2 at various stages of the cell cycle, including G_1 phase and the G_1/S and G_2/M transitions.[18,19] In other types of tumors, such as prostatic and head and neck carcinomas,

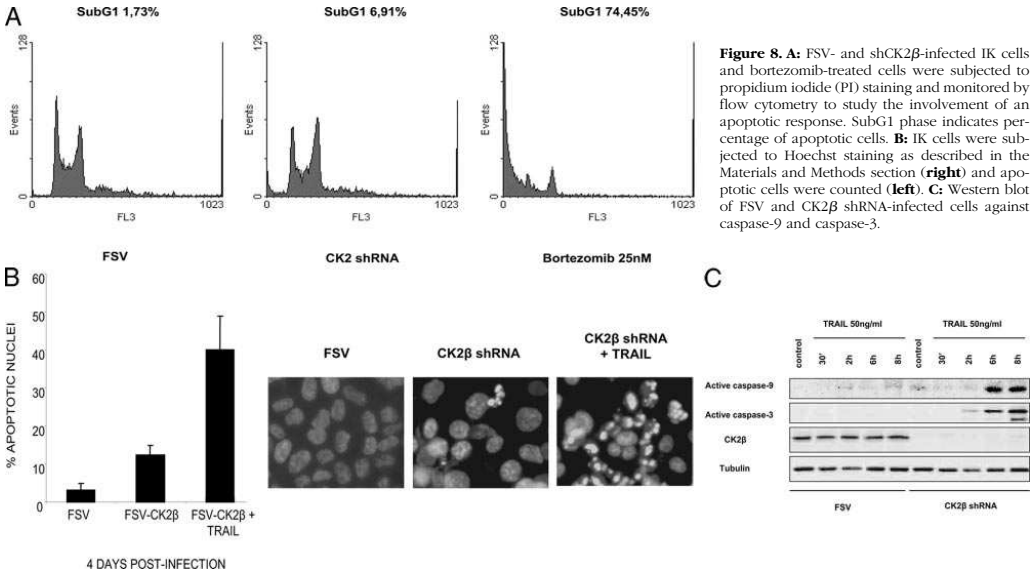

Figure 8. A: FSV- and shCK2β-infected IK cells and bortezomib-treated cells were subjected to propidium iodide (PI) staining and monitored by flow cytometry to study the involvement of an apoptotic response. SubG1 phase indicates percentage of apoptotic cells. **B:** IK cells were subjected to Hoechst staining as described in the Materials and Methods section (**right**) and apoptotic cells were counted (**left**). **C:** Western blot of FSV and CK2β shRNA-infected cells against caspase-9 and caspase-3.

the proliferating front of the neoplasm showed higher CK2 immunoexpression.[16,20] Recent studies have suggested that overexpression of CK2 in tumor cells may not be simply a reflection of cell proliferation alone but additionally may reflect some pathobiological characteristics of the neoplasm, such as the grade of differentiation of the tumor. In this regard, in our study CK2 immunoexpression had a tendency to increase in nonendometrioid EC and in stage II and III tumors. However, these differences were not statistically significant.

A very interesting result of the present study is the relationship between CK2β and the PI3K and Wnt pathways, two signaling pathways frequently activated in EC. CK2β showed a significant positive correlation with AKT and a nearly significant negative correlation with PTEN immunoexpression. The tumor suppressor gene PTEN is frequently abnormal in EC; loss of heterozygosity at chromosome 10q23 occurs in 40% of cases[21,22] and somatic PTEN mutations are also common and occur in 37 to 61% of EC.[23–26] Our results support a role for PTEN, and the PI3K/AKT pathway in the regulation of CK2β expression. It is known that decreased expression of PTEN leads to increased levels of phospho-AKT, which results in activation of antiapoptotic proteins as well as inactivation of proteins involved in cycle progression (p27 and p21). Moreover, Torres and colleagues, 2001, demonstrated that CK2 is able to decrease PTEN stability and increase its proteasome-mediated degradation through the phosphorylation of a cluster of Ser/Thr residues in its C-terminus.[27] Moreover, inhibition of PTEN phosphorylation by a CK2 inhibitor diminishes the PTEN protein content. Additional evidence relating CK2 and the PI3K pathway were obtained from the Jurkat cell line, which is PTEN-null. In these cells, down-regulation of CK2 catalytic activity correlated with decreased AKT activity, measured by reduced phosphorylation of typical AKT targets.[28]

We also found a significant correlation between CK2β immunoexpression and β-catenin nuclear staining. CK2 is a positive regulator of Wnt signaling.[29] The Wnt signaling is important in EC, particularly in endometrioid (type I) tumors, because mutations in exon 3 of CTNNB1 are found in 25 to 40% of endometrioid EC. CTNNB1 mutations lead to nuclear accumulation of β-catenin, which has an important impact in transcription of target genes of the Wnt pathway, such as cyclin D1 or MMP-7. CK2 is present in β-catenin complexes and activated in Wnt-signaling cells.[30] Recent studies have shown that CK2 enhances β-catenin through phosphorylation of hLEF-1 stimulating the binding and transactivation of β-catenin *in vitro*.[31] In breast cancer cells, CK2 is capable of phosphorylating β-catenin, and regulating its turnover[15]; in these cells, CK2 activity is essential for maintenance of β-catenin and Drl protein levels.

Also, we found a statistical association between FLIP and CK2β cytoplasmic immunostaining. Such results support the hypothesis that FLIP may be under the regulation of CK2. In a previous study, we demonstrated a role for FLIP in resistance to TRAIL and Fas-induced apoptosis in EC.[13] We also demonstrated that FLIP was frequently expressed in EC samples. FLIP is a well-established regulator of TRAIL- and FasL-triggered apoptosis in many cell types, which is constitutively or highly expressed in some tumors such as prostate cancer, Hodgkin's lymphoma, gastric cancer, bladder carcinoma, and malignant mesothelial cell lines. Moreover, in a very recent study, we have also shown that CK2 is an important regulator of death receptor-induced apoptosis, by regulating the levels of FLIP. We demonstrated that in

EC both pharmacological inhibition and CK2 knock-down reduced the levels of FLIP. We also showed that inhibition of CK2 plus addition of either TRAIL- or a Fas-activated caspase-8 as initiator caspase of the extrinsic pathway.[9]

Several studies have implicated dysregulation of the CK2 activity with enhanced survival of tumor cells. In the rat ventral prostate, CK2 has been implicated in the androgenic regulation of the cells and receptor-mediated apoptosis.[32–34] In this model loss of CK2 from the cell lead to a cessation of cell growth activity and to induce apoptosis. Other studies have shown that transient overexpression of CK2-α and CK2-$\alpha\beta$ in tumor cells resulted in a significant protection against etoposide-mediated apoptosis.[7] Recent reports in the carcinoma cell lines PC-3 and ALVA-41, suggest a role for FLIP in regulation of CK2 sensitivity to TRAIL-induced apoptosis.[35] Treatment of the cell lines with TRAIL plus a CK2 inhibitor (TBB) lead to a down-regulation of FLIP expression and concomitant caspase-8 activation. Moreover, overexpression of CK2α restored FLIP expression and TRAIL resistance to apoptosis cell death.

Increased CK2 activity is associated with cell growth and proliferation in many types of tumors displaying aberrant or increased CK2 activity.[3,36,37] To ascertain whether CK2 regulates proliferation of EC cells *in vitro*, Ishikawa cells were infected with lentiviruses carrying CK2β shRNA. Knock-down of CK2β protein caused a marked decrease in expression of cyclin D1, phosphorylation of ERK-1/2, decrease of BrdU incorporation, and a marked decrease in proliferation rate that was not caused by a consistent increase in number of apoptotic cells. Interestingly, results from the TMA showed a relationship between CK2 and cyclin D1 that did not reach statistical signification. It is important to keep in mind that cyclin D1 is also a target of other molecular alterations frequently detected in EC (microsatellite instability, mutations in CTNNB1, activation of NF-κB).[10,38,39] The presence of these alterations in a certain percentage of tumors might have interfered in the correlation between CK2 and cyclin D1. Finally the Ishikawa EC cell line cells infected with lentivirus carrying CK2β shRNA failed to develop colonies, clearly suggesting that CK2 regulates both cell proliferation and anchorage-independent cell growth. The fact that CK2 has become a highly pleiotropic protein makes the study of the exact mechanism that orchestrates its control over cell growth and proliferation difficult. Many mechanisms have been proposed, as it has been observed that CK2 regulates by phosphorylation many transcription factors such as c-Myc, c-Myb, AP-1, and steroid hormone receptors among others. Moreover, it has also been reported that CK2 can modulate NF-κB activity by phosphorylation of Rel-A (p65) NF-kappa B subunit or by promoting the degradation of its inhibitory subunit IkB.[40] Recently, Yde and collaborators[41] have suggested that CK2β regulates G_2/M transition by controlling stabilization of Wee1 and its subsequent inhibitory phosphorylation of CDK1 on Tyr15 pointing to CK2 as a key regulator of cell cycle progression in lung cancer cells. Our results add important information to the complex network of cell proliferation and cell cycle controlled by CK2 and further research efforts will be required to uncover the precise mechanism.

References

1. Evans MP, Podratz K: Endometrial neoplasia: prognostic significance of ploidy status. Clin Obstet Gynecol 1996, 39:696–706
2. Matias-Guiu X, Catasus L, Bussaglia E, Lagarda H, Garcia A, Pons C, Munoz J, Arguelles R, Machin P, Prat J: Molecular pathology of endometrial hyperplasia and carcinoma. Hum Pathol 2001, 32:569–577
3. Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K: Protein kinase CK2 signal in neoplasia. Histol Histopathol 2001, 16:573–582
4. Zhang P, Davis AT, Ahmed K: Mechanism of protein kinase CK2 association with nuclear matrix: role of disulfide bond formation. J Cell Biochem 1998, 69:211–220
5. Ahmed K: Nuclear matrix and protein kinase CK2 signaling. Crit Rev Eukaryot Gene Expr 1999, 9:329–336
6. Tawfic S, Faust RA, Gapany M, Ahmed K: Nuclear matrix as an anchor for protein kinase CK2 nuclear signalling. J Cell Biochem 1996, 62:165–171
7. Guo C, Yu S, Davis AT, Wang H, Green JE, Ahmed K: A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. J Biol Chem 2001, 276:5992–5999
8. Guo C, Yu S, Davis AT, Wang H, Green JE, Ahmed K: A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. J Biol Chem 2001, 276:5992–5999
9. Llobet D, Eritja N, Encinas M, Llecha N, Yeramian A, Pallares J, Sorolla A, Gonzalez-Tallada FJ, Matias-Guiu X, Dolcet X: CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells. Oncogene 2008, 27:2513–2524
10. Pallares J, Martinez-Guitarte JL, Dolcet X, Llobet D, Rue M, Palacios J, Prat J, Matias-Guiu X: Abnormalities in the NF-kappaB family and related proteins in endometrial carcinoma. J Pathol 2004, 204:569–577
11. Pallares J, Martinez-Guitarte JL, Dolcet X, Llobet D, Rue M, Palacios J, Prat J, Matias-Guiu X: Survivin expression in endometrial carcinoma: a tissue microarray study with correlation with PTEN and STAT-3. Int J Gynecol Pathol 2005, 24:247–253
12. Pallares J, Bussaglia E, Martinez-Guitarte JL, Dolcet X, Llobet D, Rue M, Sanchez-Verde L, Palacios J, Prat J, Matias-Guiu X: Immunohistochemical analysis of PTEN in endometrial carcinoma: a tissue microarray study with a comparison of four commercial antibodies in correlation with molecular abnormalities. Mod Pathol 2005, 18:719–727
13. Dolcet X, Llobet D, Pallares J, Rue M, Comella JX, Matias-Guiu X: FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. Lab Invest 2005, 85:885–894
14. Seldin DC, Leder P: Casein kinase II-alpha transgene-induced murine lymphoma—relation to theileriosis in cattle. Science 1995, 267:894–897
15. Landesman-Bollag E, Song DH, Romieu-Mourez R, Sussman DJ, Cardiff RD, Sonenshein GE, Seldin DC: Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol Cell Biochem 2001, 227:153–165
16. Yenice S, Davis AT, Goueli SA, Akdas A, Limas C, Ahmed K: Nuclear casein kinase-2 (Ck-2) activity in human normal, benign hyperplastic, and cancerous prostate. Prostate 1994, 24:11–16
17. Faust RA, Niehans G, Gapany M, Hoistad D, Knapp D, Cherwit D, Davis A, Adams GL, Ahmed K: Subcellular immunolocalization of protein kinase CK2 in normal and carcinoma cells. Int J Biochem Cell Biol 1999, 31:941–949
18. Pepperkok R, Lorenz P, Ansorge W, Pyerin W: Casein kinase-II is required for transition of G0/G1, early G1, and G1/S phases of the cell-cycle. J Biol Chem 1994, 269:6986–6991
19. Lorenz P, Pepperkok R, Ansorge W, Pyerin W: Cell biological studies with monoclonal and polyclonal antibodies against human casein kinase-II subunit-beta demonstrate participation of the kinase in mitogenic signaling. J Biol Chem 1993, 268:2733–2739

20. Seitz G, Munstermann U, Schneider HR, Issinger OG: Characterization of casein kinase-II in human colonic carcinomas after heterotransplantation into nude-mice. Biochem Biophys Res Commun 1989, 163:635–641
21. Nagase S, Sato S, Tezuka F, Wada Y, Yajima A, Horii A: Deletion mapping on chromosome 10q25-q26 in human endometrial cancer. Br J Cancer 1996, 74:1979–1983
22. Peiffer SL, Herzog TJ, Tribune DJ, Mutch DG, Gersell DJ, Goodfellow PJ: Allelic loss of sequences from the long arm of chromosome-10 and replication errors in endometrial cancers. Cancer Res 1995, 55:1922–1926
23. Bussaglia E, del Rio E, Matias-Guiu X, Prat J: PTEN mutations in endometrial carcinomas: a molecular and clinicopathologic analysis of 38 cases. Hum Pathol 2000, 31:312–317
24. Kong D, Suzuki A, Zou TT, Sakurada A, Kemp LW, Wakatsuki S, Yokoyama T, Yamakawa H, Furukawa T, Sato M, Ohuch N, Sato S, Yin J, Wang S, Abraham JM, Souza RF, Smolinski KN, Meltzer SJ, Horii A: PTEN1 is frequently mutated in primary endometrial carcinomas. Nat Genet 1997, 17:143–144
25. Risinger JI, Hayes AK, Berchuck A, Barrett J: PTEN/MMAC1 mutations in endometrial cancers. Cancer Res 1997, 57:4736–4738
26. Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH: Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 1997, 57:3935–3940
27. Torres J, Pulido R: The tumor suppressor PTBN is phosphorylated by the protein kinase CK 2 at its C-terminus. J. Biol Chem 2001 276:993–998
28. Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F, Pinna LA, Ruzzene M: Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death Differ 2005, 12:668–677
29. Song DH, Dominguez I, Mizuno J, Kaut M, Mohr SC, Seldin D: CK2 phosphorylation of the armadillo repeat region of beta-catenin potentiates Wnt signaling. J Biol Chem 2003, 278:24018–24025
30. Gao Y, Wang HY: Casein kinase 2 is activated and essential for Wnt/beta-catenin signaling. J Biol Chem 2006, 281:18394–18400
31. Wang S, Jones KA: CK2 controls the recruitment of Wnt regulators to target genes in vivo. Curr Biol 2006, 16:2239–2244
32. Ahmed K: Significance of the casein kinase system in cell growth and proliferation with emphasis on studies of the androgenic regulation of the prostate. Cell Mol Biol Res 1994, 40:1–11
33. Tawfic S, Goueli SA, Olson MOJ, Ahmed K: Androgenic regulation of phosphorylation and stability of nucleolar protein nucleolin in rat ventral prostate. Prostate 1994, 24:101–106
34. Tawfic S, Olson MOJ, Ahmed K: Role of protein-phosphorylation in posttranslational regulation of protein B23 during programmed cell-death in the prostate-gland. J Biol Chem 1995, 270: 21009–21015
35. Wang G, Ahmad KA, Ahmed K: Role of protein kinase CK2 in the regulation of tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis in prostate cancer cells. Cancer Res 2006, 66:2242–2249
36. Ahmed K, Gerber DA, Cochet C: Joining the cell survival squad: an emerging role for protein kinase CK2. Trends Cell Biol 2002, 12:226–230
37. Litchfield DW: Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. Biochem J 2003, 369:1–15
38. Machin P, Catasus L, Pons C, Munoz J, Matias-Guiu X, Prat J: CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. Hum Pathol 2002, 33:206–212
39. Moreno-Bueno G, Rodriguez-Perales S, Sanchez-Estevez C, Hardisson D, Sarrio D, Prat J, Cigudosa JC, Matias-Guiu X, Palacios J: Cyclin D1 gene (CCND1) mutations in endometrial cancer. Oncogene 2003, 22:6115–6118
40. Landesman-Bollag EL, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC: Protein kinase CK2 in mammary gland tumorigenesis. Oncogene 2001, 20:3247–3257
41. Yde CW, Olsen BB, Meek D, Watanabe N, Guerra B: The regulatory β-subunit of protein kinase CK2 regulates cell-cycle progression at the onset of mitosis. Oncogene 2008, 27:4986–4997

Objectiu 2

4. EL SORAFENIB INDUEIX APOPTOSI I SENSIBILITZA LES CÈL·LULES CANCEROSES D'ENDOMETRI A TRAIL VIA MECANISMES MOLECULARS DIFERENTS.

4.1 El sorafenib indueix mort cel·lular en cèl·lules de carcinoma d'endometri.

4.2 El sorafenib sensibilitza les cèl·lules de carcinoma d'endometri a l'apoptosi induida per TRAIL i Fas.

4.3 La sensibilització a TRAIL és independent de l'activitat cinasa de B-Raf i MEK/ERK.

4.4 El sorafenib indueix la degradació via proteasoma de FLIP i Mcl-1.

4.5 L'expressió forçada de Mcl-1 però no de FLIP preven l'apoptosi induida per el sorafenib

4.6 La sobreexpressió de FLIP però no la de Mcl-1 restaura la resistència a TRAIL i Fas.

4.7 El sorafenib sensibilitza les cèl·lules de cultius primaris de carcinoma d'endometri a l'apoptosi induida per TRAIL.

***(Llobet D**, Eritja N, Yeramian A, Pallarés J, Sorolla A, Santacana M, González-Tallada FJ, Matias-Guiu X, Dolcet X. Sorafenib induces apoptosis and sensitizes endometrial cancer cells to TRAIL by different molecular mechanisms. [sotmès a Journal of Cellular and Molecular Medicine].)*

SORAFENIB INDUCES APOPTOSIS AND SENSITIZES ENDOMETRIAL CANCER CELLS TO TRAIL BY DIFFERENT MOLECULAR MECHANISMS.

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Running title: Molecular mechanisms of Sorafenib-incuced apoptosis in ECCs.

Keywords: endometrial carcinoma, apoptosis, death receptor, Sorafenib, ERK/MAPK, Mcl-1, FLIP.

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ABSTRACT.

Sorafenib induces apoptosis and enhances TRAIL induced cell killing of tumoral cells. We have investigated the effects of the multikinase inhibitor Sorafenib alone or in combination with TRAIL and agonistic Fas antibodies on endometrial carcinoma cells. Then, we have focused in the search of the differential molecular mechanisms by which Sorafenib induces cell death and the ones involved in sensitization to TRAIL. In the present study, we show that Sorafenib induces apoptosis of both endometrial cancer cell lines and human primary cultures and sensitized these cells to TRAIL and aFas-induced apoptosis. However, Raf/MEK/ERK inhibition by Sorafenib was not responsible for Sorafenib cell death or TRAIL sensitization of endometrial cancer cells. Sorafenib treatment correlated with a downregulation of both FLIP and Mcl-1, caused by a proteasomal degradation of both proteins. We evaluated the contribution of FLIP and Mcl-1 downregulation in apoptosis triggered by Sorafenib alone or Sorafenib plus TRAIL. Interestingly, cell death caused by Sorafenib was mediated by downregulation of Mcl-1, but not by FLIP. In contrast, we found that Sorafenib sensitization of endometrial carcinoma cells to TRAIL-and Fas-induced apoptosis was dependent on FLIP but not on Mcl-1 downregulation. Altogether, we discern the dual mechanisms by which Sorafenib causes cell death from those involved in death receptor sensitization.

INTRODUCTION

The Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and Fas Ligand (FasL) belong to the pro-apoptotic cytokines of the Tumor Necrosis Factor (TNF) superfamily. TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells (Ashkenazi et al., 1999) (Walczak et al., 1999) indicating that it may become a promising anticancer agent (Srivastava, 2001) (Takeda et al., 2007). TRAIL-based therapies are under current clinical trials in Phase I and II (Johnstone et al., 2008). However, an increasing number of tumoral cell types display resistance to TRAIL-mediated cell killing. To circumvent such resistance, combinatorial therapies to inhibit molecular determinants involved in inhibition of apoptosis triggered by TRAIL would be very important. TRAIL triggers apoptosis by binding one of its functional receptors DR4 or DR5 (LeBlanc & Ashkenazi, 2003). Engagement of DR4 or DR5 receptors leads to the formation of a Death-Inducing Signaling Complex (DISC). The Death Domain (DD) of these receptors recruits Fas Associated DD-containing protein (FADD) which in turn binds pro-caspase-8. After recruitment to the DISC, pro-caspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signaling (Bodmer et al., 2000; Kischkel et al., 2000; Sprick et al., 2000). One of the key regulators of apoptosis triggered by either FasL and TRAIL is the FLICE-Inhibitory Protein (FLIP) (Irmler et al., 2000; Thome et al., 1997). High levels of FLIP are found in many tumoral tissues including endometrial carcinoma. We have previously shown that FLIP plays a critical role in the regulation of sensitivity of endometrial carcinoma cells (ECC) to TRAIL-induced apoptosis. In this previous work, we demonstrated that siRNA mediated inhibition of FLIP sensitized endometrial cancer cells to TRAIL-induced apoptosis (Dolcet et al., 2005). FLIP shares a high degree of homology with caspase-8, and contains two Death Efector Domains (DED) and a defective caspase-like domain that lacks proteolytic activity. Thus, high levels of FLIP compete with caspase-8 and displace its binding to FADD, which results in inhibition of apoptosis.

Sorafenib (also known as Bay 43-9006, Nexavar) was initially identified as a Raf-1, but subsequent studies revealed that Sorafenib is a multikinase inhibitor with activity over several kinases, including B-Raf on its wild type and V600 mutated forms; tyrosine

kinase receptors such as plateled –derived growth factor, vascular-endothelial growth factors 1 and 2, c-Kit, FLT3 or Ret (Flaherty, 2007; Wilhelm et al., 2006). Sorafenib is currently administrated as chemotherapeutic agent to patients with advanced renal cell carcinoma, and there are ongoing clinical trials for melanoma, hepatocellular carcinoma and non-small lung cancer (Flaherty, 2007; Wilhelm et al., 2006).

Recent findings evidence that Sorafenib may enhance TRAIL-induced cell killing on cancer cells (Meng et al., 2007; Ricci et al., 2007; Rosato et al., 2007). The proposed molecular mechanisms by which Sorafenib sensitizes cancer cells to TRAIL include downregulation of the myeloid cell leukemia-1 (Mcl-1) (Meng et al., 2007), downregulation of Mcl-1 together with FLIP protein levels (Rosato et al., 2007) or a transcriptional reduction of c-IAP2 and Mcl-1 (Ricci et al., 2007). Moreover, the role of Raf kinase activity and their downstream kinases MAPK/ERK kinase (MEK) and Mitogen-Activated Protein Kinase/extrecellular-Regulated Kinase (ERK/MAPKs) as a mechanistic effector of Sorafenib anti-tumour effects is uncertain.

Here, we demonstrated that Sorafenib induced apoptosis in endometrial carcinoma cell (ECC) lines and sensitized ECC and primary cultures from endometrial carcinoma patients to TRAIL-induced apoptosis. Long-term exposure to Sorafenib alone triggered apoptosis of ECC. However, short-exposure periods to Sorafenib had no killing effects, but dramatically enhanced TRAIL- and agonistic Fas antibody- (aFas) induced apoptosis. Then, we focused in the search of the differential molecular mechanisms by which Sorafenib induces cell death and the ones involved in sensitization to TRAIL. Sorafenib sensitization to TRAIL was independent of B-Raf kinase activity or MEK/ERK inhibition. Sorafenib sensitization correlated with downregulation of FLIP protein levels. Sorafenib mediated FLIP reduction was not caused by transcriptional repression of FLIP but by proteasome degradation, since co-treatment with proteasome inhibitors completely prevented reduction of FLIP levels. Accordingly, FLIP overexpression was sufficient to inhibit Sorafenib sensitization to TRAIL. In contrast, overexpression of Mcl-1, which effectively prevents apoptosis induced by Sorafenib, did not prevent cells from TRAIL plus Sorafenib-induced apoptosis. Because of the given importance of Sorafenib and TRAIL in cancer therapy, we exposed primary cultures obtained from biopsies of patients with endometrial carcinoma to TRAIL plus Sorafenib. Accordingly to the results obtained in cell lines, Sorafenib sensitized such cancer cells to apoptosis and reduced both Mcl-1 and FLIP levels.

RESULTS

Sorafenib induces apoptotic cell death of ECCs .

To begin with, we explored the sensitivity of endometrial carcinoma cell lines to Sorafenib-induced cell killing. For this purpose, we exposed Ishikawa (IK), HEC-1A, RL-95/2 and KLE endometrial carcinoma cell lines to increasing doses of Sorafenib and we evaluated cytotoxicity by LDH release after 24 or 48 hours. Sorafenib induced a dose-dependent release of LDH of all four cell lines. It is worth to mention that Ishikawa, RL-95/2 and HEC-1A displayed maximum cytotoxicity at 24 hours of exposure whereas KLE did not show significant increase on cytotoxicity after 48 hours (Fig 1A). Because we observed similar effects on cytotoxicity over all cell lines we chose IK cells to further analyze caspase activation and PARP processing. A time course treatment of IK cells induced a detectable caspase-3, caspase-9 and PARP processing after 12 and 24 hours of exposure to 20 μM Sorafenib (Fig 1B). The above results indicate that Sorafenib induces apoptotic cell death of endometrial cell lines.

Sorafenib sensitizes endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis.

Next, we investigated whether Sorafenib may sensitize resistant cells to TRAIL- and Fas-induced apoptosis. As demonstrated above, Sorafenib alone triggered apoptosis at 24 or 48 hours of treatment. However, 8 hours of treatment with Sorafenib alone caused a slightly increase of cytotoxicity (Supplementary Fig 1A). To analyze whether Sorafenib may sensitize ECCs to death receptor-induced apoptosis, we exposed IK cells to 20μM Sorafenib in presence or absence of aFas or TRAIL. After 8 hours of treatment, we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining and we assessed caspase processing and activation by western blot to initiator caspases -8, -9, and the effector caspase-3. After 8 hours, Sorafenib alone caused only a slightly increase on apoptotic IK cells, but co-treatment with either aFas or TRAIL plus Sorafenib induced a marked increase on the number of nuclei displaying apoptotic morphology as assessed by Hoechst (Fig 1C, Supplementary Fig 1B) and processing of caspases-8, -9 and -3 (Fig 1C). Similar results were obtained with KLE cells (Supplementary Fig 1C, Fig 1D).

These results demonstrate that Sorafenib not only induces apoptosis but also sensitizes endometrial cancer cells to TRAIL and aFas apoptosis.

Sorafenib sensitization to TRAIL is independent of B-Raf and MEK/ERK kinase activity.

One of the substrates of Sorafenib inhibitory activity is B-Raf, which regulates the activation of MAPK/ERK pathway. Therefore, we examined whether Sorafenib sensitization to TRAIL was caused by inhibition of the ERK/MAPKs. Treatment of both IK and KLE cells resulted in a significant decrease of ERK phosphorylation, suggesting that Sorafenib inhibited ERK/MAPK kinase signaling (Fig 2A). Next, we analyzed whether apoptosis sensitization by Sorafenib was the result of inhibition of B-Raf kinase activity or the downstream MEK/ERK kinases. To assess this point, we first infected IK cells with a plasmid encoding a wild type B-Raf or a kinase-dead B-Raf K483M mutant (B-Raf KD). After 24-48 hours, cells were exposed to TRAIL or aFas and we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining, and we assessed caspase processing and activation by western blot to initiator caspases -8, -9, and the effector caspase-3. B-Raf K483M did not cause neither increase in the number of apoptotic nuclei (Fig 2B, Supplementary Figure 2A) nor activation of any of the caspases analyzed (Fig 2C). As a control for sensitization to TRAIL, we infected parallel cultures with lentiviruses carrying shRNA against FLIP. Similarly, treatment of cultures with the specific MEK inhibitor UO126 failed to sensitize IK cells to TRAIL- or aFas-induced apoptosis as assessed by Hoechst staining (Fig 2D, Supplementary Figure 2B) or caspase activation (Fig 2E). As a control, we treated parallel cultures with DRB which we have previously demonstrated to sensitize ECCs to TRAIL and aFas (Llobet et al., 2008).

The above results suggest that the mechanism of sensitization to TRAIL or aFas is independent of inhibition of B-Raf kinase activity or inactivation of the MEK/ERK signaling cascade.

Sorafenib triggers proteasome-mediated degradation of FLIP and Mcl-1.

Having demonstrated that the effects of Sorafenib on ECC seem to be independent of MEK/ERK signaling, we focused our investigations in the search of mechanisms by which Sorafenib kills ECC and sensitizes to death receptor apoptosis. Recent evidences point to Mcl-1 as an important molecule involved in regulation of both apoptosis induced by Sorafenib and apoptosis triggered by combination of Sorafenib plus TRAIL. Moreover, we have previously demonstrated that FLIP is critical in regulation of

TRAIL-induced apoptosis of ECC (Dolcet et al., 2005; Llobet et al., 2008). These evidences enabled us to check whether Soranefib may regulate FLIP and Mcl-1. For this purpose, we performed a time-course analysis of expression of both FLIP and Mcl-1 of IK cells treated with Sorafenib. Both Mcl-1 and FLIP expression was markedly reduced within the first 24 hours of treatment with Sorafenib (Fig 3A, 3B). In contrast, the levels of Bcl-XL did not change at any time-point analyzed. Noteworthy, decrease of FLIP expression was a rapid event and it became evident as soon as 3 hours of Sorafenib treatment. Such downregulation coincided with the rapid sensitization of IK cells to TRAIL and aFas. Similar results were obtained when KLE cells were treated for 24 or 48 hours with Sorafenib (Fig 3A, 3B).

Next, we investigated the mechanisms by which Sorafenib regulates FLIP and Mcl-1 levels. The levels of endogenous FLIP protein can be controlled transcriptionally but, recent evidences also suggest that endogenous FLIP protein levels may be regulated by the ubiquitin proteasome system. To ascertain whether FLIP levels are transcriptionally regulated, we performed real-time PCR on mRNA extracted from IK cells treated with Sorafenib for 2 or 9 hours. As a control, parallel cultures were treated for 9 hours with DRB or apigenin which we have recently demonstrated that reduce FLIP mRNA levels (Llobet et al., 2008). Sorafenib treatment did not reduce the levels of FLIP mRNA, suggesting that Sorafenib regulates FLIP protein at post-transcriptional level (Fig 3C).

Both Mcl-1 (Liu et al., 2005; Shmueli & Oren, 2005; Warr et al., 2005; Zhong et al., 2005) and FLIP (Chang et al., 2006; Kim et al., 2002; Poukkula et al., 2005) protein levels are also regulated by ubiquitin-proteasome-mediated degradation. To determine whether proteasomal degradation was also involved in downregulation of Mcl-1 and FLIP after Sorafenib treatment, we treated IK cells with Sorafenib in presence or absence of the proteasome inhibitor MG-132. As we show in figure 3, addition of MG-132 completely inhibited the reduction in FLIP (Fig 3D) and Mcl-1 protein (Fig3E) caused by Sorafenib. These results suggest that Sorafenib triggers Mcl-1 and FLIP degradation through the proteasome.

Expression of Mcl-1 but not FLIP prevents Sorafenib-induces apoptosis.

Next, we evaluated the contribution of FLIP and Mcl-1 downregulation in apoptosis induced by Sorafenib alone. For this purpose, we infected IK cells with lentiviruses a carrying a plasmid encoding Flag-tagged FLIP or, we IK transfected cells pCDNA3 plasmid expressing Mcl-1. After 3 days to allow FLIP or Mcl-1 expression, cells were

treated with Sorafenib and we quantified nuclei displaying apoptotic morphology. FLIP expression did not inhibit Sorafenib-induced apoptosis at any time-point studied (Fig 4A). In contrast to FLIP, Mcl-1 overexpression significantly reduced the number of apoptotic nuclei (Fig 4B) as well as processing of caspases and the caspase substrate PARP (Fig 4C). Consistently, ectopic expression of Mcl-1 did not restore FLIP levels (Fid 4D). These results suggest that Mcl-1, but not FLIP downregulation is involved in apoptosis triggered by Sorafenib.

Expression of FLIP but not Mcl-1 restores TRAIL and aFas resistance

Both FLIP and Mcl-1 have been involved in the regulation of TRAIL sensitivity of cancer cells. Then, we examined the contribution of each of these proteins in Sorafenib-induced sensitization to TRAIL. To ascertain whether downregulation of endogenous FLIP triggered by Sorafenib was responsible for TRAIL induced apoptosis, we infected IK cells with lentiviruses carrying a plasmid encoding FLIP cDNA. After 3 days to allow FLIP expression, cells were treated with TRAIL in presence or absence of Sorafenib. Apoptotic nuclei were then visualized by Hoechst staining and caspase processing by western blotting. As shown in Figure 5A, overexpression of FLIP resulted in a significant reduction of apoptotic nuclei caused by Sorafenib plus either TRAIL or aFas. Consistent with this observation, FLIP overexpression inhibited processing of the caspases-8, -9, and -3 caused by TRAIL or aFas in presence of Sorafenib (Fig 5B).

In contrast to FLIP, expression of Mcl-1 did not prevent apoptosis triggered by treatment of ECCs with Sorafenib plus TRAIL as assessed by LDH cytotoxicity assay, Hoechst staining of apoptotic nuclei (Fig 5C) or caspase activation (Fig 5D). Interestingly, expression of FLIP restored TRAIL and aFas resistance in presence of Sorafenib but the levels of Mcl-1 remained low (Fig 5E).

Sorafenib sensitizes primary endometrial carcinoma explants to TRAIL-induced apoptosis.

TRAIL is a potential anti-cancer agent, because of its ability to trigger apoptosis in cancer cells without affecting normal cells. Humanized anti-DR4 and anti-DR5 are in advanced clinical trials (Johnstone et al., 2008; Reed, 2006). However, an increasing number of tumoral cells display mechanisms of TRAIL resistance to apoptosis. Such resistance has increased the interest of combinatorial therapies (Johnstone et al., 2008;

Takeda et al., 2007). We decided to test whether Sorafenib could be effective to kill primary endometrial carcinoma explants treated with TRAIL. We cultured different endometrial carcinoma explants obtained from biopsies of patients with endometrial carcinoma. We have previously characterized these explants to be of epithelial origin by means of cytokeratin and β-catenin expression (Dolcet et al., 2006).

First, we analyzed the levels of phosphorylated ERK by western blot in 3 different primary explants treated or not with Sorafenib. As we observed for endometrial cancer cell lines, we found that Sorafenib reduced ERK phosphorylation (Fig 6A). In agreement with the results observed in endometrial carcinoma cell lines, treatment of parallel primary culture explants with Sorafenib caused a marked downregulation of both FLIP and Mcl-1 protein levels (Fig 6B). Moreover, Sorafenib alone caused activation of caspase-3 which was further increased after addition of TRAIL or aFas (Fig 6B). Accordingly, treatment of parallel explants with Sorafenib plus either TRAIL or aFas caused an increased cytotoxicity and nuclei displaying apoptotic morphology (Fig 6C). Moreover, Sorafenib plus TRAIL treatment activated capases-8, -9, and -3 (Fig 6D).

All the above results suggest that co-treatment with TRAIL and Sorafenib may be useful strategy to induce apoptosis of endometrial cancer cells.

DISCUSSION

In the present study we have assessed the effects of the multikinase inhibitor Sorafenib on endometrial carcinoma cell lines and primary cultures. We provide evidence of the differential mechanisms underlying Sorafenib-induced apoptosis from those involved in sensitization or enhancement of TRAIL-induced apoptosis.

First, we have demonstrated that Sorafenib causes a dose-dependent killing of endometrial carcinoma cells. Such cell death displayed features of apoptosis as cells had typical apoptotic morphology and activation of caspases 3 and 9. Moreover, we showed that at time points were Sorafenib alone did not induce apoptosis; it sensitized ECCs to apoptosis induced by TRAIL. TRAIL plus Sorafenib treatment resulted in activation of the extrinsic apoptotic pathway with concomitant caspase-8 processing.

Among other kinases, B-Raf is a target of kinase inhibitory activity of Sorafenib. Raf isoforms are top of Raf/MEK/ERK signaling and activation of serine/threonine kinase of Raf results in phosphorylation of MEK which in turn phosphorylate ERK/MAPKs. Inhibition of ERK by specific inhibitors sensitizes or enhances TRAIL-induced apoptosis of melanoma or breast cancer cells (Grosse-Wilde et al., 2008; Lee et al., 2006; Ortiz-Ferron et al., 2006; Tran et al., 2001; Zhang et al., 2003). In contrast, others have shown that ERK inhibition does not change the apoptotic response of TRAIL resistant cells (Vaculova et al., 2006) or that ERK2 activation is even required for induction of apoptosis by TRAIL (Frese et al., 2003). Because of this duality about the role of ERKs in TRAIL apoptosis, our first question was whether sensitization to TRAIL by Sorafenib could be caused by inhibition of the MAPK pathway. We have found that inhibition of ERK/MAPK signaling pathway does not result in sensitization to TRAIL, suggesting that the inhibitory effects of Sorafenib on ERK/MAPK activity was not responsible for sensitization to TRAIL. Recent reports have demonstrated that Sorafenib enhances TRAIL induced apoptosis in other cell types (Meng et al., 2007; Ricci et al., 2007; Rosato et al., 2007). However, it is not fully demonstrated whether inhibition of kinase activity of B-Raf by Sorafenib is involved in the sensitization of cancer cells to TRAIL- or FasL- apoptosis. In line with the results obtained with U0126-mediated inhibition of ERK, we have found that expression of Kinase inactive form of B-Raf did not sensitized ECCs to TRAIL apoptosis. These results demonstrate that Sorafenib sensitizes ECCs cells to TRAIL and aFas apoptosis by a Raf/MEK/ERK

independent mechanism. To this regard, increasing evidences support the hypothesis that Raf isoforms may promote survival independent of MAPK signaling (Hindley & Kolch, 2002; Kolch et al., 2002). Moreover, mice lacking Raf-1 are embryonic lethal, but mice expressing kinase inactive form display a normal phenotype which strongly suggest kinase-independent effects of Raf proteins (Huser et al., 2001). Interestingly, Raf-1 can control proapoptotic proteins such as MST2 independently of its MEK kinase activity (O'Neill & Kolch, 2005; O'Neill et al., 2004)

Next, we found that apoptosis triggered by Sorafenib correlated with downregulation of both Mcl-1 and FLIP. Both Mcl-1 and FLIP have been associated with Sorafenib-induced cytotoxicity. To date, Sorafenib induces downregulation of Mcl-1 though inhibition of its translation (Rahmani et al., 2005) or Mcl-1 stabilization (Ding et al., 2008; Yu et al., 2005). Recent findings also demonstrate that Sorafenib in combination with Vorinostat induces autonomous cell death by decreasing FLIP levels and increasing CD95 activation (Zhang et al., 2008). We have found that overexpression of Mcl-1 but not FLIP reduced apoptosis triggered by Sorafenib. Noteworthy, although we were able to inhibit Sorafenib induced apoptosis by overexpressing Mcl-1, FLIP protein remained at low levels. Previous works from our laboratory demonstrated that FLIP protein levels are the main regulator of live/dead decisions of ECCs after exposure to TRAIL and aFas (Dolcet et al., 2005; Llobet et al., 2008). If FLIP levels are really important, Sorafenib treated cells should undergo apoptosis after TRAIL treatment even in cells with overexpressed Mcl-1. Indeed, we have found that Mcl-1 overexpression did not protect from Sorafenib plus TRAIL-induced apoptosis. In contrast, FLIP overexpression restored TRAIL resistance in presence of Sorafenib. The fact that Mcl-1 protein was kept at low levels when FLIP was ectopically expressed, reinforces the hypothesis that downregulation of Mcl-1 is not responsible for sensitization to TRAIL caused by Sorafenib.

The role of FLIP in cancer has been widely demonstrated. FLIP is constitutively or highly expressed in different types of malignancies such as prostate cancer (Zhang et al., 2004), Hodgkin lymphoma (Dutton et al., 2004), gastric cancer (Lee et al., 2003), bladder carcinoma (Korkolopoulou et al., 2004), malignant mesothelial cell lines (Rippo et al., 2004) and endometrial carcinoma (Dolcet et al., 2005). We previously demonstrated that siRNA to FLIP is enough to sensitize IK cells to TRAIL-induced apoptosis (Dolcet et al., 2005), suggesting that FLIP levels are critical in sensitization to TRAIL-induced apoptosis. We also explored the mechanism by which Sorafenib may

regulated FLIP protein levels. Recent findings have demonstrated that Sorafenib regulates FLIP by inhibition of translation (Rosato et al., 2007). Our results suggest that Sorafenib induces downregulation of FLIP by inducing its ubiquitin-proteasome degradation, without changing FLIP mRNA levels. FLIP amount of protein can be controlled at different points. FLIP can be transcriptionally downregulated by some anti-neoplasic drugs such as 5-fluorouracil, oxaliplatin and irinotecan in colon carcinoma cells (Galligan et al., 2005). Such FLIP mRNA downregulation has been shown to sensitize these cells to TRAIL induced apoptosis. FLIP levels are also regulated by ubiquitin-proteasome-mediated degradation (Chang et al., 2006; Kim et al., 2002; Poukkula et al., 2005). In fact, some anticancer drugs such as the cyclooxygenase-2 inhibitor celecoxib (Liu et al., 2006) or the flavonoids (Son et al., 2007) and flavopiridol (Palacios et al., 2006) can sensitize cancer cells to TRAIL-induced apoptosis by inducing a proteasome-mediated degradation of FLIP. Moreover, we have recently found that in endometrial cancer cells, FLIP levels can be regulated both transcriptionally and through its degradation by the ubiquitin-proteasome system (Llobet et al., 2008).

Finally, we demonstrated that Sorafenib sensitized primary endometrial carcinoma explants to TRAIL-induced apoptosis. Recombinant TRAIL or agonistic anti-TRAIL receptor antibodies are in current clinical trials for treatment of both solid and haematological malignancies (Johnstone et al., 2008). Although these agents show some anti-tumoral activity as a monotherapy, increasing evidences demonstrate that these agents may be more effective used in combination with other anti-cancer treatments. These observations may be because there are many tumoral cell types that display resistance to apoptosis after TRAIL exposure. Our previous data support the hypothesis that most of endometrial cancer cell lines and primary cultures are insensitive to TRAIL (Dolcet et al., 2005; Llobet et al., 2008). Therefore, combination of treatments may be a useful tool to sensitize ECCs to TRAIL. Here, the data obtained in both cell lines and primary explants suggest that treatments with TRAIL together with Sorafenib may be interesting for combinatorial therapies for endometrial carcinomas.

In summary, our results evidence that the mechanistic effectors of apoptosis triggered by Sorafenib or by combination of Sorafenib with TRAIL or aFas are different. Whereas Mcl-1 is important for Sorafenib-induced apoptosis, FLIP but not Mcl-1 is involved in sensitization to TRAIL- or aFas- induced apoptosis by Sorafenib. Such molecular duality may be useful to induce apoptosis in cancer cells displaying

resistance to apoptosis. That is, if a cancer cell type display resistance to Sorafenib treatment due to increased Mcl-1 expression, combination of TRAIL plus Sorafenib can be useful to reduce FLIP levels and sensitize these cells to apoptosis triggered by TRAIL. On the other hand, FLIP is constitutively expressed in many tumours conferring to these cells resistance to death receptor-induced apoptosis. In this scenario, Sorafenib treatment can bypass apoptosis resistance by reducing Mcl-1 levels.

MATERIAL AND METHODS

Reagents, plasmids and antibodies - 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to Tubulin and anti-Flag M2 were from Sigma (St Louis, MO). Kinase inhibitors PD98059, DRB and apigenin, proteasome inhibitor MG-132, monoclonal antibody to caspase-8 and human recombinant TRAIL were from Calbiochem (La Jolla, CA). Antibody to caspase 9 and cleaved caspase-3 were obtained from Cell Signalling (Beverly, MA). Monoclonal antibody to FLIP (NF6) and agonistic Fas antibody (aFas) were purchased from Alexis Corp (Lausen, Switzerland). Antibody to Mcl-1 was purchased from BDbiosciences (San Jose, CA). Antibody to PARP was from Neomarkers. Anti-B-Raf antibody was from SantaCruz Biotechnology, Inc (SantaCruz, CA). Peroxidase-congugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden). BAY 43-9006 (Sorafenib) was provided by Bayer Pharmaceuticals (New Haven CT).

Lentiviral vector containing Flag-tagged mouse FLIP cDNA was a gift from Dr. Joan Comella (Dept de Bioquímica i Biologia Molecular, Universitat Autonoma de Barcelona, Barcelona). The pCDNA3 vector encoding Mcl-1 cDNA was a generous gift from Dr Isabel Marzo

Cell lines, Culture Conditions and Transfection - The Ishikawa 3-H-12 cell line (IK) was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95/2 and HEC-1-A cells were a gift from Dr Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbeco's modified Eagles Medium (DMEM) (Sigma) supplemented with 10% Foetal Bovine Serum (Invitrogen, Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicilin/streptomicin (Sigma) at 37ºC with saturating humidity and 5% CO_2 .

When indicated, transfections plasmids constructs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.

Explant culture of endometrial adenocarcinoma - Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (JP). A specific informed consent was obtained from each patient, and the study was approved by the local Ethic Committee. Tissue was collected in DMEM, chopped in 1 mm pieces and incubated

with collagenase in DMEM for 1,5 hours at 37ºC with periodic mixing. Digested tissue was mechanically dissociated through a 10 ml pipette and a 1 ml blue tip and resuspended in 2 ml of fresh DMEM medium. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM medium and tissue was allowed to sediment by gravity for 5 minutes. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% Foetal Bovine Serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% of penicilin/streptomicin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37ºC with saturating humidity and 5% CO_2..

Lentiviral production and infection - Oligonucleotides to produce FLIP plasmid based shRNA were cloned into the FSV vector using AgeI-BamHI restriction sites. shRNA target sequence was CCTCACCTTGTTTCGGACTA. To produce infective FLIP overexpressing lentiviral particles, 293T cells were co-transfected by calcium phosphate method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA producing vector (FSV) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3-4 days in same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 minutes at 1000 rpm and filtered through a 0.45 μM filter (Millipore). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24-48 hours in presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for two additional days to allow endogenous protein knock-down or protein overexpression.

Cell viability assays and assessement of apoptosis - Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated on M96 well plates at $15x10^3$ cells per well. After the indicated treatments, the cells were incubated for 2-3 hours with 0.5 mg/ml of MTT reagent and lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmon, CA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0,5 mg/ml to each M96 well. Cells were counted under epifuorescence microscope (Leica Microsystems).

Cytotoxicity assay kit was pucharsed from Roche Diagnostics (Indianapolis, IN, USA). Cells were first plated as described previously in materials and methods cell culture section. After treatment, 100 ul of supernatant were transfered in a M96 multiwell plate.

To determine LDH activity 100 ul of freshly prepared reaction mixture were added to each well and incubated for up to 30 minutes at 15-25 grades as described by manufacturer's instructions. Absorbance was measured at 490nm with a reference wavelenght of 600nm. Percentage of cytotoxicity for each well was referenced to positive control cells permeabilized with triton 1%.

Western Blot analysis **-** Endometrial adenocarcinoma cell lines were washed with cold PBS and lysed with lysis buffer (2% SDS, 125 mM Tris-HCL pH6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad,). Equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST (20 mM Tris-Hcl pH7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4ºC. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

RNA extraction, reverse transcription and Real-time PCR- Total RNA was extracted Ik cells treated as indicated using Trizol reagent (Invitrogen). One microgram of total RNA was converted into cDNA using Taqman Reverse Transcription Reagents (Applied biosystems P/N N808-0234) according to manufacturer's recommendations. Two microliters of the reverse transcrption reaction were used as template for a for real-time detection of human FLIP expression using TaqMan Tecnology on an Applied Biosystems 7000 sequence detection system. Gene expression quantitation was performed in separate tubes (singleplex) for both target gene and endogenous control gene using the primer and probe sequences for human FLIP and GUSB obtained commercially from Applied biosystems Assay-on-demand™ Gene (ABI P/N 4331182 :Hs00236002_m1, and ABI P/N 4326320E). The reaction was performed with 10 µl Taqman® Universal PCR Master Mix No AmpErase® UNG 2X (P/N 4324018) , 1 µl 20X Assay-on-demand™ Gene and 2 µL of complementary DNA (cDNA) diluted in RNase-free water adjusted to 20 µl volume reaction. The thermal cycler conditions was UNG activation 2 min at 50ºC, AmpliTaq activation 95ºC for 10 min, denaturation 95ºC for 15 s, and annealing/extension 60ºC for 1 minute (repeat 40 times) on ABI7000. Triplicate Ct values were analyzed with Quantitative Relative software using de comparative CT (ΔΔCT) method as described by the manufacturer. The amount of target($2^{-\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference gene (GUSB). Results are presented as a relative mRNA amount compared to the untreated samples.

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REFERENCES

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104:** 155-62.

Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, et al. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2:** 241-3.

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, et al. (2006). The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124:** 601-13.

Ding Q, Huo L, Yang JY, Xia W, Wei Y, Liao Y, et al. (2008). Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Res* **68:** 6109-17.

Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA, et al. (2006). Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* **281:** 22118-30.

Dolcet X, Llobet D, Pallares J, Rue M, Comella JX,Matias-Guiu X. (2005). FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Lab Invest* **85:** 885-94.

Dutton A, O'Neil JD, Milner AE, Reynolds GM, Starczynski J, Crocker J, et al. (2004). Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci U S A* **101:** 6611-6.

Flaherty KT. (2007). Sorafenib: delivering a targeted drug to the right targets. *Expert Rev Anticancer Ther* **7:** 617-26.

Frese S, Pirnia F, Miescher D, Krajewski S, Borner MM, Reed JC, et al. (2003). PG490-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis requires activation of ERK2. *Oncogene* **22:** 5427-35.

Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K,Johnston PG. (2005). Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* **4:** 2026-36.

Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, et al. (2008). TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* **118:** 100-10.

Hindley A,Kolch W. (2002). Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases. *J Cell Sci* **115:** 1575-81.

Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S, et al. (2001). MEK kinase activity is not necessary for Raf-1 function. *Embo J* **20:** 1940-51.

Irmler M, Steiner V, Ruegg C, Wajant H,Tschopp J. (2000). Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* **468:** 129-33.

Johnstone RW, Frew AJ,Smyth MJ. (2008). The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* **8:** 782-98.

Kim Y, Suh N, Sporn M,Reed JC. (2002). An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* **277:** 22320-9.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ,Ashkenazi A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12:** 611-20.

Kolch W, Kotwaliwale A, Vass K,Janosch P. (2002). The role of Raf kinases in malignant transformation. *Expert Rev Mol Med* **4:** 1-18.

Korkolopoulou P, Goudopoulou A, Voutsinas G, Thomas-Tsagli E, Kapralos P, Patsouris E, et al. (2004). c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. *Urology* **63:** 1198-204.

LeBlanc HN,Ashkenazi A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10:** 66-75.

Lee SH, Kim HS, Kim SY, Lee YS, Park WS, Kim SH, et al. (2003). Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. *Apmis* **111:** 309-14.

Lee TJ, Lee JT, Park JW,Kwon TK. (2006). Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun* **351:** 1024-30.

Liu H, Peng HW, Cheng YS, Yuan HS,Yang-Yen HF. (2005). Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. *Mol Cell Biol* **25:** 3117-26.

Liu X, Yue P, Schonthal AH, Khuri FR,Sun SY. (2006). Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* **66:** 11115-9.

Llobet D, Eritja N, Encinas M, Llecha N, Yeramian A, Pallares J, et al. (2008). CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells. *Oncogene* **27:** 2513-24.

Meng XW, Lee SH, Dai H, Loegering D, Yu C, Flatten K, et al. (2007). Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *J Biol Chem* **282:** 29831-46.

O'Neill E,Kolch W. (2005). Taming the Hippo: Raf-1 controls apoptosis by suppressing MST2/Hippo. *Cell Cycle* **4:** 365-7.

O'Neill E, Rushworth L, Baccarini M,Kolch W. (2004). Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science* **306:** 2267-70.

Ortiz-Ferron G, Tait SW, Robledo G, de Vries E, Borst J,Lopez-Rivas A. (2006). The mitogen-activated protein kinase pathway can inhibit TRAIL-induced apoptosis by prohibiting association of truncated Bid with mitochondria. *Cell Death Differ* **13:** 1857-65.

Palacios C, Yerbes R,Lopez-Rivas A. (2006). Flavopiridol Induces Cellular FLICE-Inhibitory Protein Degradation by the Proteasome and Promotes TRAIL-Induced Early Signaling and Apoptosis in Breast Tumor Cells. *Cancer Res* **66:** 8858-69.

Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson MS, et al. (2005). Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* **280:** 27345-55.

Rahmani M, Davis EM, Bauer C, Dent P,Grant S. (2005). Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem* **280:** 35217-27.

Reed JC. (2006). Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol* **3:** 388-98.

Ricci MS, Kim SH, Ogi K, Plastaras JP, Ling J, Wang W, et al. (2007). Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* **12:** 66-80.

Rippo MR, Moretti S, Vescovi S, Tomasetti M, Orecchia S, Amici G, et al. (2004). FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. *Oncogene* **23:** 7753-60.

Rosato RR, Almenara JA, Coe S,Grant S. (2007). The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res* **67:** 9490-500.

Shmueli A,Oren M. (2005). Life, death, and ubiquitin: taming the mule. *Cell* **121:** 963-5.

Son YG, Kim EH, Kim JY, Kim SU, Kwon TK, Yoon AR, et al. (2007). Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* **67:** 8274-84.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, et al. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12:** 599-609.

Srivastava RK. (2001). TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* **3:** 535-46.

Takeda K, Stagg J, Yagita H, Okumura K,Smyth MJ. (2007). Targeting death-inducing receptors in cancer therapy. *Oncogene* **26:** 3745-57.

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, et al. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386:** 517-21.

Tran SE, Holmstrom TH, Ahonen M, Kahari VM,Eriksson JE. (2001). MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* **276:** 16484-90.

Vaculova A, Hofmanova J, Soucek K,Kozubik A. (2006). Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. *FEBS Lett* **580:** 6565-9.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5:** 157-63.

Warr MR, Acoca S, Liu Z, Germain M, Watson M, Blanchette M, et al. (2005). BH3-ligand regulates access of MCL-1 to its E3 ligase. *FEBS Lett* **579:** 5603-8.

Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, et al. (2006). Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* **5:** 835-44.

Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH, et al. (2005). The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* **24:** 6861-9.

Zhang G, Park MA, Mitchell C, Hamed H, Rahmani M, Martin AP, et al. (2008). Vorinostat and sorafenib synergistically kill tumor cells via FLIP suppression and CD95 activation. *Clin Cancer Res* **14:** 5385-99.

Zhang X, Jin TG, Yang H, DeWolf WC, Khosravi-Far R,Olumi AF. (2004). Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* **64:** 7086-91.

Zhang XD, Borrow JM, Zhang XY, Nguyen T,Hersey P. (2003). Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene* **22:** 2869-81.

Zhong Q, Gao W, Du F,Wang X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* **121:** 1085-95.

LEGENDS TO FIGURES.

Figure1. Sorafenib induces apoptosis and sensitizes endometrial cancer cells to TRAIL and aFas. A, IK, KLE, HEC1A and RL-95/2 cells were exposed to increasing concentrations of Sorafenib for 24 or 48 hours. Supernatant lactate deshydrogenase (LDH) was quantified to measure cell death. Results are expressed as percent of cytotoxicity. B, IK cells were treated for the indicated times with Sorafenib. Western blots to active caspase-9, -3 and PARP cleavage were performed to characterize an apoptotic response. Inmunodetection of tubulin was performed to ensure equal protein amounts. C, IK cells were treated with Sorafenib or with no additives (NA) in presence or absence of TRAIL (UN). Quantification of percentage of IK cells displaying nuclei with apoptotic morphology after Hoechst staining (top) and immunodetection of activated caspases and PARP cleavage were achieved by western blot (bottom). D, KLE cells were treated with Sorafenib or with no additives (NA) in presence or absence of TRAIL (UN). Quantification of percentage of KLE cells displaying nuclei with apoptotic morphology after Hoechst staining (top) and immunodetection of activated caspases and PARP cleavage were achieved by western blot (bottom).

Figure2. Sensizitation of ECCs to TRAIL and aFas by Sorafenib is independent of ERK/MAPKs. A, Sorafenib inhibits ERK/MAPK phosphorylation. IK (left) and KLE (right) cells were treated with Sorafenib alone or in combination with TRAIL 50 ng/ml or aFas 50ng/ml respectively for 8 hours. Cells were lysed and activated ERK1/2 was analyzed by western blot. B, IK cells were infected with lentiviruses carrying either an empty vector (FCIV), a wild type form of B-Raf (B-Raf WT) or a inactive form –kinase death- of B-Raf (B-Raf KD). As a control parallel cultures were infected with FLIP shRNA lentiviruses. After 3 days to allow expression of constructs, TRAIL or aFas were added to culture media for 12 hours. Cells were stained with Hoechst and the number of nuclei displaying apoptotic morphology was quantified. C, parallel IK cell cultures were lysed and caspase activation, ERK phosphorylation, B-Raf and FLIP levels were analyzed by western blot. Membranes were reprobed with tubulin and anti-pan-ERK to ensure equal protein loading. D, Inhibition of p-ERK1/2 does not sensitize endometrium cancer cells to TRAIL and aFas-induced apoptosis. IK cells were pretreated 30’ with MEK1 inhibitor U0126 20uM or DRB (used as positive control). Next, TRAIL or aFas were added to culture media for 12 hours. Cells were stained with

Hoechst and the number of nuclei displaying apoptotic morphology was quantified. E, parallel IK cell cultures were lysed and caspase activation,ERK phosphorylation, and FLIP levels were analyzed by western blot.

Figure 3. Soranenib causes a proteasome-mediated downregulation of Mcl-1 and FLIP. A, IK cells were exposed to Sorafenib 20uM for the indicated times. Cells were lysed and protein extracts were analyzed for the expression of Mcl-1 and Bcl-XL proteins (left). Western blot showing Mcl-1 and Bcl-XL expression in KLE cells treated with Sorafenib treated for 24 and 48 hours (right). B, IK cells were exposed to Sorafenib 20uM for the indicated times. Cells were lysed and protein extracts were analyzed for the expression of FLIP protein. (left). Western blot showing FLIP downregulation in KLE cells treated with Sorafenib treated for 24 and 48 hours (right). C, Real-time PCR was performed to examine FLIP mRNA levels of IK cells treated with Sorafenib 20uM for 2 and 9 hours respectively. IK cells treated with Apigenin and DRB 50uM were performed in parallel and were considered as positive controls. D, Proteasome inhibition restores FLIP protein levels in IK cells treated with Sorafenib. Top, IK cells were pretreated with MG-132 5uM for 30 min and thereafter cells were treated with Sorafenib 20uM for the indicated periods of time. After each condition cells were lysed and FLIP protein levels were detected by western blot. Bottom, Positive control of IK cells exposed to Apigenin 50 uM alone or in combination with MG-132 5uM were executed in parallel to ensure correct MG-132 function. E, Downregulation of Mcl-1 involves proteasome-mediated degradation. IK cells were preincubated with MG-132 5uM for 30 min. Thereafter cells were coestimulated with either MG-132 in combination with Sorafenib or with Sorafenib alone during a time course. After each time point cells were lysed and protein extracts were used to determine Mcl-1 modulation by western blot.

Figure 4. Mcl-1 but not FLIP overexpression protects from Sorafenib-induced apoptosis.

A, IK cells were infected with lentiviruses containing either an empty vector (FCIV) or a vector encoding full lenght mouse Flag-tagged FLIP (FLIP ovex). After three days cells were left untreated (UN) or treated for the indicated periods of time with Sorafenib 20uM. Percentage of apoptotic nuclei was assessed by Hoechst staning. B, IK cells were transfected using lipofectamine 2000 with pCDNA3 empty vector or a pCDNA3 coding

vector for Mcl-1. IK cells were exposed to a time course treatment of Sorafenib 20uM and Hoescht staining was performed. Cells were stained with Hoechst and the percentage of apoptotic nuclei was quantified. Parallel cultures were analyzed by western blot to ensure Mcl-1 overexpression. C, Mcl-1 overexpression impedes caspase activation and PARP cleavage after Sorafenib 20 uM exposure for 48 hours. IK cells were transfected with pCDNA3 empty vector or a pCDNA3 coding vector for Mcl-1. IK cells were exposed to Sorafenib 20uM and cell lysates were analyzed by western blot with antibodies to caspases and PARP cleveage. Membranes were reprobed with Mcl-1 antibody to ensure protein overexpression and with antibodies to tubulin to ensure equal protein loading. D, FLIP is kept downregulated in IK overexpressing Mcl-1. IK cells were transfected with pCDNA3 empty vector or a pCDNA3 coding vector for Mcl-1. IK cells were exposed to Sorafenib 20uM and cell lysates were analyzed by western blot with antibodies to FLIP. Membranes were reprobed with Mcl-1 antibody to ensure protein overexpression and with antibodies to tubulin to ensure equal protein loading.

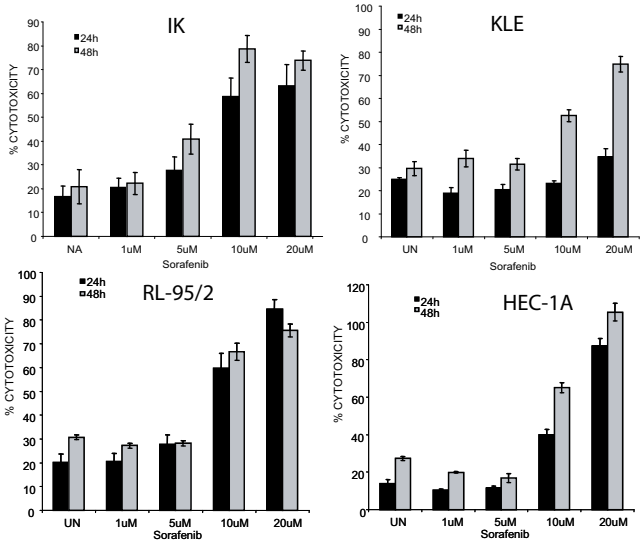
Figure 5. FLIP but not Mcl-1 protects from apoptosis triggered by Sorafenib plus TRAIL and aFas. A, IK cells were infected with lentiviruses containing either an empty vector (FCIV) or a vector encoding full lenght mouse Flag-tagged FLIP (FLIP ov). After three days cells were exposed to medium without additives (NA) or medium containing Sorafenib 20uM alone or in combination with TRAIL 50ng/ml or aFas 50ng/ml or left untreated (UN) for 8 hours. Top, quantification of nuclei displaying apoptotic cell death were visualized by Hoechst staining. Bottom, representative microphotographs of cultures stained with Hoechst. B, Parallel IK cultures were lysed and resulting protein extracts were analyzed by western blot to the indicated caspases. Immunodetection of FLAG epitope reveals correct FLIP overexpression. Membranes were reprobed with tubulin to ensure equal protein loading. C, Overexpression of Mcl-1 cannot impede cell death triggered by combination of Sorafenib plus TRAIL. IK cells were transfected with either an empty PCDNA3 vector or a PCDNA3 vector coding for Mcl-1. After two days post-transfection cells were exposed to Sorafenib for 8 hours and cytotoxicity was assessed by LDH (left) of by quantification of apoptotic nuclei visualized by Hoechst staning (right). D, parallel cultures were lysed and caspase activation was analyzed by western blot. Membranes were reprobed with Mcl-1 antibody to ensure overexpression and with tubulin to monitor equal protein loading. E,

FLIP overexpressing cells display equal Mcl-1 protein decrease compared to control infected IK cells exposed to Sorafenib 20uM.

Figure 6. Sorafenib 20uM exposure render endometrium adenocarcinoma primary explants sensitive to TRAIL-induced apoptosis. A, Three different endometrial adenocarcinoma explants (EC,1 EC2, EC3) were cultured for 2 two days and treated with Sorafenib 20uM for 12 hours. Cells were lysed and p-ERK1/2 status was checked by western blot. Inmunodetection of total ERK was performed to ensure equal protein amounts. B, Left, same endometrial carcinoma explants were cultured for two days and then exposed to Sorafenib for 12 hours. Cell lysates were used to determine FLIP protein levels. Right, EC4 endometrial carcinoma explant was pre-treated with Sorafenib for 30'. Thereafter, TRAIL and aFas were added to culture media to a final concentration of 150 ng/ml. After 12 hours cells were lysed and protein extracts were used to determine activation of executor caspase-3 or FLIP protein levels. C, EC4 culture explant was treated as in C and LDH (left) and percentage of apoptotic nuclei (right) were quantified. Top right, representative microphotographs of Hoechst stained culture explant. D, EC5 explant culture was treated with medium without additives (NA) or medium containing Sorafenib in presence or absence of TRAIL (UN). Cell lysates were analyzed by western blot showing activation of indicated caspases and reduction of FLIP protein levels. Membranes were reprobed with tubulin antibody to ensure equal loading.

FIGURE 1

A



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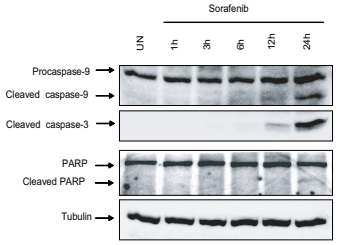

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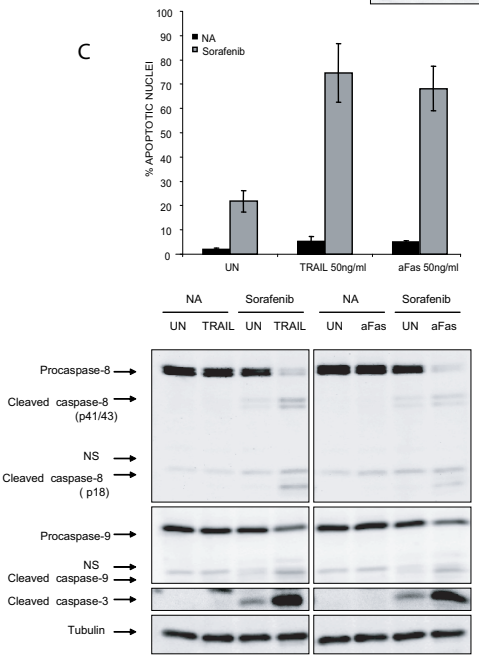

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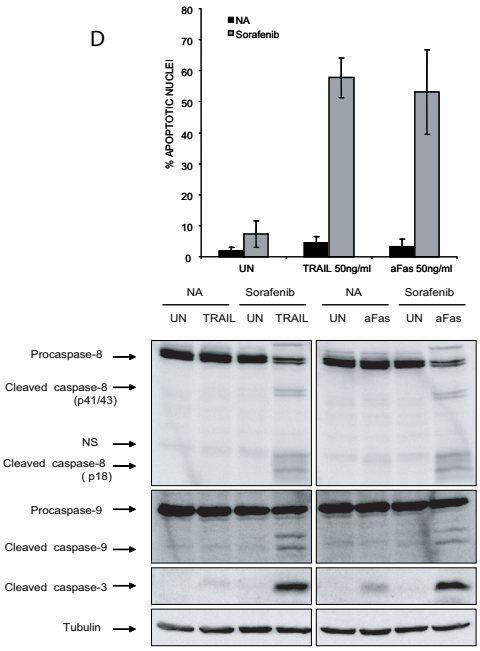
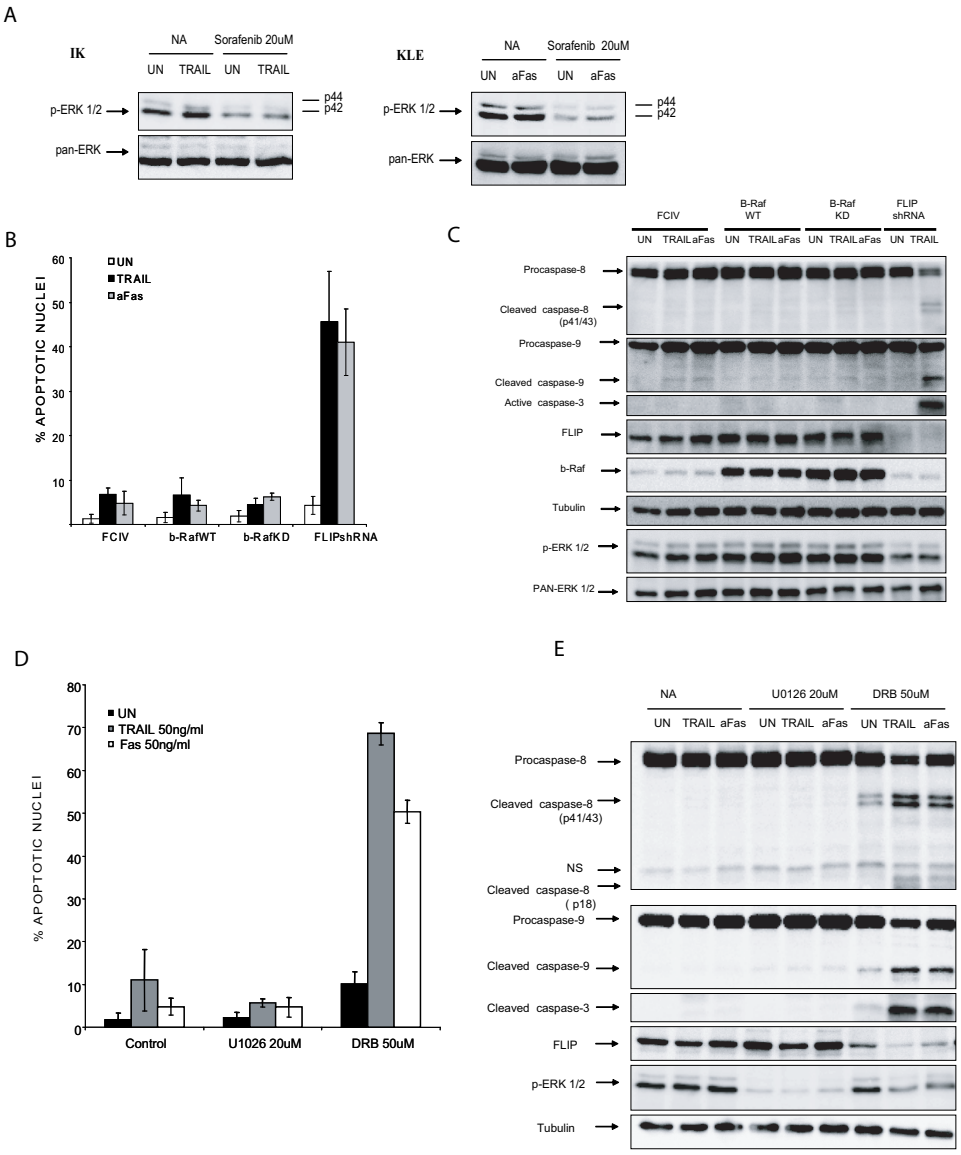


Figure 2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pan-ERK
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b-RafWT
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FLIPshRNA
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B-Raf WT
B-Raf KD
FLIP shRNA
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Procaspase-8
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Procaspase-9
Cleaved caspase-9
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b-Raf
Tubulin
p-ERK 1/2
PAN-ERK 1/2
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FLIP
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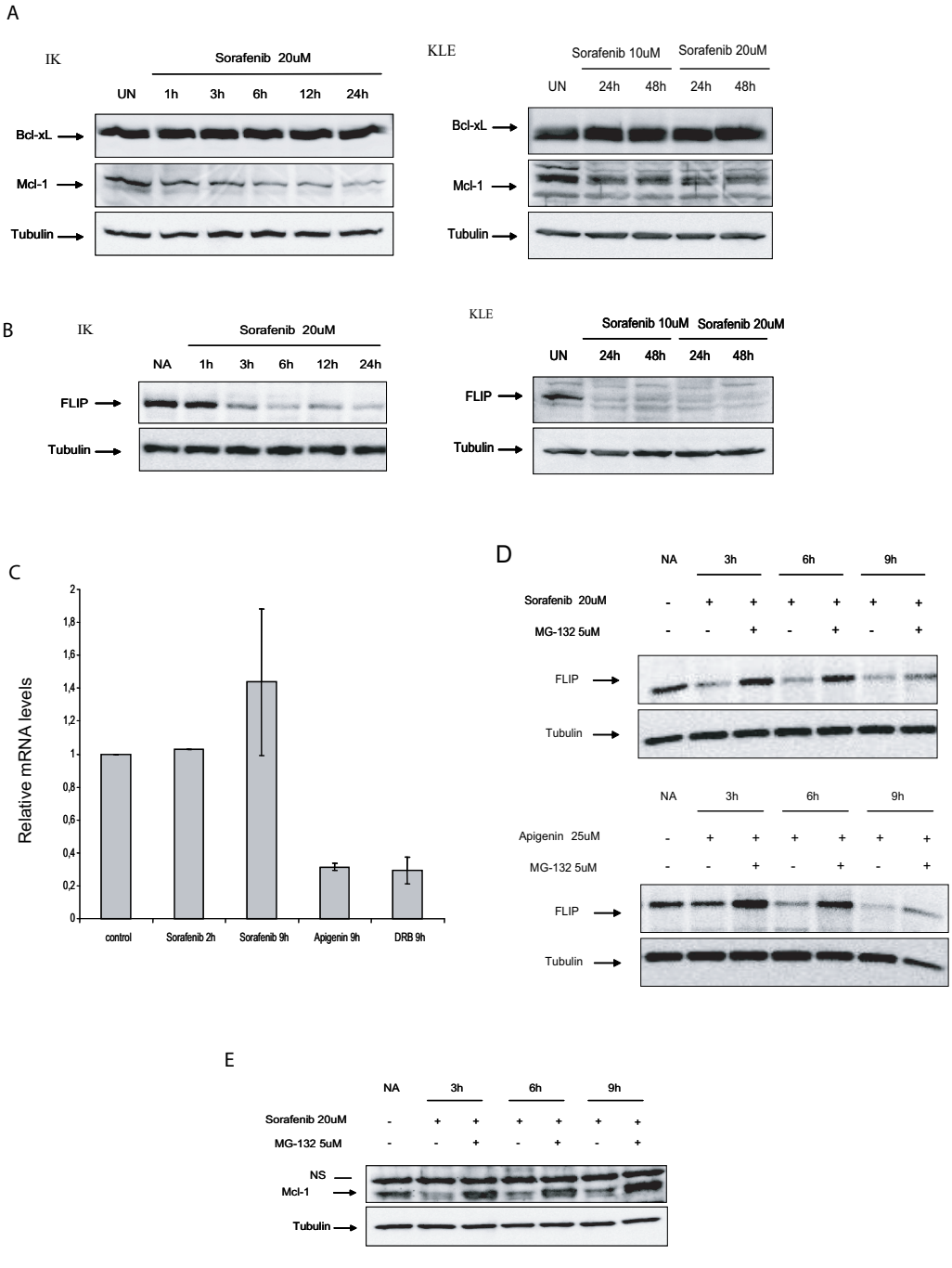
Figure 3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Apigenin 9h
DRB 9h
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Sorafenib 20uM
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Figure 4

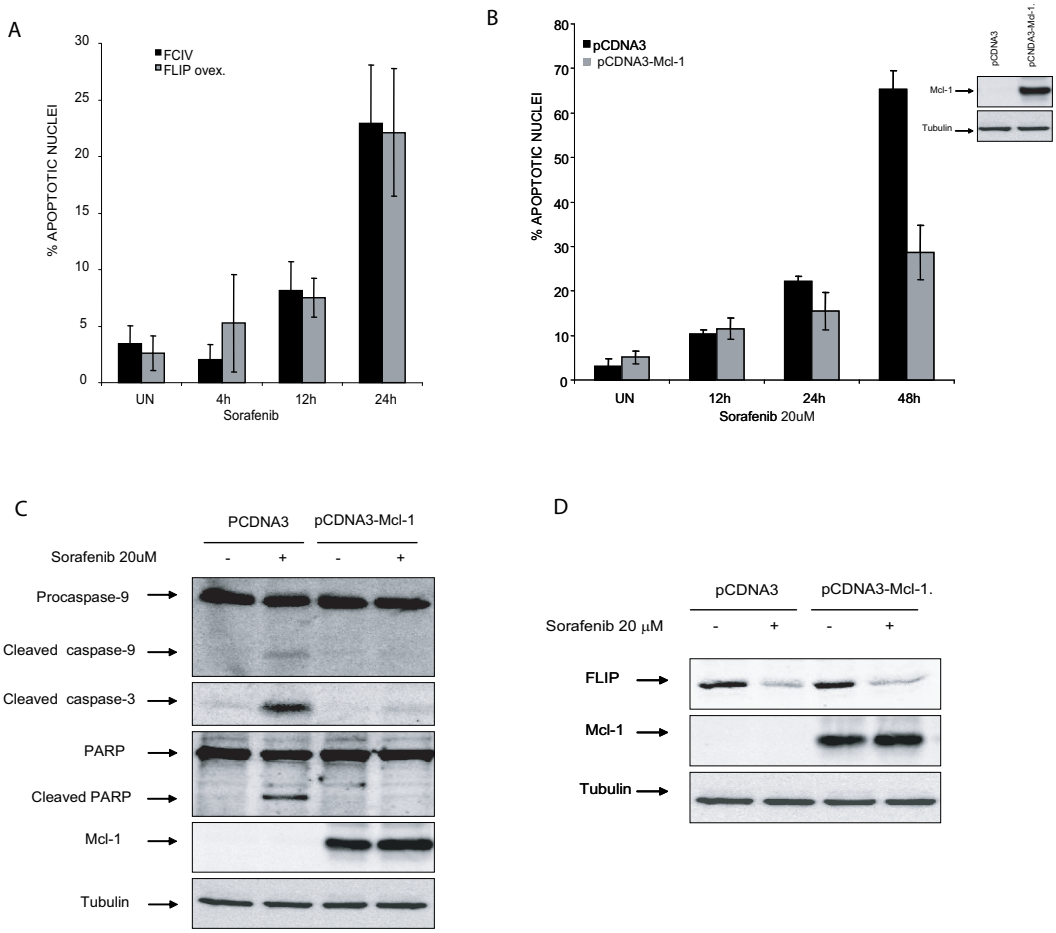
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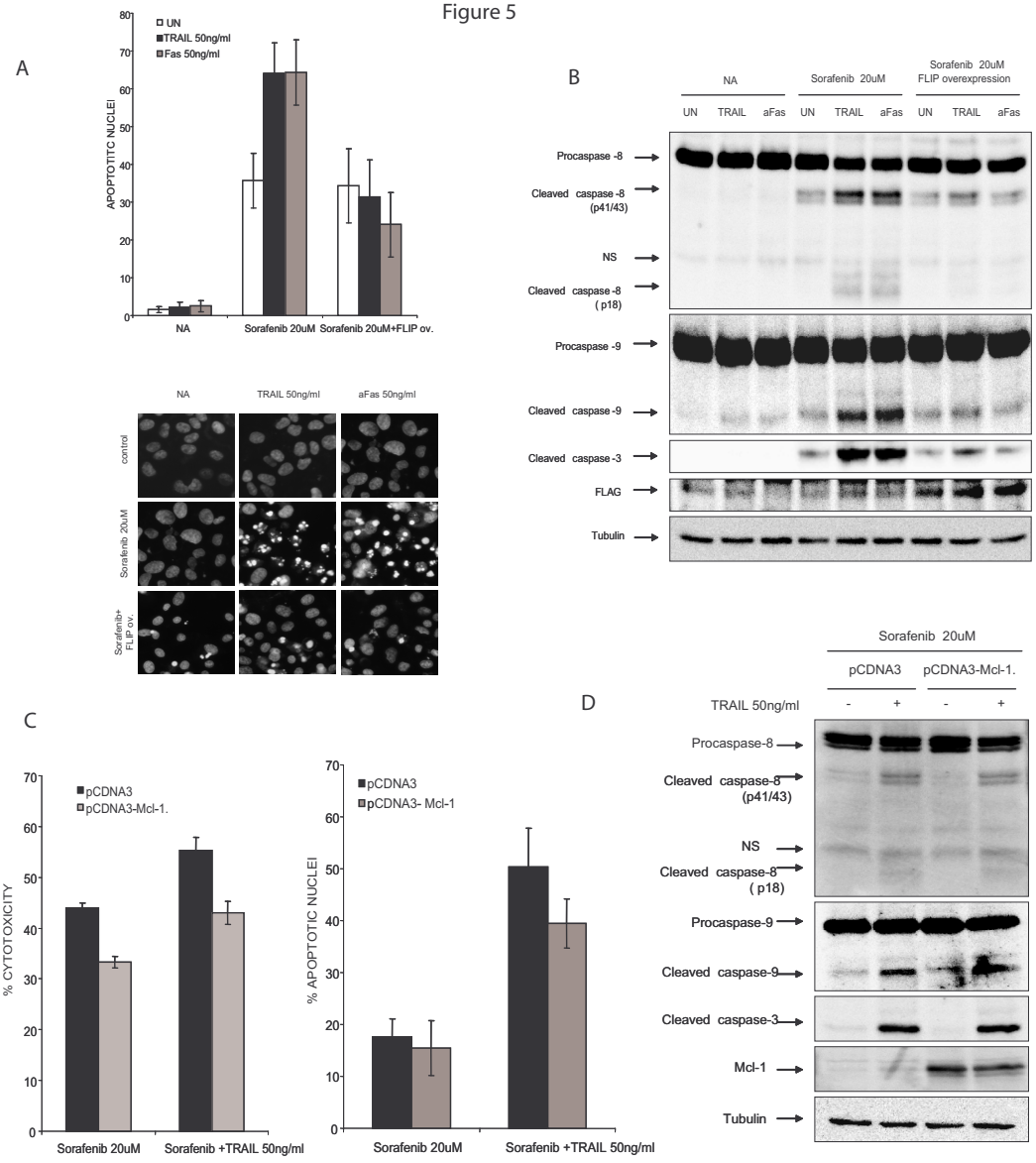
Figure 5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Sorafenib 20uM+FLIP ov.
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aFas 50ng/ml
control
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Sorafenib+ FLIP ov.
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Sorafenib 20uM
Sorafenib 20uM FLIP overexpression
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TRAIL
aFas
Procaspase -8
Cleaved caspase -8 (p41/43)
NS
Cleaved caspase -8 (p18)
Procaspase -9
Cleaved caspase -9
Cleaved caspase -3
FLAG
Tubulin
C
pCDNA3
pCDNA3-Mcl-1.
% CYTOTOXICITY
Sorafenib 20uM
Sorafenib +TRAIL 50ng/ml
pCDNA3
pCDNA3- Mcl-1
% APOPTOTIC NUCLEI
Sorafenib 20uM
Sorafenib +TRAIL 50ng/ml
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Cleaved caspase-8 (p41/43)
NS
Cleaved caspase-8 (p18)
Procaspase-9
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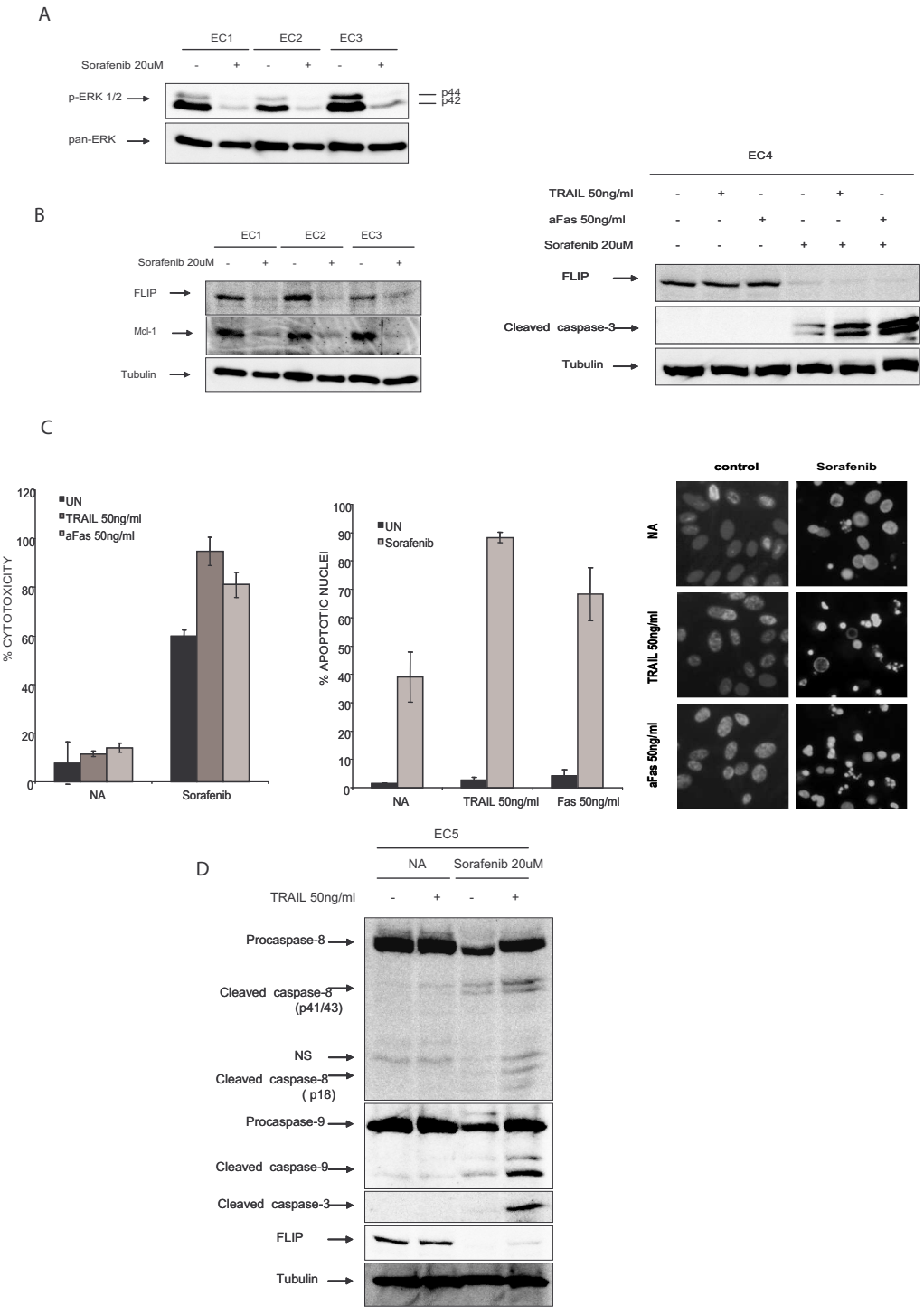
Figure 6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Tubulin
EC4
TRAIL 50ng/ml
aFas 50ng/ml
Sorafenib 20uM
Cleaved caspase-3
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TRAIL 50ng/ml
Fas 50ng/ml
control
Sorafenib
D
EC5
NA
Sorafenib 20uM
Procaspase-8
Cleaved caspase-8 (p41/43)
NS
Cleaved caspase-8 (p18)
Procaspase-9
Cleaved caspase-9
Cleaved caspase-3

Objectiu 3

5. ELS INHIBIDORS DEL PROTEASOMA INDUEIXEN MORT CEL·LULAR PERÒ ACTIVEN NF-KB EN LINIES CEL·LULARS I CULTIUS PRIMARIS DE CARCINOMA D'ENDOMETRI.

5.1 Els inhibidors del proteasoma causen apoptosi en línies cel·lulars de càncer d'endometri.

5.2 La inhibició del proteasoma resulta en la fosforilació i posterior degradació de IκB-α.

5.3 Els inhibidors del proteasoma indueixen translocació nuclear de NF-κB, unió al DNA, activitat transcripcional i fosforilació de la subunitat p65 en el residu serina 536.

5.4 La sobreexpressió de la forma superrepressora de IκB-α (SR-IκB-α) bloqueja l'activitat NF-κB induïda pels inhibidors del proteasoma.

5.5 L'activitat de NF-κB observada requereix de les proteïnes cinases IKKα i IKKβ.

5.6 IKKα i IKKβ són requerides per a que la fosforilació de p65 tingui lloc però no participen en la degradació de IκB-α.

5.7 El tractament amb els inhibidors del proteasoma indueixen apoptosi i activen NF-κB en cultius primaris d'explants de carcinoma d'endometri.

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Proteasome Inhibitors Induce Death but Activate NF-κB on Endometrial Carcinoma Cell Lines and Primary Culture Explants*[S]

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Proteasome inhibitors are currently used as chemotherapeutic drugs because of their ability to block NF-κB, a transcription factor constitutively activated in many different types of human cancer. In the present study, we demonstrate that proteasome inhibitors induce cell death in endometrial carcinoma cell lines and primary explants but, instead of blocking NF-κB, they increase its transcriptional activity. Proteasome inhibitors induce phosphorylation of IKKα/β, phosphorylation and degradation of IκBα, and phosphorylation of the p65 NF-κB subunit on serine 536. Proteasome inhibitor-induced NF-κB activity can be blocked by a non-degradable form of IκBα or dominant negative forms of either IKKα or IKKβ. Lentiviral delivery of shRNAs to either IKKα or IKKβ cause blockade of NF-κB transcriptional activity and inhibit phosphorylation of p65 on serine 536, but has no effect on IκBa degradation. These results suggest a role for p65 phosphorylation in proteasome inhibitor-induced NF-κB activation. Accordingly, siRNA knockdown of p65 inhibits proteasome inhibitor-induced NF-κB transcriptional activity. Our results demonstrate that proteasome inhibitors, including bortezomib, induce cell death on endometrial carcinoma cells and primary explants. However, they activate NF-κB instead of blocking its transcriptional potential. Therefore, the concept that proteasome inhibitors are blockers of NF-κB activation should be carefully examined in particular cell types.

The proteasome represents a novel putative target for cancer therapy. PS-341 (Velcade/bortezomib) is a dipeptidyl boronic acid inhibitor with high specificity for the proteasome (1, 2). It is currently used in the treatment of patients with multiple myeloma (3–7). Preclinical studies have suggested that proteasome inhibitors show antitumor activity against solid tumors, including carcinomas of the breast (8), lung (9), colon (10), bladder (11), ovary, prostate (12), pancreas (13), and glioblastoma multiforme. Furthermore, evidence has shown that transformed cells appear to be more susceptible to proteasome inhibitor-induced apoptosis than nontransformed cells. Finally, these inhibitors can sensitize cancer cells to death induced by members of the tumor necrosis family (TNF)[5] family such as TRAIL.

The antitumoral effects of bortezomib have been extensively studied in multiple myeloma, inactivation of NF-κB being one of the proposed mechanisms of action. NF-κB is a pleiotropic transcription factor, which is activated by a broad variety of stimuli such as growth factors, cytokines, ionizing radiation, ultraviolet light, or chemotherapeutic drugs (14, 15). NF-κB regulates the expression of a large number of genes, which carry important functions in inflammation, apoptosis, proliferation, and angiogenesis. NF-κB shows constitutive or increased activity in a wide variety of tumors (16, 17), including endometrial carcinoma (18) and plays a crucial role in neoplastic transformation (16, 19). In resting cells, NF-κB is held inactive in the cytoplasm, bound to the inhibitors of NF-κB (IκB). Activation of the canonical NF-κB pathway depends on stimuli-induced phosphorylation of the IκB kinase (IKK) complex, which includes the kinases IKKα, IKKβ, and the regulatory subunit IKKγ. Activated IKKs induce phosphorylation of IκBα on serines 32 and 36, and the subsequent ubiquitination and degradation by a proteasome-dependent pathway. Free NF-κB is then translocated to the nucleus where it regulates the transcription of several sets of genes. Optimal activation of NF-κB transcriptional activity requires phosphorylation of NF-κB subunits such as p65. In addition, NF-κB activity can be further modulated by phosphorylation of the p65 subunit. p65 sequence contains several serine residues that can be phosphorylated (20). One such residues is serine 536, which can be

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This work is dedicated to Eric and Laura.

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[5] The abbreviations used are: TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; shRNA, short hairpin RNA; siRNA, small interfering RNA; fmk, fluoromethyl ketone; DMEM, Dulbecco's modified Eagle's medium; BAF, Boc-D-fmk; DN, dominant negative; ER, endoplasmic reticulum.

phosphorylated by several kinases in different signaling pathways. Phosphorylation of p65 at serine 536 is accomplished by several different stimuli such as lymphotoxin β (21), TNF (22), lipopolysaccharide (23), or interleukin 1 (24).

The main goal of the present study was to demonstrate that bortezomib and other three proteasome inhibitors induce apoptosis in endometrial carcinoma cell lines and primary culture explants obtained from endometrial carcinoma tumor samples. However, we have found that such inhibitors activate NF-κB. Proteasome inhibitors induced phosphorylation and reduction of the levels of IκBα protein and phosphorylation of p65 subunit on serine 536, leading to increased NF-κB transcriptional activity. p65 phosphorylation depended on either IKKα and IKKβ. Proteasome inhibitor-induced activation of NF-κB required degradation of IκBα, and also functional expression of IKKα, IKKβ, and the p65 NF-κB subunit. Proteasome inhibitors have been widely used as pharmacological inhibitors of NF-κB. However, our results demonstrate that this may not always be the case. Therefore, an accurate study of the effects of proteasome inhibitors in signaling pathways may be needed in specific cell types. More importantly, our results may also have clinical relevance because proteasome inhibitors are currently used as anticancer drugs.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and Antibodies—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and monoclonal antibody to tubulin were from Sigma. Proteasome inhibitors MG-132, epoxomicin, and ALLN and antibodies to IKKα and IKKβ were from Calbiochem (La Jolla, CA). Bortezomib (Millenium Pharmaceuticals, Cambridge, MA) was obtained from the Department of Pharmacy (Hospital Arnau de Vilanova, Lleida). The broad specificity caspase inhibitor Boc-D-fmk (BAF) was purchased from Calbiochem (La Jolla, CA). Antibodies to anti-p65-phosphoserine 536, anti-phospho-IκBα, and active caspase-3 were obtained from Cell Signaling (Beverly, MA). Antibody to anti-pantothenate-cytokeratin and cytokeratin-7 were from DAKO (Glostrup, Denmark). Antibody to IκBα, p65, and siRNA targeting p65 or c-Rel were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to β was from BD Biosciences. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham Biosciences.

Dominant negative forms of IKKα and IKKβ were a generous gift from Dr. Alun M. Davies. Plasmid containing 5 NF-κB sites and the luciferase reporter gene (NFκB-LUC) was a gift from Dr. Giles Hardingham. Plasmid encoding β-galactosidase was a gift from Mari Carmen Ruiz Ruiz.

Cell lines, Culture Conditions, and Transfection—The Ishikawa 3-H-12 cell line was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr. Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95 and HEC-1-A cells were a gift from Dr. Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma), and 1% of penicillin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% CO_2. Transfections of both plasmid constructs and siRNAs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

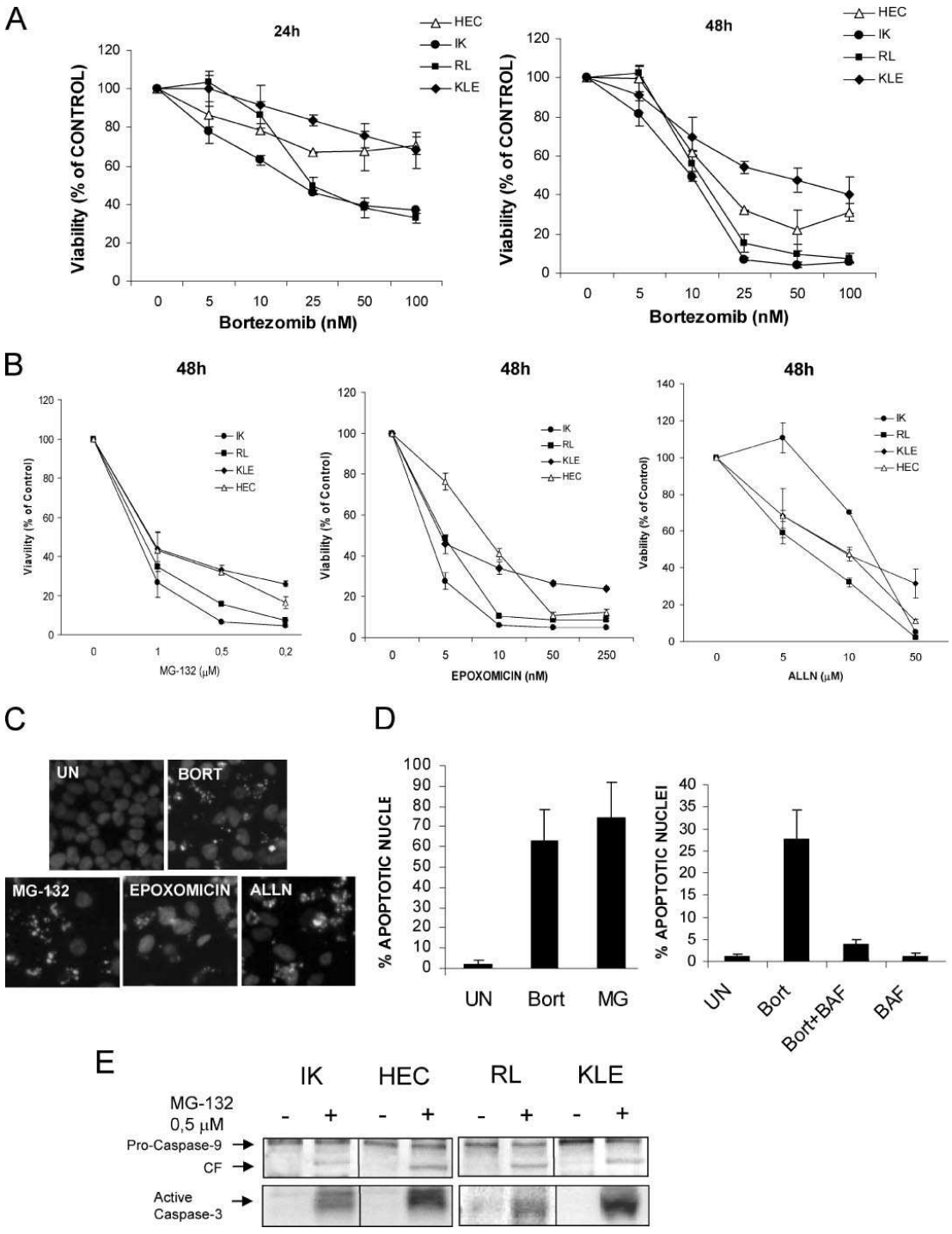
Explant Culture of Endometrial Adenocarcinoma—Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (J.P.). A specific informed consent was obtained from each patient, and the study was approved by the local Ethics Committee. Tissue were collected in DMEM, minced in 1-mm pieces, and incubated with collagenase in DMEM for 1.5 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 10-ml pipette and a 1-ml blue tip and resuspended in 2 ml of fresh DMEM. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM and tissue was allowed to sediment by gravity for 5 min. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% of penicillin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37 °C with saturating humidity and 5% CO_2. After 2 days in culture, explants were treated with the indicated concentrations of proteasome inhibitors.

Lentiviral Production and Infection—Oligonucleotides to produce plasmid-based shRNA were cloned into the FSFsi vector using AgeI-BamHI restriction sites. shRNA target sequence to IKKα was GCAGGCTCTTTCAGGGACA and target sequence to IKKβ was AAAGTGTCAGCTGTATCCT. To produce infective lentiviral particles, 293T cells were co-transfected by the calcium phosphate method with the virion packaging elements (VSV-G and D8.9) and the shRNA producing vector (FSPsi) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3–4 days in the same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 min at 1000 × *g*, and filtered through a 0.45-μm filter (Millipore). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24–48 h in the presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for two additional days to allow endogenous protein knockdown or protein overexpression.

Cell Viability Assays and Assessment of Apoptosis—Cell viability was determined by the MTT assay. Endometrial adenocarcinoma cells were plated on M96-well plates at 15×10^3 cells per well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent and lysed with Me_2SO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

Western Blot Analysis—Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and



lysed with lysis buffer (2% SDS, 125 mM Tris-HCl, pH 6.8). Protein concentrations were determined with the protein assay kit (Bio-Rad). Equal amounts of proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by incubation with TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) plus 5% nonfat milk. Membranes were incubated with primary antibodies overnight at 4 °C. Signal was detected with ECL Advance (Amersham Biosciences).

Luciferase Assays—Endometrial carcinoma cell lines were plated in M24 multiwell plates and transfected using either calcium phosphate or Lipofectamine 2000 following the manufacturer's instructions, with the reporter NFκB-LUC construct together with a plasmid encoding β-galactosidase. After 24 h, cells were treated as indicated in each experiment and cells were lysed with 60 ml of luciferase lysis buffer (25 mM glycylglycine, pH 7.8, 15 mM Mg_2SO_4, 1% Triton X-100, 5 mM EGTA) and rocked on ice for 15 min. 30 ml of lysates were transferred to M96 multiwell plates and 30 ml of luciferase assay buffer was added to a final concentration of (25 mM glycylglycine, 15 mM $KHPO_4$, pH 7.8, 15 mM Mg_2SO_4, 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol containing, 2 mM ATP, 100 mM acetyl-coenzyme A, and 100 mM luciferine). Luciferase was measured using a microplate luminometer. After luciferase measuring 60 ml of 2× β-galactosidase buffer (200 mM $NaPO_4$, 20 mM KCl, 2 mM $MgSO_4$, 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside) was added to each well and measured on a microplate reader at 415 nm.

Electrophoretic Motility Shift Assay—After appropriate treatment, nuclear and cytoplasmic extracts were obtained from 2×10^6 IK and HEC cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce). Electrophoretic mobility shift assay was carried out using the Light-Shift Chemiluminiscent kit (Pierce), 5–10 μg of nuclear protein extracts were incubated with 20 fmol of 5′ biotin-labeled NF-κB consensus oligonucleotide (MWG-Biotech, Ebersberg, Germany) for 20 min at room temperature. DNA complexes were electrophoresed on a 5% acrylamide gel, and transferred to a nylon membrane (Amersham Biosciences). Light signal was developed following the manufacturer's instructions. For supershift experiments, 2 μg of p65 antibody was added to the binding reaction and incubated for additional 45 min.

RESULTS

Proteasome Inhibitors Induce Cell Death on Endometrial Adenocarcinoma Cell Lines—Bortezomib is currently used as a chemotherapeutic agent in patients with relapsed multiple myeloma. It is under ongoing clinical trials for evaluation of efficacy in the treatment of some solid tumors. However, some tumoral cell types undergo apoptosis or cell growth arrest after proteasome inhibitor treatment, whereas others are insensitive to them, or require co-treatment with other drugs or factors. To investigate whether bortezomib and the other three proteasome inhibitors were able to induce cell death on endometrial adenocarcinoma cells, we treated four endometrial carcinoma (EC) cell lines, Ishikawa (IK), KLE, RL-95 (RL), and HEC-1-A (HEC) with bortezomib and three different proteasome inhibitors (MG-132, epoxomicin, or ALLN). All proteasome inhibitors tested caused a dose-dependent decrease on cell viability as assessed by MTT (Fig. 1, *A* and *B*). The reduction on viability was accompanied by an increase of apoptotic nuclei as seen by Hoechst staining (Fig. 1*C*) suggesting apoptotic cell death. Treatment with MG-132 resulted in processing of caspase-3 and caspase-9. (Fig. 1*D*). Co-treatment of IK with bortezomib or MG-132 plus BAF, a broad specificity caspase inhibitor almost completely abolished cell death (Fig. 1*E*). Altogether these data indicate that proteasome inhibitors are effective in inducing apoptotic cell death on endometrial carcinoma cell lines.

Proteasome Inhibitors Induce Phosphorylation and Degradation of IκBα—Inhibition of proteasome prevents NF-κB activation and causes cell cycle arrest or cell death on many different types of cell. The antitumoral effects of bortezomib have been proposed to involve NF-κB inhibition as a main mechanism of action. To investigate whether this was the case in our cell lines, we assessed the levels of IκBα, after treatment with proteasome inhibitors. Time course treatment of IK, HEC, or RL cells with 25 nM bortezomib resulted in a marked increase on phosphorylation of serine 32, followed by reduction of IκBα protein levels over time (Fig. 2*A*). To rule out the possibility that IκBα phosphorylation and reduction were nonspecific effects of bortezomib in our particular cell lines, we treated IK cells with the other three proteasome inhibitors. Treatment of IK cells with MG-132 resulted in phosphorylation and reduction of IκBα, similar to that observed with bortezomib (Fig. 2*B*). Although each cell line showed different basal levels of IκBα, treatment with MG-132 resulted in a similar reduction on IκBα levels in all of them (Fig. 2*C*). We also observed a significant reduction of IκBα with epoxomicin or ALLN (Fig. 2*D*).

Proteasome Inhibitors Induce NF-κB Nuclear Translocation, DNA Binding, Transcriptional Activity, and Phosphorylation of p65 on Serine 536—The results described above stimulated us to investigate whether the reduction of IκBα resulted in an activation of NF-κB transcriptional activity. To address this point, we used different experimental approaches. We carried out a transcriptional activity assay of NF-κB by luciferase reporter assay. Cells were transfected with NF-κB-dependent luciferase reporter construct and treated with the indicated doses of proteasome inhibitors for 14–16 h. IK cells treated with 10 or 25 nM bortezomib displayed a marked increase on the basal luciferase activity (Fig. 3*A*). Such an increase was also observed

FIGURE 1. **Proteasome inhibitors trigger apoptosis on endometrial carcinoma cell lines.** *A*, Ishikawa (*IK*), KLE, RL-95 (*RL*), and HEC-1-A (*HEC*) cells were treated with increasing doses of bortezomib for 24 or 48 h and cell viability was assessed by MTT. Results are expressed as percent of the control values. *B*, Ishikawa (*IK*), KLE, RL-95 (*RL*), and HEC-1-A (*HEC*) cells were treated for 48 h with the indicated doses of MG-132, epoxomicin, or ALLN and cell viability was assessed by MTT. Results are expressed as percent of the control values. *C*, micrographs of IK cells left untreated (*UN*) or treated for 36 h with 25 nM bortezomib, 1 μM MG-132, 50 nM epoxomicin, or 50 μM ALLN and stained with Hoechst dye. *D*, quantification of apoptotic nuclei after 36 h treatment with 25 nM bortezomib (*Bort*) or 0.5 μM MG-132 (*left graph*). Quantification of Hoechst-stained apoptotic nuclei of IK cells treated with 25 nM bortezomib alone (*Bort*), 100 μM BAF, or a combination (*Bort* + *BAF*) (*right graph*). *E*, IK, RL, KLE, or HEC cells were treated with 0.5 μM MG-132 for 24 h and lysates were subjected to Western blot with antibodies to active caspase-3 or antibody to caspase-9.

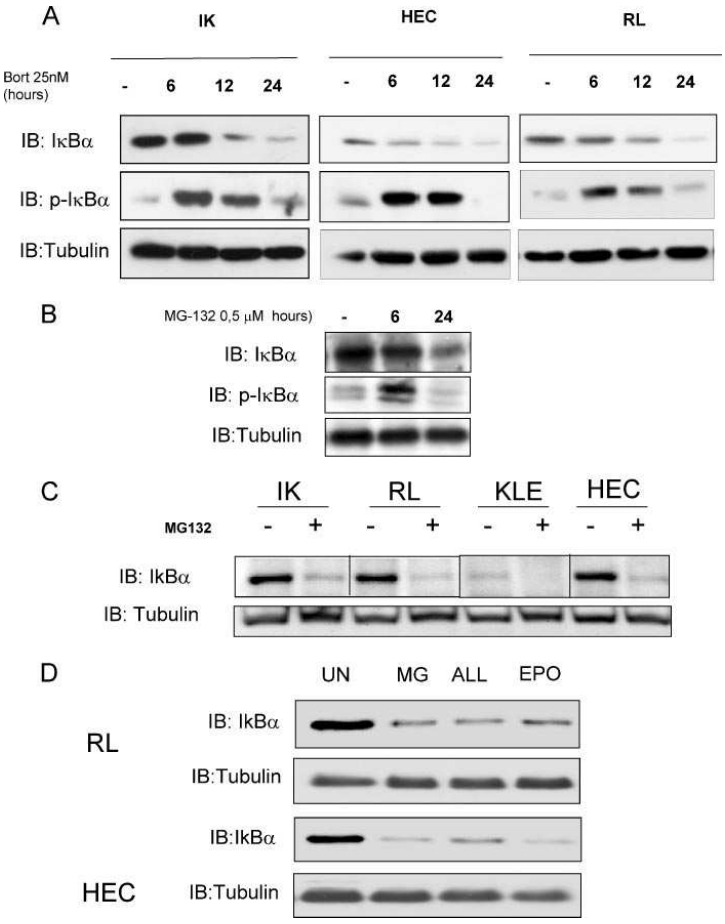


FIGURE 2. **Proteasome inhibitors induce phosphorylation and degradation of IκBα.** *A*, Ishikawa (*IK*), HEC-1-A (*HEC*), and RL-95 (*RL*) cells were treated for the indicated times with 25 nM bortezomib and cell lysates were subjected to Western immunoblot (*IB*) with antibodies to phosphorylated IκBα, IκBα, or tubulin. *B*, IK cells were treated for 6 or 24 h with 0.5 mM MG-132 and cell lysates were analyzed by Western blot with antibodies to phosphorylated IκBα, IκBα, or tubulin. *C*, IK, RL, KLE, or HEC cells were treated with 0.5 μM MG-132 or left untreated for 24 h and cell lysates were subjected to Western blot with antibodies to IκBα (*top panel*) or tubulin (*bottom panel*). *D*, RL and HEC cells were treated for 24 h with 0.5 μM MG-132 (*MG*), 50 nM epoxomycin (*EPO*), or 50 μM ALLN (*ALL*) and cell lysates were subjected to Western blot antibodies to IκBα (*top panel*) or tubulin (*bottom panel*).

when IK cells were treated with MG-132, epoxomicin, or ALLN. Consistent with this result, we also observed that treatment with MG-132 or bortezomib resulted in the formation of a DNA·NF-κB complex as assessed by electrophoretic mobility shift assay (Fig. 3*B*). This complex showed a similar pattern of migration to that observed with TNF. To provide a control of proteasome inhibitor action, we stimulated a melanoma cell line (M16) and endometrial carcinoma cell line RL-95 with TNF. TNF treatment triggers the canonical, proteasome-dependent, IκBα degradation and p65 NF-κB binding activity in both cell lines. As expected, both bortezomib and MG-132 reduce NF-κB binding to DNA in M16 cells. Similar results are obtained in RL cells with MG-132 (Fig. 3*C*). A supershift analysis using a p65 antibody caused a marked reduction on the NF-κB complex and the appearance of a supershifted band in both MG and TNF stimulated cells (Fig. 3*B*). Accordingly, Western blot analysis on nuclear and cytoplasmic extracts revealed that treatment with either MG-132 or bortezomib induced p65 nuclear translocation on IK cells. As control we treated HEC cells with TNF, which is known to induce p65 nuclear translocation (Fig. 3, *D* and *E*). Finally, we also analyzed phosphorylation of the p65 subunit of NF-κB on serine 536, which has been associated with increased transactivation potential. Treatment with bortezomib or other proteasome inhibitors resulted in increased phosphorylation on serine 536 of p65 in all the cell lines that were tested (Fig. 3*F*).

SR-IκBα Blocks Proteasome Inhibitor-induced NF-κB Activity —To ascertain whether IκBα degradation was dependent on serine phosphorylation, we transfected IK and HEC cells with a construct encoding a form of IκBα carrying serine to alanine mutations at residues 32 and 36, named SR-IκBα. These mutations prevent IκBα phosphorylation and its subsequent proteasome-mediated degradation, thereby preventing release and nuclear translocation of NF-κB (46). Expression of SR-IκBα caused a marked reduction on the activation of NF-κB transcriptional activity by either bortezomib or MG-132 in HEC and IK cells (Fig. 4). This data supports the hypothesis that proteasome inhibitors require phosphorylation and degradation of IκBα.

NF-κB Activity Induced by Proteasome Inhibitors Requires Functional IKKα and IKKβ—The major upstream kinases involved in NF-κB activation are IKKα and IKKβ. We investigated whether these kinases were involved in activation of NF-κB by proteasome inhibitors. Treatment of IK, HEC, or RL cells with bortezomib caused increased phosphorylation of IKKα/β, as assessed by Western blot analysis of lysates with an antibody that specifically recognizes the phosphorylated forms of both proteins (Fig. 5*A*).

To determine the requirement of either or both IKKα or IKKβ subunits, we co-transfected IK or HEC cells with the NF-κB-luciferase reporter construct plus dominant negative forms of either IKKα (DN-IKKα) or IKKβ (DN-IKKβ). Both DN-IKKα and DN-IKKβ blocked NF-κB transcriptional activ-

ity on IK cells treated with 25 nM bortezomib (Fig. 5*B*, *left*) and in IK and HEC cells treated with MG132 (Fig. 5*B*, *right*).

To further demonstrate the role of IKKα or IKKβ subunits, we designed lentiviral shRNAs targeting each subunit to knockdown endogenous expression. We designed shRNAs targeting two sequences for each kinase. Infection of IK cells with lentiviruses carrying shRNAs to either IKKα or IKKβ subunits revealed that each shRNA selectively knocked down the expression of the corresponding protein (Fig. 5*C*). These two shRNAs were selected for subsequent experiments. We infected IK cells with the functional IKKα or IKKβ shRNAs for 3 days to allow protein knockdown, and cells were subsequently treated with bortezomib. As shown in Fig. 5*D*, both IKKα shRNA and IKKβ shRNA blocked NF-κB activity with similar efficiency to the dominant negative forms. Our data suggest that phosphorylation and expression of both IKKα and IKKβ subunits are required for proteasome inhibitor-induced NF-κB activity. Neither IKKα nor IKKβ knockdown increased the cell viability in proteasome inhibitor-treated cells (supplemental Fig. 1).

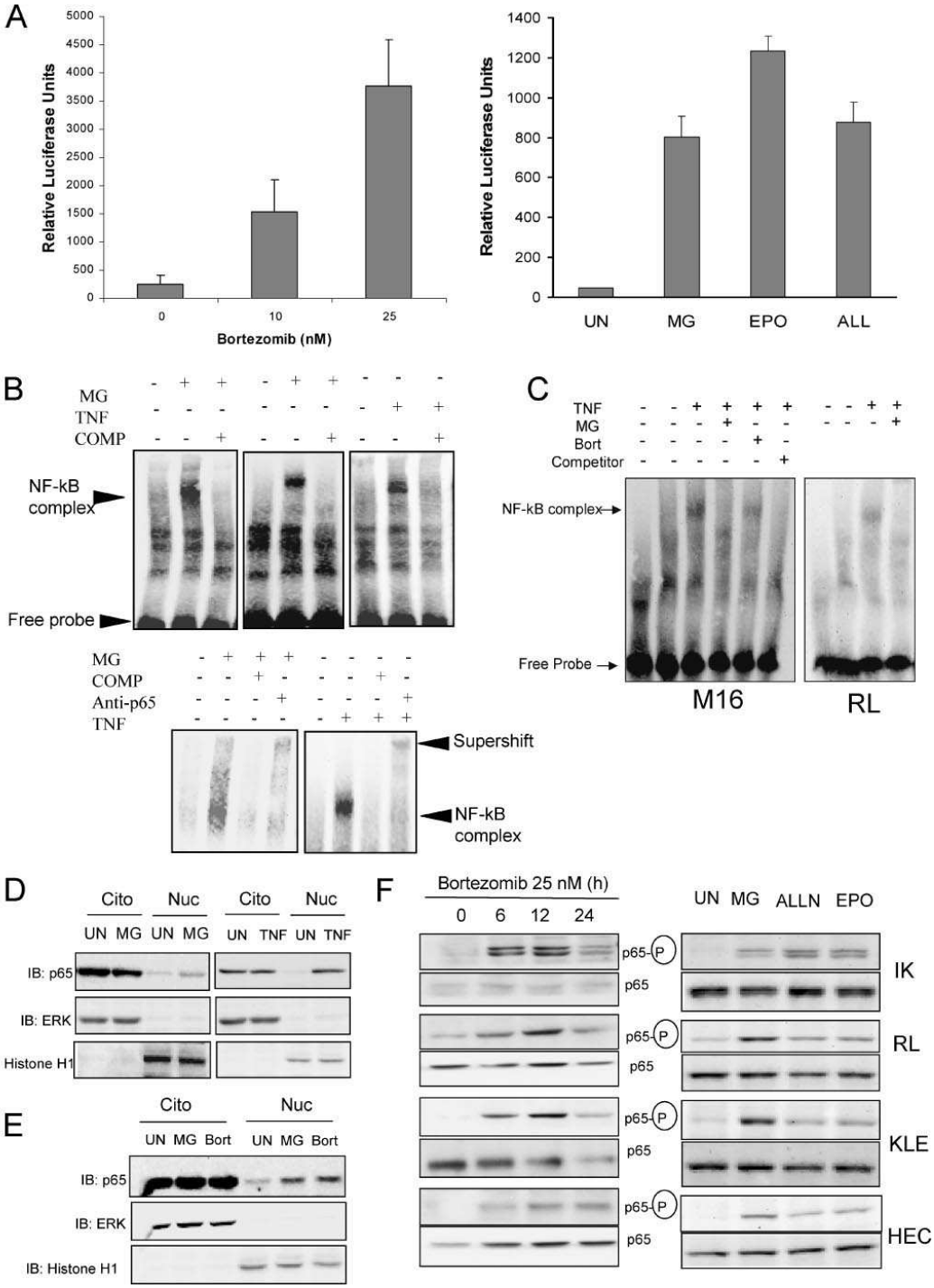
IKKα and IKKβ Are Required for p65 Phosphorylation but Not for IκBa Degradation after Proteasome Inhibitor Treatment—To determine whether IKK was responsible for proteasome inhibitor-induced p65 phosphorylation, HEC cells were infected for 3 days with lentiviruses carrying either IKKα or IKKβ shRNAs. HEC cells were treated for 6 or 24 h with 25 nM bortezomib and cell lysates were analyzed by Western blot (Fig. 6*A*). As shown in Fig. 6, both IKKα and IKKβ shRNA strongly inhibited p65 phosphorylation. The cells transfected with IKKβ shRNA showed a reduction of phosphorylated IκBα, but also a decrease on total IκBα protein. However, the molecular weight shift on migration of IκBα and degradation of IκBα by bortezomib remained unaffected (Fig. 6*A*). Our findings suggest that p65 phosphorylation is mediated by either IKKα or IKKβ, but also show that IκBα degradation is independent of IKKs. Similar results were obtained with IK cells (data not shown).

These results suggest that proteasome inhibitors increase NF-κB activity, by involving the p65 subunit of NF-κB. To determine whether transcriptional activity of NF-κB was really dependent of p65 subunit, we co-transfected p65 siRNA or c-Rel siRNA with the NF-κB reporter construct, and cells were exposed to bortezomib. Transfection of p65 siRNA resulted in a significant decrease of NF-κB transcriptional activity in both IK and HEC cells (Fig. 6*B*). Such a result was not observed when c-Rel siRNA was transfected.

Proteasome Inhibitors Induce Cell Death and Activate NF-κB in Primary Culture Explants from Endometrial Adenocarcinoma Samples—To further demonstrate the effect of proteasome inhibitors on endometrial carcinoma, we assessed the effects of such inhibitors on primary explants obtained from human endometrial carcinomas. After enzymatic digestion, glandular-like structures were plated on 24-well dishes and the epithelial origin of the samples was assessed by immunofluorescence to a wide spectrum cytokeratin, cytokeratin-7 and β-catenin, two proteins that are only expressed on endometrial cells of epithelial origin, but not on stromal cells (Fig. 7*A*). To ascertain whether proteasome inhibitors induced apoptosis on primary explants as well as on cell lines, we treated endometrial explant cultures with 0.5 μM MG-132 or 25 nM bortezomib and we assessed caspase-3 activation by immunofluorescence with an antibody that recognizes its active form (Fig. 7*B*). Both MG-132 and bortezomib increased the number of cytokeratin positive cells displaying active caspase-3. These results suggest that bortezomib and MG-132 induce apoptotic cell death on primary endometrial carcinoma explants. To determine whether MG-132 and bortezomib also were able to activate NF-κB, we analyzed IκBα phosphorylation and degradation after treatment with these two inhibitors at doses that induced IκBα degradation and phosphorylation on endometrial carcinoma cell lines. As shown in Fig. 7*C*, both MG-132 and bortezomib induced phosphorylation and degradation of IκBα and phosphorylation of p65 at serine 536 in a similar way to that observed in all cell lines tested. Altogether, these results demonstrate that proteasome inhibitors are able to activate NF-κB signaling in primary endometrial carcinoma explants.

DISCUSSION

Bortezomib and other proteasome inhibitors trigger cell growth arrest or apoptosis on several tumors (2). Bortezomib is a modified dipeptidyl boronic acid that creates compounds that form covalent complexes with the proteasome. It is used as a chemotherapeutic agent for treatment of relapsed and refractory multiple myeloma (3–7). It is also a promising anti-cancer agent for treatment of solid tumors. In many different types of tumor cells, proteasome inhibition cause cell death by blocking NF-κB activity. In this article, we demonstrate for the first time that bortezomib and other three proteasome inhibitors (MG-132, epoxomicin, and ALLN) may induce apoptosis in endometrial cancer cell lines and primary culture explants from endometrial carcinomas. Cell death was accompanied by activation of caspases and apoptotic nuclear morphology. However, in contrast to that observed on other cancer cells, this cell death is not related with NF-κB blockage. In endometrial cancer cells, bortezomib and other proteasome inhibitors increase NF-κB activity rather than its inhibition. NF-κB is a family of transcription factors involved in the regulation of genes encoding cytokines, cytokine receptors, and cell adhesion molecules, which drive immune and inflammatory responses (14–16). However, NF-κB is also related to carcinogenesis, by regulating genes involved in apoptosis, the cell cycle, differentiation, invasion, and cell migration (16, 19). Therefore, inhibition of NF-κB is a promising target for treatment of cancer. Because of their ability to block IκBα degradation, proteasome inhibitors have been widely used as inhibitors of NF-κB. In fact, inhibition of NF-κB activity has been reported as the main mediator of cytotoxic effects of bortezomib. One of the main goals of this study was to investigate the effect of proteasome inhibition on NF-κB in endometrial carcinoma. Treatment of endometrial carcinoma cell lines with bortezomib did not stabilize or inhibit IκBα, but induced phosphorylation and reduction of IκBα levels. Similar results were obtained when the endometrial adenocarcinoma cell lines were exposed to the other three proteasome inhibitors (MG-132, epoxomicin, or ALLN) that inhibit the proteasome by other chemical mechanisms. Although these results seem to contradict the well established mechanism of IκBα degradation by proteasome inhibition, it is worth mentioning that some recent evidence suggests that proteasome inhibitors may acti-



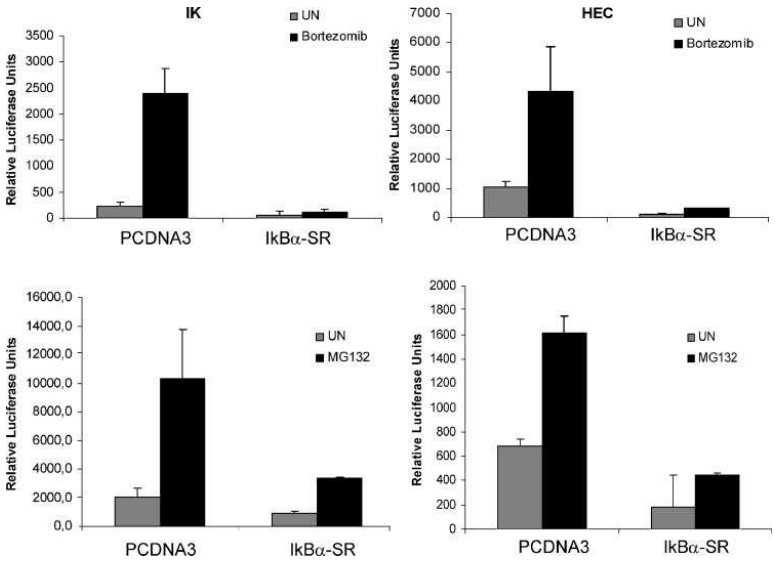


FIGURE 4. **IκBα superrepressor blocks bortezomib and MG132-induced NF-κB transcriptional activity.** IK or HEC cells were co-transfected with the NFκB-luciferase reporter construct and either the empty vector (PCDNA3), or a IκBα superrepressor, which cannot be phosphorylated on serines 32 and 36 (SR-IκBα). After 48 h, cells were stimulated with 25 nM bortezomib or 0.5 μM MG132 and luciferase activity was assayed 16 h later. Results are expressed in relative luciferase units.

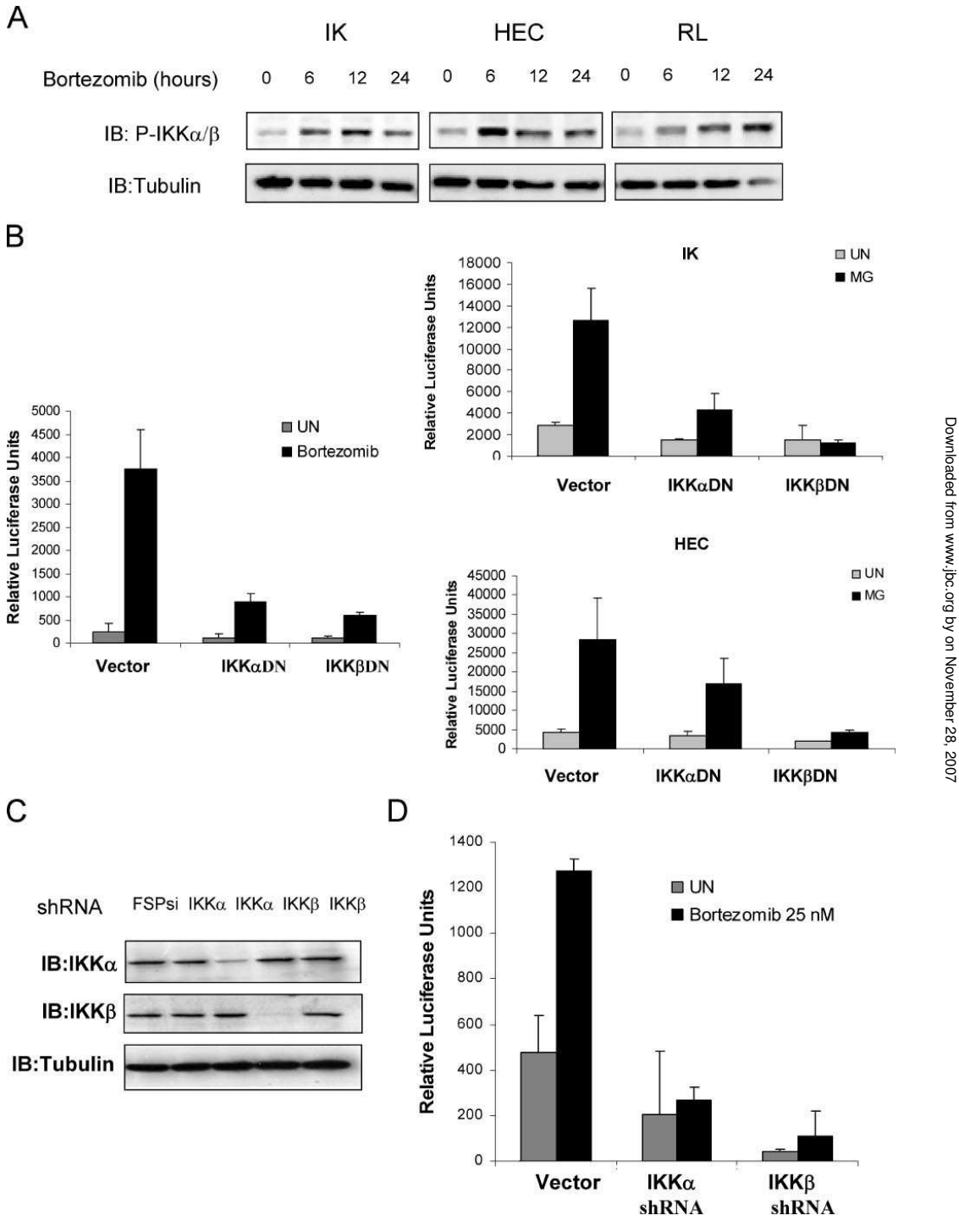
vate NF-κB in some cancer cell lines. This was the case of the colon adenocarcinoma cell line HT-29 after treatment with MG-132 (25). In agreement with this report, we have found IκBα degradation, IKK phosphorylation, and increased transcriptional activity of NF-κB, as a result of exposure to the four different proteasome inhibitors.

Phosphorylation and degradation of IκBα is followed by an increase of NF-κB activity. We found that proteasome inhibitors induced the formation of a DNA·NF-κB complex displaying similar migration properties than that observed after stimulation with TNF. TNF is a well known activator of the canonical NF-κB pathway that results in nuclear translocation of p65 subunits. Therefore, this result suggests that p65 may be involved in NF-κB activity induced by such inhibitors. Accordingly, we have found supershift of the NF-κB complex using p65 antibody, nuclear translocation of p65, and more importantly, p65 siRNA reduced the transcriptional activity induced by proteasome inhibitors. Moreover, we have found that all proteasome inhibitors tested, including bortezomib, increase p65 phosphorylation on serine 536. Recent evidence suggest that phosphorylation on this serine is critical for p65 transcriptional activity after different stimuli such as lymphotoxin β (21), TNF (22), lipopolysaccharide (23), or interleukin 1 (24) stimulation. Moreover, a recent report also shows that MG-132 can also enhance serine 536 phosphorylation on HeLa cells (24). Therefore, phosphorylation of p65 is consistent with the increased transcriptional activity observed after treatment with proteasome inhibitors.

Recent reports describe IKK-independent mechanisms that can also activate NF-κB upon certain stimuli. Recently, it has been shown that DNA damaging agents such as topoisomerase inhibitors require IKK complex to induce NF-κB (26, 27), whereas others suggest that NF-κB activation can take place independently of IKKs (28). To address the involvement of IKKα or IKKβ in proteasome inhibitor effects on endometrial carcinoma cell lines, we transfected IK or HEC cells with the dominant negative forms of IKKα or IKKβ or with lentiviral-transduced shRNAs, and we assessed NF-κB activity after bortezomib or MG-132 exposure. These experiments clearly demonstrated that, in contrast to topoisomerase inhibitors, NF-κB activation by proteasome inhibitors in endometrial carcinoma cell lines requires functional IKKs.

Reporter experiments performed with the IκBα supperrepressor indicate that IκBα phosphorylation and degradation are required for proteasome inhibitor-induced NF-κB activity. However, shRNA to either IKKα or IKKβ did not block IκBα degradation by proteasome inhibitors. IKKβ shRNA diminished IκBα phosphorylation but such reduction can be explained by the reduction of total IκBα protein levels.

FIGURE 3. **Proteasome inhibitors increase NF-κB nuclear translocation, DNA binding, transcriptional activity, and phosphorylate p65 subunit at serine 536.** *A*, IK cells were transfected with the NFκB-luciferase reporter construct and treated with 10 and 25 nM bortezomib (*left graph*), or treated with 0.5 μM MG-132 (*MG*), 50 nM epoxomycin (*EPO*), or 50 μM ALLN (*ALL*) (*right graph*) for 16 h, and cell lysates were assayed for luciferase activity. Results are expressed as relative luciferase units. *B*, IK cells were treated with bortezomib MG-132 (*MG*) for 16 h, and HEC cells were treated with MG-132 for 16 h or with TNF for 30 min, and nuclear lysates were incubated with 20 fmol of labeled probe. A 200 M excess of unlabeled competitor was included in the indicated lanes to demonstrate the specificity (*upper panel*). For supershift experiments HEC cells were treated with MG-132 or TNF and nuclear lysates were incubated with 200 M excess of the unlabeled competitor antibody to p65 as indicated (*lower panel*). *C*, RL endometrial carcinoma cells and the melanoma cell line M16 were treated for 30 min in the presence or absence of proteasome inhibitors and nuclear lysates were incubated with 20 fmol of biotin-labeled probe. *Arrows* indicate the proteasome inhibitor-induced NF-κB complex and the free probe. A 200 M excess of unlabeled competitor was included in the indicated lanes to demonstrate the specificity. *D*, HEC cells were treated with 50 ng/ml TNF for 30 min or with 0.5 mM MG-132 for 16 h. Cytoplasmic (*Cito*) and nuclear (*Nuc*) cell lysates were analyzed by Western blot with antibodies to p65 NF-κB subunit. To determine the purity of cytoplasmic and nuclear lysates, membranes were reprobed with anti-ERK antibody (marker of cytoplasmic fraction) or with histone H1 antibody (nuclear fraction). *IB*, immunoblot. *E*, IK cells were treated for 16 h with either 25 nM bortezomib (*Bort*) or 0.5 mM MG-132 and nuclear and cytoplasmic lysates were analyzed with p65 antibodies. Membranes were reprobed with antibodies to ERK and histone H1. *F*, IK cells were treated for 0, 6, 12, or 24 h with 25 nM bortezomib (*left panel*) and IK, RL, HEC, and KLE cells were treated for 16 h with 0.5 μM MG-132, 50 nM epoxomycin, or 50 μM ALLN (*right panel*) and cell lysates were subjected to Western blot with antibodies that specifically recognize serine 536 only when it is phosphorylated. Membranes were also incubated with antibodies to total p65 to ensure equal amounts of protein.



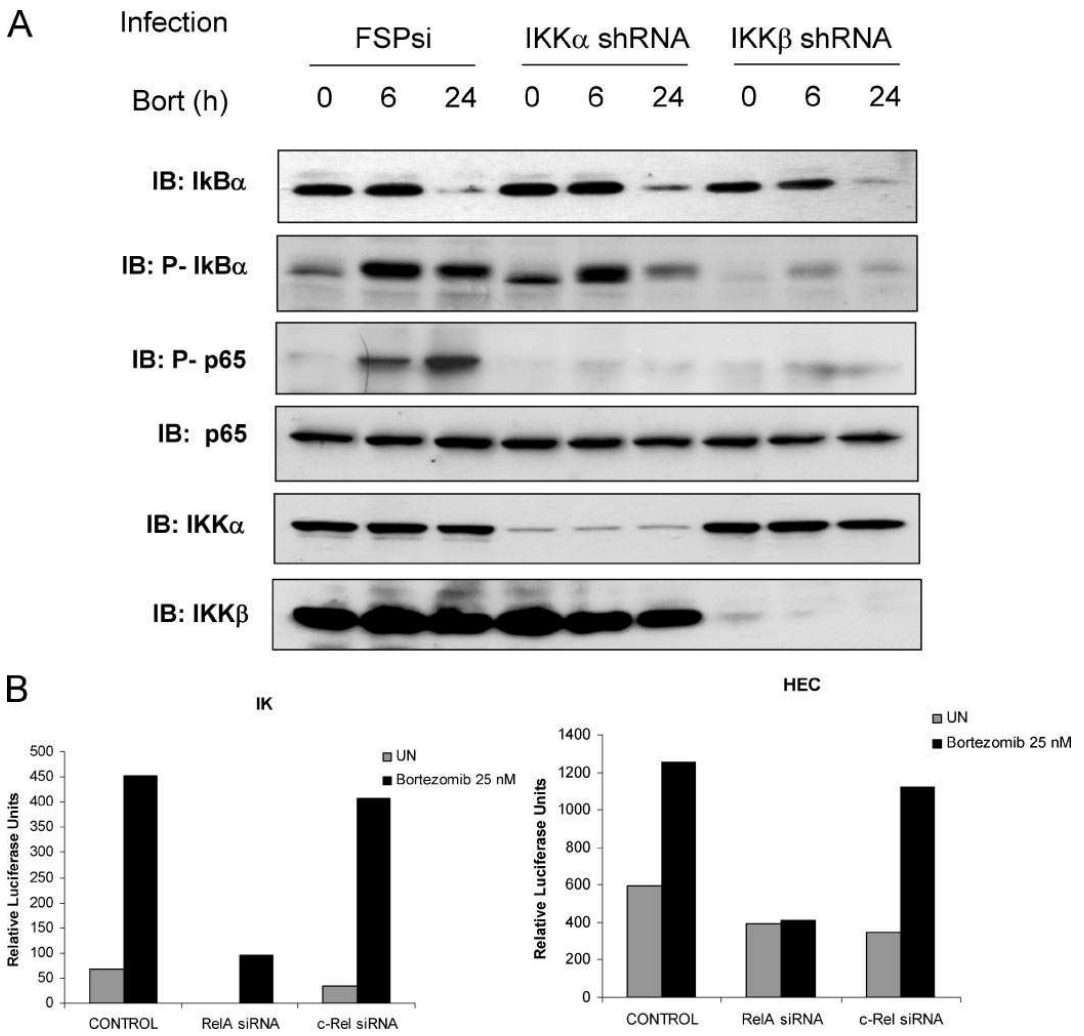


FIGURE 6. **Down-regulation of IKKα and IKKβ inhibit p65 phosphorylation and activity but not IκBα degradation.** *A*, HEC cells were infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ to allow protein knockdown and then stimulated for 6 or 24 h with 25 nM bortezomib, and lysates were analyzed by Western blot. Blots were incubated with antibodies to IκBα, phospho-IκBα, and phospho-Ser^{536}-p65. Membranes were also blotted with antibodies to IKKα or IKKβ to ensure down-regulation of target proteins by shRNAs. *B*, *bar chart* showing HEC co-transfected with the NFκB-luciferase reporter construct and either siRNAs targeting p65 (RelA) or c-Rel subunits of NF-κB. After 48 h to allow siRNA to down-regulate protein expression, cells were stimulated with 25 nM bortezomib and luciferase activity was assayed 16 h later. Results are expressed in relative luciferase units. *IB*, immunoblot.

Although both IKKα and IKKβ shRNAs did not block IκBα degradation, they markedly reduced NF-κB activity. These results suggest that NF-κB activation by proteasome inhibitors is further regulated downstream of IκBα degradation. Increasing evidence supports that NF-κB phosphorylation may be an important mechanism of NF-κB regulation (20). As mentioned above, we found that all proteasome inhibitors phosphorylated p65 at serine 536. Such phosphorylation can be achieved by

FIGURE 5. **IKKα and IKKβ are required for bortezomib and MG132-induced NF-κB activity.** *A*, Western blot showing IK, HEC, and RL cell lines stimulated for 6, 12, or 24 h with 25 nM bortezomib and incubated with an antibody that recognizes IKKα/IKKβ phosphorylated or with tubulin to ensure equal protein loading (*bottom panel*). *B*, *bar charts* showing NF-κB transcriptional inhibition by either IKKα or IKKβ dominant negative forms on IK cells treated with bortezomib (*left chart*) or in IK and HEC cells treated with MG-132 (*right charts*). *C*, Western blot analysis of IKKα and IKKβ expression on IK cells infected for 3 days with lentiviruses carrying two different shRNAs targeting IKKα or IKKβ. *D*, IK cells infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ for 3 days to allow protein knockdown and then transfected with the NFκB-luciferase reporter construct. 24 h later they were treated with 25 nM bortezomib and luciferase activity was assayed. *IB*, immunoblot.

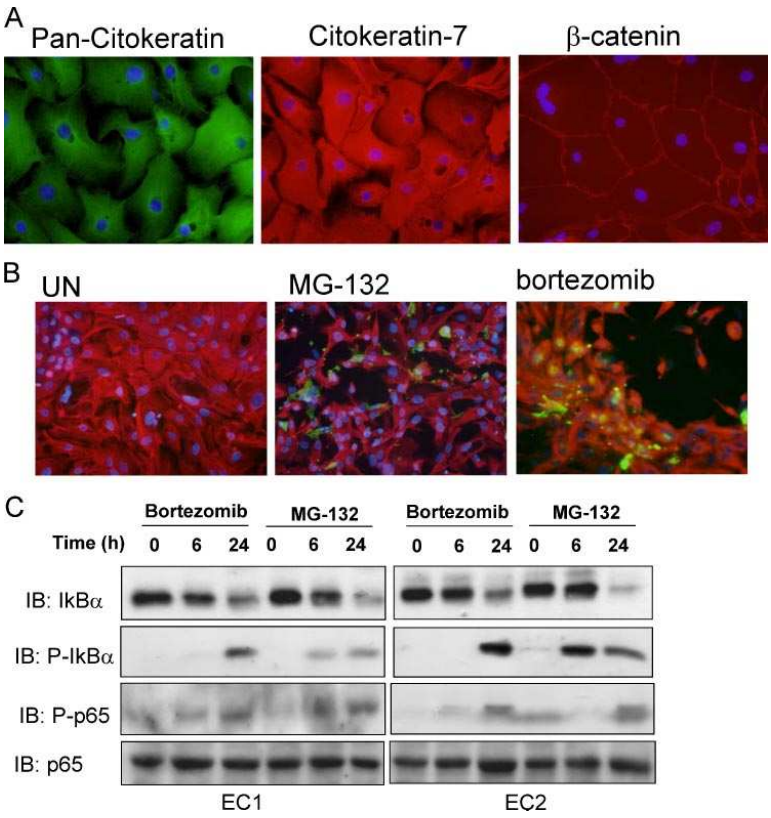


FIGURE 7. **Proteasome inhibitors induce cell death and activate NF-κB on primary endometrial carcinoma explants.** *A*, endometrial carcinoma explants after 3 days in culture stained with antibodies against pantothenate-cytokeratin, cytokeratin-7, or β-catenin. Nuclei were visualized by Hoechst staining. *B*, endometrial carcinoma explants were treated with 25 nM bortezomib, 0.5 mM MG-132, or left untreated for 24 h, fixed, and stained with anti-active caspase-3 antibody (*green*), citokeratin-7 (*red*), and Hoechst dye to visualize nuclei (*blue*). *C*, two different endometrial carcinoma explants were treated with 25 nM bortezomib or 0.5 μM MG-132 and cell lysates were analyzed by Western blot by IκBα, phosphorylated IκBα, phosphorylated p65, or tubulin. *IB*, immunoblot.

different upstream kinases. Phosphorylation on serine 536 is triggered by IKKα after TNFα (29) and lymphotoxin β (30) or by IKKβ after T cell co-stimulation (47) or lipopolysaccharide (30) but also by TBK1, Akt, or IKKε (20, 24). In this study, we have found that shRNA inhibition of either IKKα and IKKβ blocked p65 at serine 536 and NF-κB transcriptional activity, suggesting a major role for the p65 subunit in proteasome inhibitor-induced activation. Consistent with this hypothesis, we have found p65 nuclear translocation and reduced NF-κB transcriptional activity in p65 siRNA-transfected cells, indicating a main role for p65 in NF-κB activation. As discussed above, IκBα is degraded even after IKK knockdown by shRNA, but both serine phosphorylation of p65 and NF-κB transcriptional activity is inhibited. Altogether the mechanism by which proteasome inhibitors induce NF-κB activity seems to be tightly regulated. Such activation requires phosphorylation and degradation of IκBα but also an IKK-mediated phosphorylation of p65.

Despite the activation of NF-κB, proteasome inhibitors are able to induce cytotoxic effects on endometrial carcinoma cell lines. Although it may be surprising, these features are also observed after treatment of cancer cells with other stress signals such as ionizing radiation, hypoxia, or ultraviolet light and chemotherapeutic drugs such as vincristine, vinblastine, etoposide, adriamycin, cisplatin, daunorubicin, etc. (31). For example, topoisomerase inhibitors such as camptothecin or doxorubicin activate NF-κB but induce cell death (32–34). However, the NF-κB activation observed after proteasome inhibitor treatment has a particular relevance because they are widely used as pharmacological blockers of IκBα degradation and subsequent NF-κB inhibition. In fact, as a control of correct proteasome inhibition, we have found that such inhibitors are effective to block NF-κB activation by TNF in both a melanoma cell line and the RL-95 endometrial cell lines, indicating that such drugs are inhibiting proteasome and, as a consequence, the NF-κB activation by the canonical pathway. These results indicate that proteasome inhibitors are effective to block NF-κB by TNF, but long treatment (hours) lead to induction of NF-κB by themselves.

NF-κB inhibition has been proposed as the main mechanism by which proteasome inhibitors are used as anticancer drugs. Although in some cell types NF-κB activation trigger cell death (35, 36), NF-κB does not mediate cytotoxic effects of proteasome inhibition. To test this hypothesis endometrial cancer cell lines were infected with lentiviruses carrying shRNA either to IKKα or IKKβ and treated with cytotoxic doses of bortezomib or MG-132 and we assessed cell viability by MTT assay. Neither IKKα nor IKKβ shRNA increased cell viability after treatment with such inhibitors (supplementary Fig. 1). Rather, we observed a slight decrease. We observed that IKKα or IKKβ knockdown reduced basal proliferation and viability of endometrial cancer cells (data not shown), which is consistent with an anti-apoptotic role of NF-κB in endometrial carcinoma cell lines.

Because of the use of bortezomib on treatment of multiple myeloma and the promising effects on some solid tumors we decided to test the ability of this inhibitor to induce apoptosis on primary explants from tumor samples from patients with endometrial carcinoma. Both MG-132 and bortezomib

induced activation of caspase-3, suggesting that both inhibitors triggered apoptotic cell death. Similar to that observed on endometrial cancer cell lines, bortezomib and MG-132 caused IκBα phosphorylation and degradation, which suggests that such inhibitors also activate NF-κB on primary endometrial carcinoma explants.

During the last few years, other molecular mechanisms, not related to NF-κB, have been involved in proteasome inhibitor cell death. Among them, it has been reported an involvement of the endoplasmic reticulum stress (ER stress) (13, 37, 39) proteins or increases in expression of death receptors (40). Other recent findings suggest that proteasome inhibitors may induce apoptosis by up-regulating BH3 only family members such as Noxa (41–43), Bik (44, 45), or Puma (45). To determine whether proteasome inhibitors produce ER stress in endometrial cancer cells we have performed reverse transcriptase-PCR analysis of *GADD153* and *HOX-1* (heme oxygenase), two stress genes that have been previously demonstrated to increase after ER stress after bortezomib treatment (13, 37, 39). We have also analyzed Noxa, BIK, and Puma expression after treatment with bortezomib (supplementary Fig. 2). In agreement with previous reports, we have found up-regulation of both *GADD153* and *HOX-1* (heme oxygenase), suggesting that ER stress may be involved in apoptosis triggered by proteasome inhibitors. We have not found significant changes in either Puma or Noxa, but a slightly and transient increase in Bik levels. Future experiments will determine whether BH3 only proteins such as Bik and ER stress regulate apoptosis induced by proteasome in endometrial carcinomas.

In summary, we have demonstrated that bortezomib and other three proteasome inhibitors induce cell death in four endometrial carcinoma cell lines as well as in primary culture explants obtained from endometrial carcinoma samples. However, all proteasome inhibitors tested activated NF-κB, a signaling pathway strongly associated in oncogenesis in many types of cancer. Our findings may have important biochemical and clinical relevance. First, because proteasome inhibitors are widely used as pharmacological inhibitors of NF-κB on the basis of their ability to block IκBα degradation but, as we show here for endometrial carcinomas, they can rather lead to activation of NF-κB. Therefore, the general use of such inhibitors as NF-κB blockers has to be carefully analyzed for particular cell types. Second, if proteasome inhibitors activate NF-κB, administration of these drugs may increase the expression of genes involved with proliferation, apoptosis resistance, or angiogenesis. The activation of these genes could explain the development of adverse effects in patients under treatment with proteasome inhibitors. Additional studies should be performed to demonstrate that the NF-κB activation shown in this report in endometrial carcinoma cell lines, and in a previous study on colonic adenocarcinoma cell lines, is also taking place in other types of tumor cells.

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REFERENCES

1. Adams, J. (2004) *Cancer Cell* **5,** 417–421
2. Adams, J. (2004) *Nat. Rev. Cancer* **4,** 349–360
3. Hideshima, T., Richardson, P., Chauhan, D., Palombella, V. J., Elliott, P. J., Adams, J., and Anderson, K. C. (2001) *Cancer Res.* **61,** 3071–3076
4. Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J., and Anderson, K. C. (2002) *J. Biol. Chem.* **277,** 16639–16647
5. LeBlanc, R., Catley, L. P., Hideshima, T., Lentzsch, S., Mitsiades, C. S., Mitsiades, N., Neuberg, D., Goloubeva, O., Pien, C. S., Adams, J., Gupta, D., Richardson, P. G., Munshi, N. C., and Anderson, K. C. (2002) *Cancer Res.* **62,** 4996–5000
6. Mitsiades, N., Mitsiades, C. S., Richardson, P. G., Poulaki, V., Tai, Y.-T., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Schlosman, R., Munshi, N. C., Hideshima, T., and Anderson, K. C. (2003) *Blood* **101,** 2377–2380
7. Orlowski, R. Z., Stinchcombe, T. E., Mitchell, B. S., Shea, T. C., Baldwin, A. S., Stahl, S., Adams, J., Esseltine, D. L., Elliott, P. J., Pien, C. S., Guerchiolini, r., Anderson, J. K., Depcik-Smith, N. D., Bhagat, R., Lehman, M. J., Novick, S. C., O'Connor, O. A., and Soignet, S. L. (2002) *J. Clin. Oncol.* **20,** 4420–4427
8. Teicher, B. A., Ara, G., Herbst, R., Palombella, V. J., and Adams, J. (1999) *Clin. Cancer Res.* **5,** 2638–2645
9. Ling, Y. H., Liebes, L., Jiang, J. D., Holland, J. F., Elliott, P. J., Adams, J., Muggia, F. M., and Perez-Soler, R. (2003) *Clin. Cancer Res.* **9,** 1145–1154
10. Cusack, J. C., Jr., Liu, R., Houston, M., Abendroth, K., Elliott, P. J., Adams, J., Baldwin, A. S., Jr. (2001) *Cancer Res.* **61,** 3535–3540
11. Johnson, T. R., Stone, K., Nikrad, M., Yeh, T., Zong, W. X., Thompson, C. B., Nesterov, A., and Kraft, A. S. (2003) *Oncogene* **22,** 4953–4963
12. Frankel, A., Man, S., Elliott, P., Adams, J., and Kerbel, R. S. (2000) *Clin. Cancer Res.* **6,** 3719–3728
13. Nawrocki, S. T., Carew, J. S., Pino, M. S., Highshaw, R. A., Dunner, K., Jr., Huang, P., Abbruzzese, J. L., and McConkey, D. J. (2005) *Cancer Res.* **65,** 11658–11666
14. Karin, M., and Lin, A. (2002) *Nat. Immunol.* **3,** 221–227
15. Aggarwal, B. B. (2004) *Cancer Cell* **6,** 203–208
16. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) *Nat. Rev. Cancer* **2,** 301–310
17. Rayet, B., and Gelinas, C. (1999) *Oncogene* **18,** 6938–6947
18. Pallares, J., Martinez-Guitarte, J. L., Dolcet, X., Llobet, D., Rue, M., Palacios, J., Prat, J., and Matias-Guiu, X. (2004) *J. Pathol.* **204,** 569–577
19. Dolcet, X., Llobet, D., Pallares, J., and Matias-Guiu, X. (2005) *Virchows Arch.* **446,** 475–482
20. Viatour, P., Merville, M. P., Bours, V., and Chariot, A. (2005) *Trends Biochem. Sci.* **30,** 43–52
21. Jiang, X., Takahashi, N., Matsui, N., Tetsuka, T., and Okamoto, T. (2003) *J. Biol. Chem.* **278,** 919–926
22. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* **274,** 30353–30356
23. Yang, F., Tang, E., Guan, K., and Wang, C. Y. (2003) *J. Immunol.* **170,** 5630–5635
24. Buss, H., Dorrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. (2004) *J. Biol. Chem.* **279,** 55633–55643
25. Nemeth, Z. H., Wong, H. R., Odoms, K., Deitch, E. A., Szabo, C., Vizi, E. S., and Hasko, G. (2004) *Mol. Pharmacol.* **65,** 342–349
26. Huang, T. T., Feinberg, S. L., Suryanarayanan, S., and Miyamoto, S. (2002) *Mol. Cell. Biol.* **22,** 5813–5825
27. Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., and Miyamoto, S. (2003) *Cell* **115,** 565–576
28. Tergaonkar, V., Bottero, V., Ikawa, M., Li, Q., and Verma, I. M. (2003) *Mol. Cell. Biol.* **23,** 8070–8083
29. O'Mahony, A. M., Montano, M., Van Beneden, K., Chen, L. F., and Geene, W. C. (2004) *J. Biol. Chem.* **279,** 18137–18145
30. Mattiolo, I., Sebald, A., Bucher, C., Charles, R. P., Nakano, H., Doi, T., Kracht, M., and Schmitz, M. L. (2004) *J. Immunol.* **172,** 6336–6344
31. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
32. Huang, T. T., Wuerzberger-Davis, S. M., Seufzer, B. J., Shumway, S. D.,

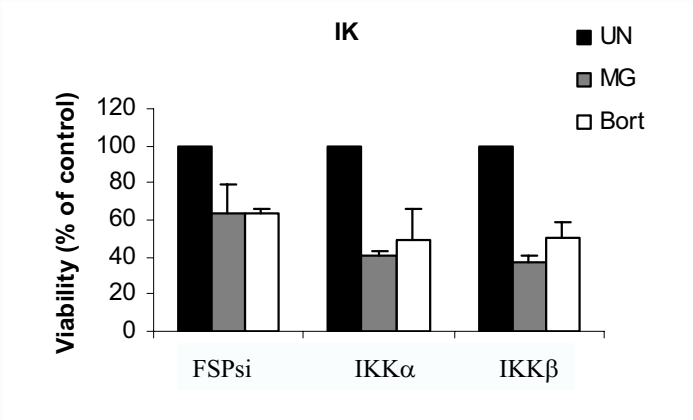
Kurama, T., Boothman, D. A., and Miyamoto, S. (2000) *J. Biol. Chem.* **275,** 9501–9509
33. Bottero, V., Busuttil, V., Loubat, A., Magne, N., Fischel, J. L., Milano, G., and Peyron, J. F. (2001) *Cancer Res.* **61,** 7785–7791
34. Tabata, M., Tabata, R., Grabowski, D. R., Bukowski, R. M., Ganapathi, M. K., and Ganapathi, R. (2001) *J. Biol. Chem.* **276,** 8029 – 8036
35. Farhana, L., Dawson, M. I., and Fontana, J. A. (2005) *Cancer Res.* **65,** 4909 – 4917
36. Jin, F., Liu, X., Zhou, Z., Yue, P., Lotan, R., Khuri, F. R., Cheng, L. W., and Sun, S. Y. (2005) *Cancer Res.* **65,** 6354 – 6363
37. Fribley, A., Zeng, Q., and Wang, C. Y. (2004) *Mol. Cell. Biol.* **24,** 9695–9704
38. Yu, J., Tiwari, S., Steiner, P., and Zhang, L. (2003) *Cancer Biol. Ther.* **2,** 694 – 699
39. Nawrocki, S. T., Carew, J. S., Dunner, K., Jr., Boise, L. H., Chiao, P. J., Huang, P., Abbruzzese, J. L., and McConkey, D. J. (2005) *Cancer Res.* **65,** 11510 –11519
40. Yoshida, T., Shiraishi, T., Nakata, S., Horinaka, M., Wakada, M., Mizutani, Y., Miki, T., and Sakai, T. (2005) *Cancer Res.* **65,** 5662–5667
41. Qin, J. Z., Ziffra, J., Stennett, L., Bodner, B., Bonish, B. K., Chaturvedi, V., Bennett, F., Pollock, P. M., Trent, J. M., Hendrix, M. J., Rizzo, P., Miele, L., and Nickoloff, B. J. (2005) *Cancer Res.* **65,** 6282– 6293
42. Perez-Galan, P., Roue, G., Villamar, N., Montserrat, E., Campo, E., and Colomer, D. (2006) *Blood* **107,** 257–264
43. Fernandez, Y., Verhaegen, M., Millar, T. P., Rush, J. L., Steiner, P., Opipari, A. W., Jr., Lowe, S. W., and Soengas, M. S. (2005) *Cancer Res.* **65,** 6294 – 6304
44. Zhu, H., Guo, W., Zhang, L., Wu, S., Teraishi, F., Davis, J. J., Dong, F., and Fang, B. (2005) *Cancer Biol. Ther.* **4,** 781–786
45. Zhu, H., Zhang, L., Dong, F., Guo, W., Wu, S., Teraishi, F., Davis, J. J., Chiao, P. J., and Fang, B. (2005) *Oncogene* **24,** 4993– 4999
46. Rodriguez, M. S., Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J. L., Hay, R. T., and Arenaza-Seisdedos, F. (1996) *Oncogene* **12,** 2425–2435
47. Mattioli, I., Sebald, A., Bucher, C., Charles, R. P., Nakano, H., Doi, T., Dracht, M., and Schmitz, M. L. (2004) *J. Immunol.* **172,** 6336 – 6344

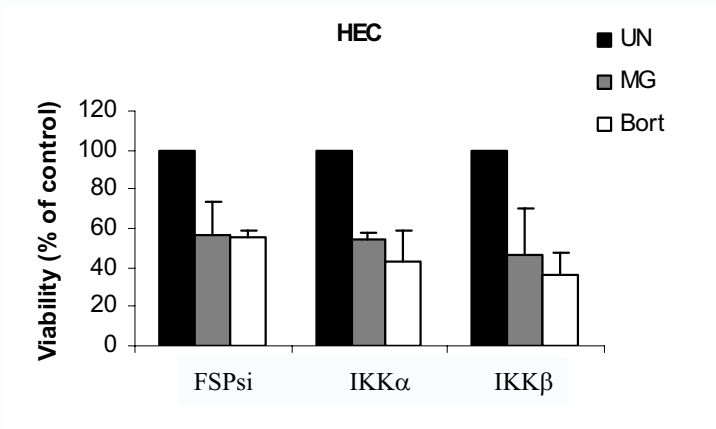
Supplementary figure legends

Supplementary figure 1. To determine the effect of NF-kB activation on apoptosis induced by proteasome inhibitors**,** Ishikawa (IK) and HEC-1-A (HEC) cells were infected with lentiviruses carrying functional shRNAs to IKKα or IKKβAfter 3 days to allow protein knock-down, cells were treated for 24 hours with 25 nM of bortezomib or 0,5 μM MG132. Cell viability was assessed by MTT. Results are expressed as percent of the control values.

Supplementary figure 2. A, To ascertain ascertain whether proteasome inhibitors may regulate the expression of BH3 only proteins, Ishikawa (IK), RL-95(RL) and HEC-1-A (HEC) cells were treated for 6, 12, 24 hours with 25 nM bortezomib. Total cell lysates were subjected to western blot analysis with antibodies to Bik, Noxa and Puma. Immunoblots were reprobed with tubulin to ensure equal protein loading. B, To determine whether proteasome inhibitors produce ER stress in endometrial cancer cells we have performed RT-PCR analysis of GADD153 and heme-oxygenase (HOX-1), two stress genes that have been previously demonstrated to increase after ER stress after bortezomib treatment.

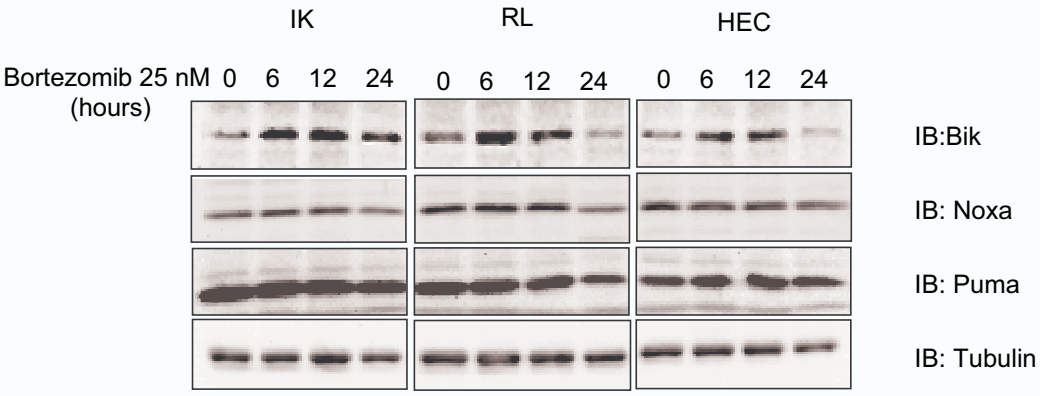
Supplemntary figure 1





Supplementary Figure 2

A



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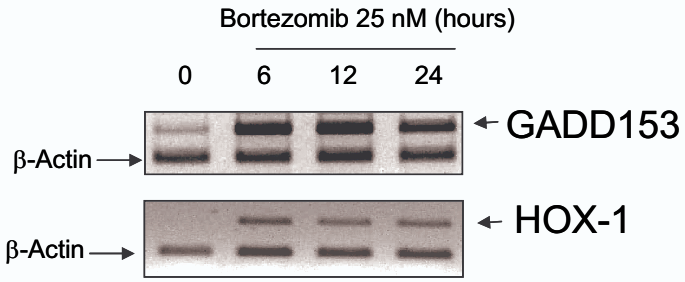

6. ELS ANTIOXIDANTS BLOQUEGEN LA FUNCIÓ DELS INHIBIDORS DEL PROTEASOMA EN CÈL·LULES DE CARCINOMA D'ENDOMETRI.

6.1 El Tiron inhibeix els efectes citotòxics del bortezomib i la edavarona inhibeix els efectes dels inhibidors del proteasoma de la família dels pèptids aldehids.

6.2 El Tiron i la edavarona inhibeixen el fenotip apoptòtic produït per el bortezomib i el MG-132 i ALLN respectivament.

6.3 L'acumulament de productes ubiquitinitzats com a conseqüència del tractament amb bortezomib, MG-132 i ALLN s'inhibeix amb la coadministració de Tiron i edavarona respectivament.

6.4 El Tiron i la edavarona bloquegen l'acció inhibitòria del bortezomib, MG-132 i ALLN sobre l'activitat del proteasoma.

6.5 Altres antioxidants com el BHA, vitamina E o la ergotioneína no exhibeixen els mateixos efectes contra cap dels inhibidors del proteasoma testats. No és el cas de la vitamina C que es comporta de forma idèntica al Tiron.

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Antioxidants block proteasome inhibitor function in endometrial carcinoma cells

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We have recently demonstrated that proteasome inhibitors can be effective in inducing apoptotic cell death in endometrial carcinoma cell lines and primary culture explants. Increasing evidence suggests that reactive oxygen species are responsible for proteasome inhibitor-induced cell killing. Antioxidants can thus block apoptosis (cell death) triggered by proteasome inhibition. Here, we have evaluated the effects of different antioxidants (edaravone and tiron) on endometrial carcinoma cells treated with aldehyde proteasome inhibitors (MG-132 or ALLN), the boronic acid-based proteasome inhibitor (bortezomib) and the epoxyketone, epoxomicin. We show that tiron specifically inhibited the cytotoxic effects of bortezomib, whereas edaravone inhibited cell death caused by aldehyde-based proteasome inhibitors. We have, however, found that edaravone completely inhibited accumulation of ubiquitin and proteasome activity decrease caused by MG-132 or ALLN, but not by bortezomib. Conversely, tiron inhibited the ubiquitin accumulation and proteasome activity decrease caused by bortezomib. These results suggest that edaravone and tiron rescue cells of proteasome inhibitors from cell death, by inhibiting blockade of proteasome caused by MG-132 and ALLN or bortezomib, respectively. We also tested other antioxidants, and we found that vitamin C inhibited bortezomib-induced cell death. Similar to tiron, vitamin C inhibited cell death by blocking the ability of bortezomib to inhibit the proteasome. Until now, all the antioxidants that blocked proteasome inhibitor-induced cell death also blocked the proteasome inhibitor mechanism of action.

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Introduction

During the last few years, a number of studies have proved that proteasome inhibitors are effective anticancer drugs. Among patients with haematological malignancies, those with multiple myeloma are currently being treated with the proteasome inhibitor bortezomib (Velcade, formerly known as PS-341) [1–6]. Ongoing preclinical studies have suggested that proteasome inhibitors show antitumour activity against solid tumours, including carcinomas of the breast [7], lung [8], colon [9], bladder [10], ovary [11], pancreas [12] and others. A recent study in our laboratory has demonstrated that proteasome inhibitors can also be effective for inducing apoptotic cell death in endometrial carcinoma cell lines and primary culture explants [13].

Proteasome inhibitors can be classified into different groups on the basis of their chemical structures and modes of action: [14] (i) the boronic acid-based proteasome inhibitors such as bortezomib, (ii) the aldehyde-based proteasome inhibitors such as MG-132, MG-115, ALLN, PSI and glyoxal or (iii) the epoxyketones such as epoxomicin. The vast majority of the currently available proteasome inhibitors preferentially block the chemotrypsin-like activity of the proteasome.

Recent evidences suggest that proteasome inhibitors might cause cell death by the induction of reactive oxygen species (ROS) in several malignancies such as non-small lung cancer cells [15], head and neck squamous carcinoma cells [16] and mantle-cell lymphoma cells [17]. These studies have also demonstrated that different antioxidants can inhibit proteasome-induced cytotoxicity in some cell types. Recent studies have, however, also demonstrated that the proteasome-inhibitor action of bortezomib on melanoma cells [18] or on different cell lines [19] might be blocked by antioxidants like tiron or vitamin C.

We have previously demonstrated that proteasome inhibitors are effective in inducing death in endometrial carcinoma cell lines and primary tumour explants.

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Here, we have assessed the effects of different antioxidants on proteasome-inhibitor cytotoxicity on endometrial carcinoma cell lines. Surprisingly, we have found a different effect of several antioxidants on different proteasome inhibitors (bortezomib, MG-132, ALLN and epoxomicin). For example, tiron completely inhibits proteasome-induced apoptosis caused by bortezomib, but has no effects on the other inhibitors. Conversely, edaravone blocks MG-132-induced and ALLN-induced apoptosis, but does not have significant effects on apoptosis induced by bortezomib/epoxomicin. The evaluation of the effects of proteasome inhibitors has demonstrated that tiron inhibits the ability of bortezomib to inhibit the proteasome. In contrast, edaravone blocks the effects of aldehyde-based proteasome inhibitors such as MG-132 and ALLN.

Materials and methods

Reagents, plasmids and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to tubulin and tiron were from Sigma (St Louis, Missouri, USA). MCI-186 (edaravone) the proteasome inhibitors MG-132, epoxomicin and ALLN and the fluorogenic proteasome substrate III were from Calbiochem (La Jolla, California, USA). Bortezomib (Millenium Pharmaceuticals, Cambridge, Massachusetts, USA) was obtained from the Department of Pharmacy (Hospital Arnau de Vilanova, Lleida, Spain). Antibodies to active caspase-3 and caspase-9 were obtained from Cell Signalling (Beverly, Massachusetts, USA). Antibody to ubiquitin was from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Peroxidase-conjugated antimouse and antirabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden).

Cell lines, culture conditions and transfection

The Ishikawa 3-H-12 cell line was obtained from the American Type Culture Collection (Manassas, Virginia, USA). KLE cells were a gift from Dr Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid, Spain). RL-95 and HEC-1-A cells were a gift from Dr Reventos (Hospital Vall d'Hebron, Barcelona, Spain). The melanoma cell lines were a gift from Rosa M. Martí. All cell lines were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal bovine serum (Invitrogen Inc., Carlsbad, California, USA), 1 mmol/l HEPES (Sigma), 1 mmol/l sodium pyruvate (Sigma), 2 mmol/l L-glutamine (Sigma) and 1% of penicillin/streptomycin (Sigma), at 37°C with saturating humidity and 5% CO_2.

Cell-viability assays and assessment of apoptosis

Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated onto M96-well plates at 15×10^3 cells/well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent and lysed with dimethyl sulphoxide. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, California, USA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Western blot analysis

Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and were lysed with lysis buffer (2% SDS; 125 mmol/l Tris–HCl, pH 6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). Nonspecific binding was blocked by incubation with Tris-buffered saline/Tween solution (20 mmol/l Tris–HCl, pH 7.4; 150 mmol/l NaCl; 0.1% Tween-20) with 5% of nonfat milk. Membranes were incubated with the primary antibodies overnight at 4°C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

Proteasome-activity assay

Endometrial carcinoma cell lines were plated onto M24 multiwell dishes at 15×10^5 cells/well. The cells were treated with the experimental conditions for 8 h. After treatment, cells were rinsed with phosphate-buffered saline and lysed in 50 µl of cytoplasmic lysis buffer (50 mmol/l HEPES, pH 8; 1% NP-40; 150 mmol/l NaCl; 1 mmol/l EDTA and 1 mmol/l ethylene glycol tetraacetic acid). Nuclei were removed by centrifugation at 10 000 rpm for 5 min. Protein concentrations were determined with the Protein Assay Kit. A total of 10–20 µg of protein was transferred to a 90-µl final volume of assay buffer (20 mmol/l HEPES, pH 7; 9, 0.5 mmol/l EDTA; 5 mmol/l $MgCl_2$ and 2 mmol/l ATP). After 10 min at 37°C, 10 µl containing 200 µmol/l of the fluorogenic proteasome substrate III Suc-Leu-Leu-Val-Tyr-AMC (final concentration of substrate 20 µmol/l) was added to each sample. Fluorescence was read every 15 min in a microplate fluorimeter at 380 nm excitation wavelength and 460 nm emission.

Results

Tiron inhibits the cytotoxic effects of bortezomib and edaravone inhibits the effects of aldehyde-based proteasome inhibitors

We have previously demonstrated that proteasome inhibitors are effective in inducing apoptosis of endometrial carcinoma cells. In contrast, some antioxidants have been shown to inhibit cell death caused by proteasome inhibitors. We first tested the effects of tiron and edaravone on cell death caused by different types of proteasome inhibitors, which use different chemical mechanisms for inhibiting the 20S proteasome. We have

used the aldehyde-based proteasome inhibitors, MG-132 and ALLN; the dipeptide boronic acid, bortezomib; and the peptide epoxyketone, epoxomicin. The ishikawa (IK) endometrial carcinoma cell line was treated with the four proteasome inhibitors in addition to increasing doses of either tiron or edaravone, and we assessed cell viability by MTT assay 24 h after treatment. The results showed that tiron treatment caused a dose-dependent inhibition of the decrease in cell viability caused by bortezomib, but had no effect on cell death caused by treatment with the other three proteasome inhibitors (Fig. 1a). Conversely, edaravone inhibited the decrease in cell viability caused by MG-132 and ALLN in a dose-dependent manner; however, it did not have any effect on cell death induced either by bortezomib or by epoxomicin (Fig. 1b).

Tiron and edaravone inhibit apoptotic features induced by bortezomib and aldehyde proteasome inhibitors, respectively

We have also addressed the effects of tiron and edaravone on apoptotic nuclear morphology caused by proteasome inhibition. IK cells were treated with the four proteasome inhibitors, alone or in the presence of either tiron or edaravone, and cells were stained with Hoechst 24 h after treatment. In agreement with the results obtained with the viability assays, the addition of tiron dramatically reduced the number of nuclei displaying apoptotic morphology in the cultures treated with bortezomib, but did not show any effect on the number of apoptotic nuclei in the cultures treated with epoxomicin, MG-132 or ALLN (Figs 2a and b). In contrast, edaravone inhibited the appearance of apoptotic nuclei on MG-132-treated and ALLN-treated cells, but it did not cause any reduction in the bortezomib-treated or epoxomicin-treated cultures (Figs 2a and b). To rule out the possibility that such effects were specific to a cell line, we performed the experiments on another endometrial carcinoma cell lines (Fig. 2b), and we obtained identical results.

We next analysed the effects of tiron and edaravone on caspase activation in endometrial carcinoma cell lines under proteasome inhibitor treatment. IK cells were treated with bortezomib, MG-132, ALLN or epoxomicin, in the presence or absence of tiron and edaravone. After 24 h, we analysed caspase activation by Western blot. All four proteasome inhibitors caused activation of caspase-3 and caspase-9, as seen by Western blot analysis of procaspase processing and generation of active fragments (Fig. 2c). Caspase activation was completely abolished in conditions containing bortezomib along with tiron, but not in conditions containing any of the other three proteasome inhibitors along with tiron (Fig. 2c). In contrast, edaravone inhibited caspase-9 and caspase-3 processing induced by MG-132 or ALLN, but did not inhibit caspase processing caused by bortezomib or epoxomicin.

Ubiquitin accumulation by bortezomib and aldehyde-based proteasome inhibitors is inhibited by tiron and edaravone, respectively

We next assayed the effects of either tiron or edaravone on the function of proteasome inhibitors by using two different readouts. First, we examined the accumulation of ubiquitinated proteins by Western blot, using an antibody that recognizes ubiquitin. Ubiquitinated product accumulation results from the inability of proteasome to degrade such products. Time-course incubation of IK cells with MG-132 resulted in an increasing amount of ubiquitinated proteins being present in cell protein extracts (Fig. 3a). Similar results were obtained with the other proteasome inhibitors.

Treatment of IK cells with each of the four proteasome inhibitors produced a similar amount of accumulation of ubiquitinated products. IK cells under cotreatment with tiron and bortezomib, however, showed amounts of ubiquitinated products that were identical to the normal levels of untreated cells (Fig. 3b). This inhibition of accumulation of ubiquitinated proteins was not observed with cotreatment with tiron or any of the other three proteasome inhibitors. Similarly, edaravone inhibited the accumulation of ubiquitinated proteins caused by MG-132 or ALLN, without any effects on ubiquitin accumulation caused by bortezomib or epoxomicin (Fig. 3b).

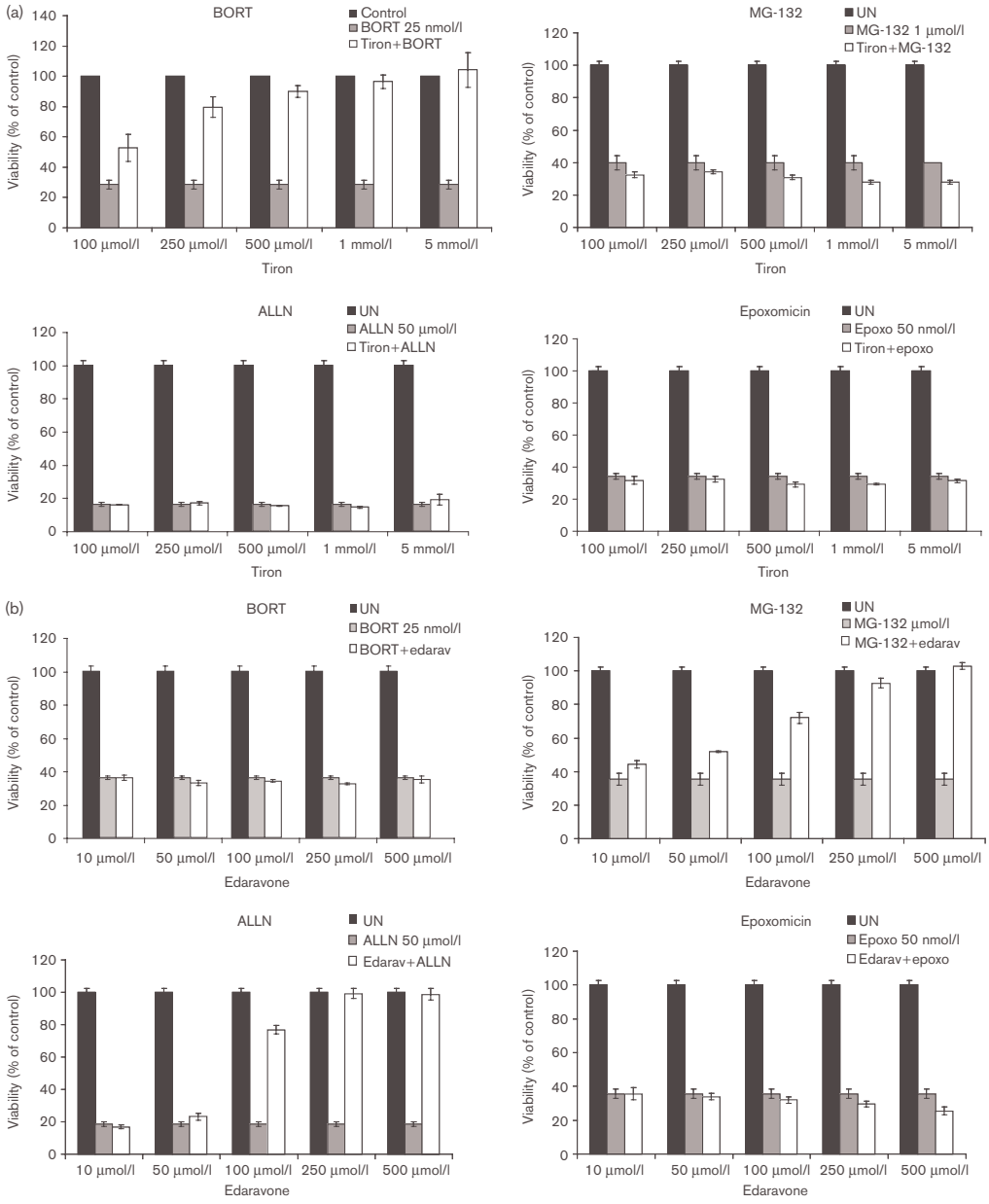
To determine whether these effects were specific for a precise tumour cell type, we extended the study to three additional endometrial carcinoma cell lines (RL-95/2, HEC-1-A and KLE) and one melanoma cell line (M36). As shown in Figs 3c and d, bortezomib was inhibited by tiron. In contrast, this action was performed on MG-132 and ALLN inhibition by edaravone, both in carcinoma and in melanoma cell lines.

Tiron and edaravone blocked proteasome-activity inhibition caused by bortezomib and aldehyde-based proteasome inhibitors

To ascertain whether the ubiquitin accumulation was the result of proteasome inhibition, we assayed the proteasome activity by incubation of cell lysates with the fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-AMC. Such a substrate is used to measure the chymotrypsin-like peptidase activity of the 20S proteasome. Treatment of IK cells with each of the four proteasome inhibitors resulted in a significant reduction of proteasome activity, by this assay. Bortezomib and epoxomicin were more efficient than MG-132 and ALLN in inhibiting the proteasome (Fig. 4a).

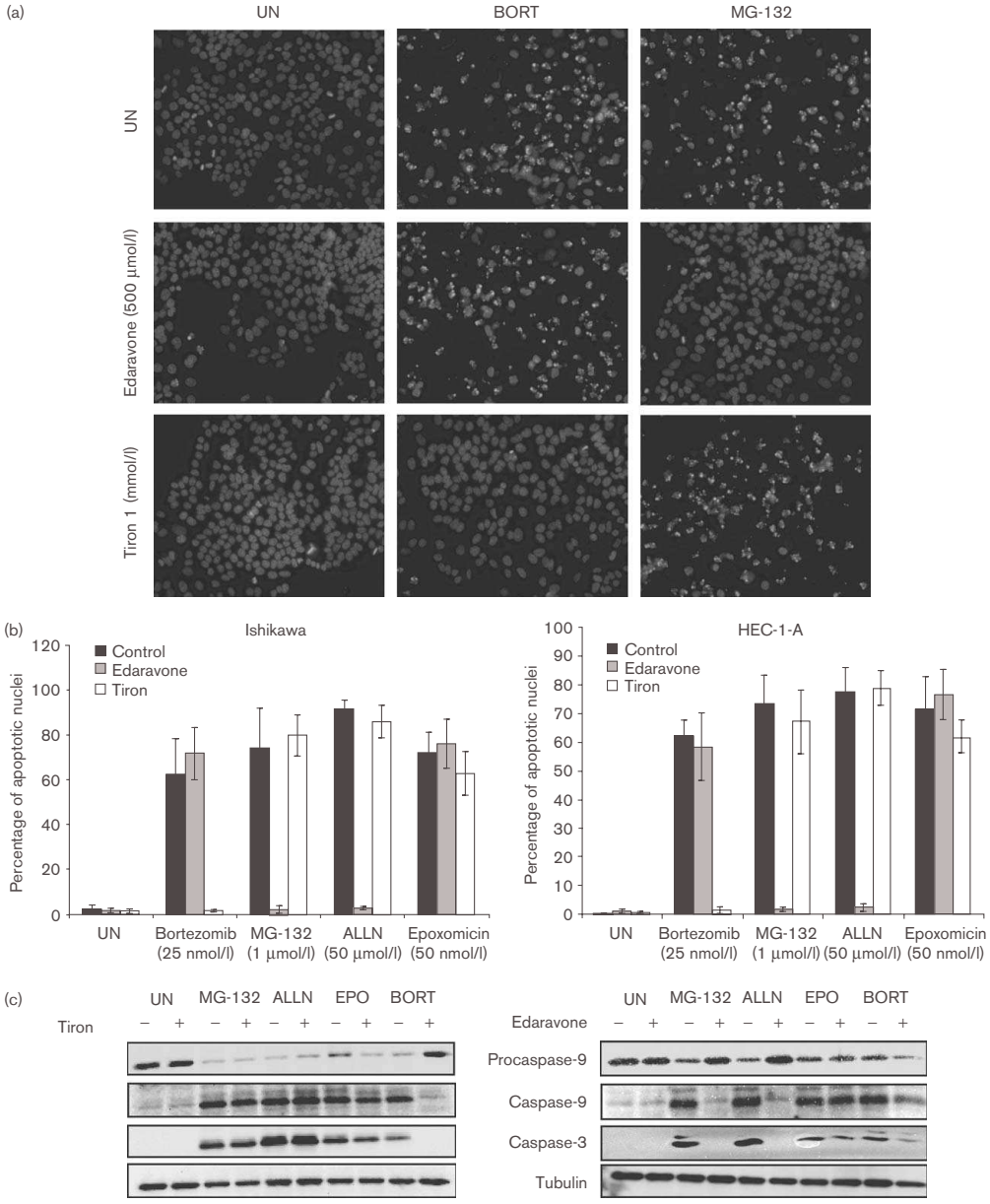
To assess the effects of antioxidants on proteasome-activity inhibition, we assayed proteasome activity in cell lysates obtained from cultures cotreated with either tiron or edaravone and with each of the proteasome inhibitors

Fig. 1



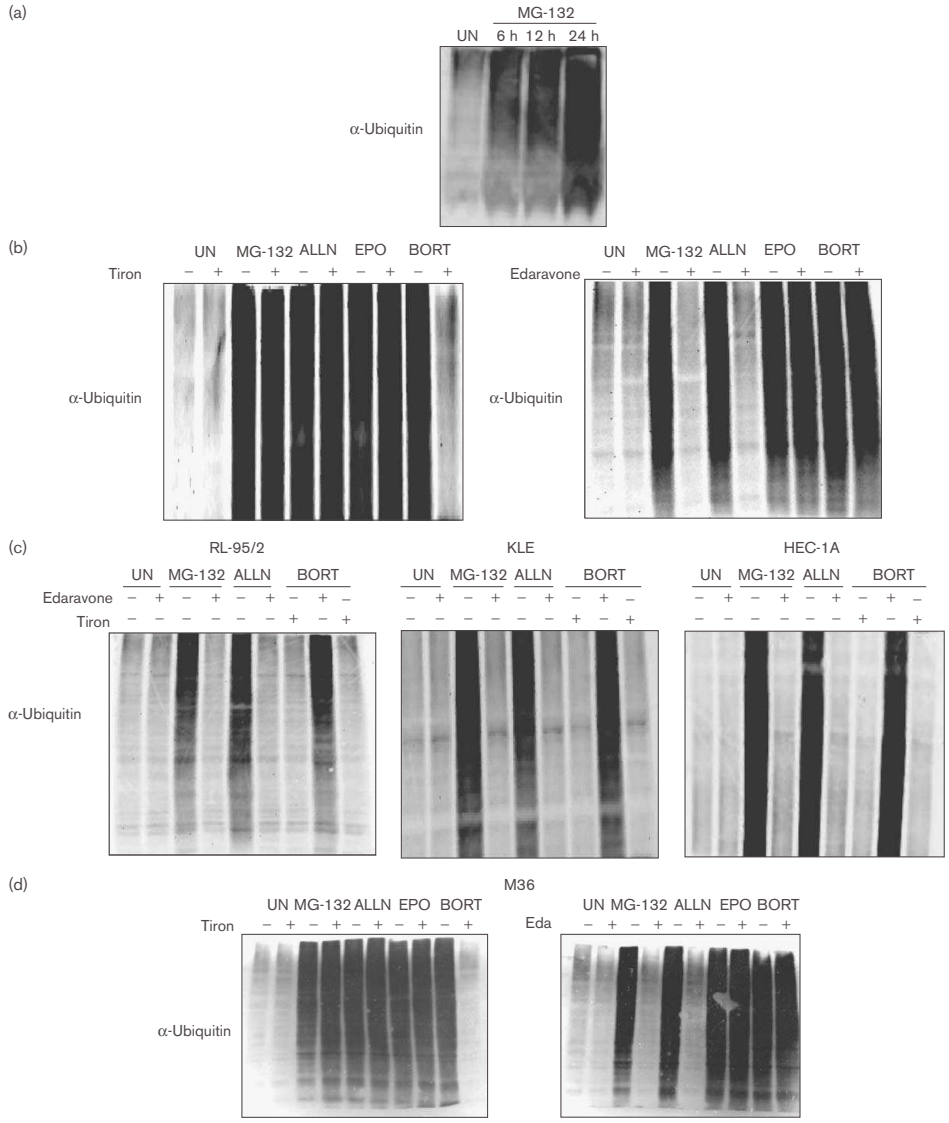
Differential effects of tiron and edaravone on reduction of cell viability caused by structurally different proteasome inhibitors. (a) IK cells were treated with 25 nmol/l bortezomib (top left), 1 μmol/l MG-132 (top right), 50 μmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of tiron. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. (b) IK cells were treated with 25 nmol/l bortezomib (top left), 1 μmol/l MG-132 (top right), 50 μmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of edaravone. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. BORT, bortezomib; IK, ishikawa; UN, untreated.

Fig. 2



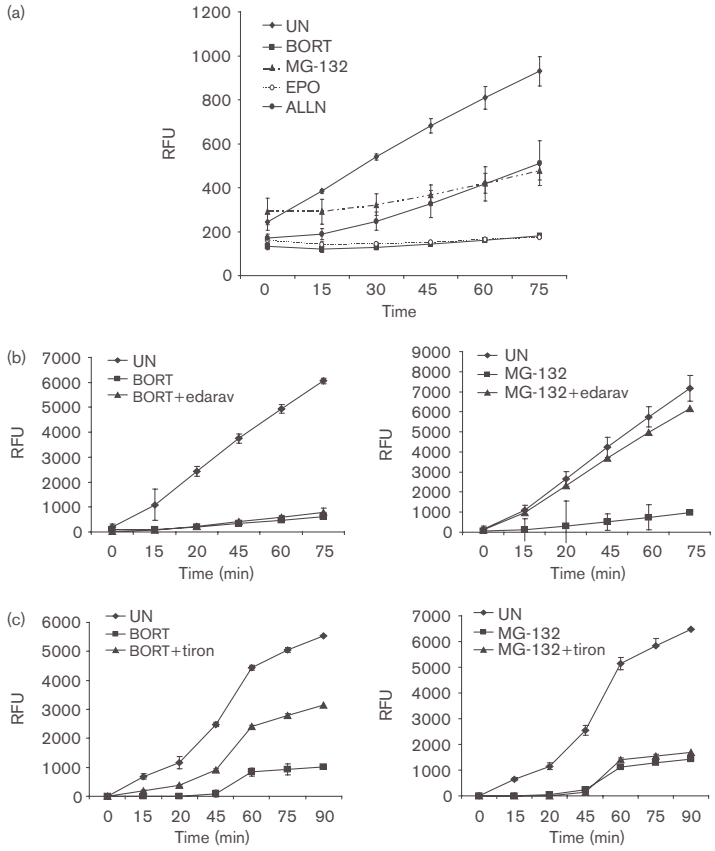
Tiron and edaravone block apoptotic nuclear morphology and caspase activation observed after treatment with proteasome inhibitors. (a) Representative micrographs showing Hoechst staining of IK cells treated for 24 h with either bortezomib 25 nmol/l or MG-132 1 μmol/l, alone or together with edaravone 500 μmol/l or tiron 1 mmol/l. (b) Quantification of IK (left) or HEC-1-A (right) nuclei displaying apoptotic morphology. IK or HEC-1-A cells were treated with the indicated proteasome inhibitors and doses, alone or in combination with edaravone or tiron. Results are expressed as percentage of apoptotic nuclei over control condition. (c) IK cells were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l of tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cell lysates were analysed by Western blot with antibodies to caspase-9, active caspase-3. Membranes were reprobed with antibodies to tubulin to ensure equal protein amounts. IK, ishikawa.

Fig. 3



Tiron and edaravone block ubiquitin accumulation induced by bortezomib-based and aldehyde-based proteasome inhibitors, respectively. (a) IK cells were treated for 6, 12 or 24 h with MG-132 and lysed. Protein lysates were analysed by Western blot with antiubiquitin antibodies. (b) IK cells were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (c) RL-95/2, KLE and HEC-1A endometrial carcinoma cell lines were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron or 500 μmol/l edaravone. After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (d) M36 melanoma cell lines were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cell lysates were analysed by Western blot with antibodies to ubiquitin. IK, ishikawa.

Fig. 4



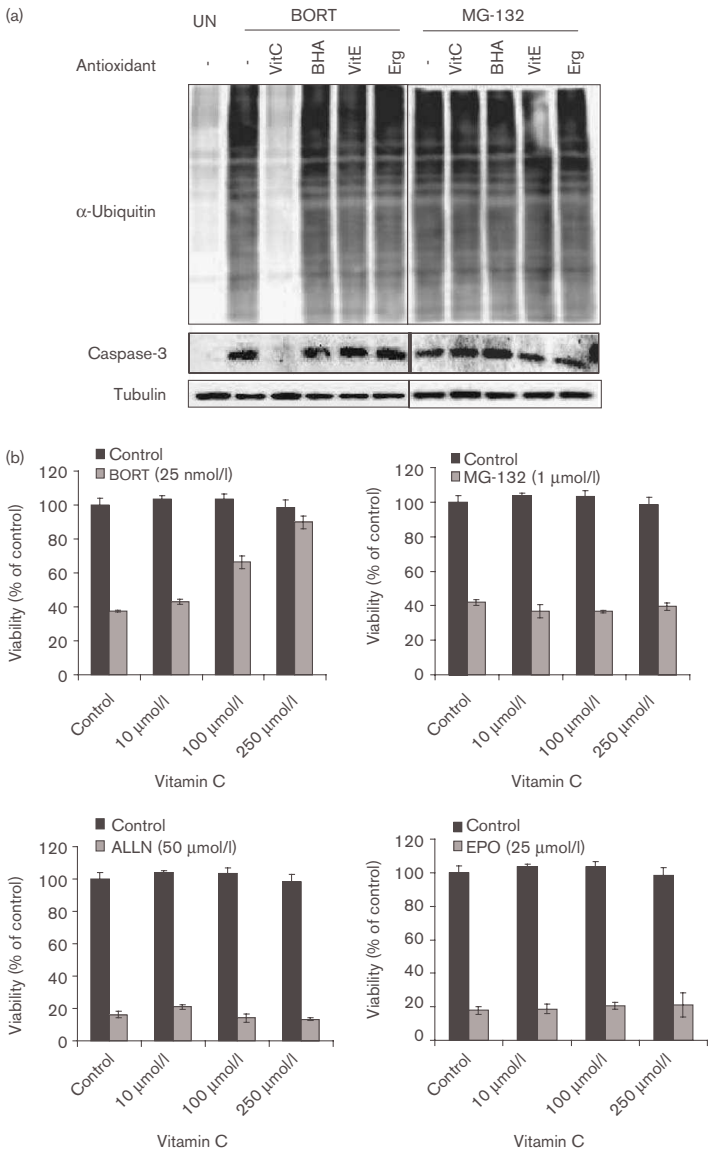
Effects of edaravone and tiron on activity of proteasome inhibitors on endometrial carcinoma cells. (a) Proteasome inhibitors reduce proteasome activity. IK cells were treated for 24 h with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). Cell lysates were incubated in the presence of the proteasome substrate as described in Materials and methods. Results are expressed as relative fluorescence units (RFUs) over time of incubation with the substrate. (b) Edaravone inhibits MG-132 blockade of proteasome activity. IK cells were treated for 24 h with 1 μmol/l MG-132 (right graph) or 25 nmol/l bortezomib (BORT) (left graph), alone or in the presence of edaravone. Cell lysates were incubated in the presence of the proteasome substrate, as described in Materials and methods. Results are expressed as RFUs. (c) IK cells were treated for 24 h with 1 μmol/l MG-132 (right graph) or 25 nmol/l bortezomib (BORT) (left graph) alone or in the presence of tiron. Cell lysates were incubated in the presence of the proteasome substrate, as described in Materials and methods. Results are expressed as RFUs. IK, ishikawa.

included in this study. Treatment of IK cell with MG-132 in addition to edaravone returned the activity of the proteasome to normal levels, indicating that edaravone blocks the proteasome inhibitory function of aldehyde-based proteasome inihibitors (Fig. 4b). In contrast, edaravone had no effects on inhibition caused by bortezomib. Tiron also insignificantly reduced the blockade of the proteasome caused by the bortezomib treatment of cells, but not that caused by the MG-132 treatment of cells (Fig. 4c).

Effects of other antioxidants on cell killing caused proteasome inhibition on endometrial carcinoma

To generalize the effects of antioxidants on proteasome inhibitors, we extended our study to other antioxidants. IK cells were treated with bortezomib or MG-132 in the presence or absence of the following antioxidants: vitamin C, vitamin E, butylated hydroxyanisole or ergothioneine. Cell lysates were collected 24 h later, and protein extracts were analysed by Western blot for ubiquitin accumulation

Fig. 5



Effects of antioxidants on the viability and activity of proteasome inhibitors. (a) IK cells were treated with 1 μmol/l MG-132 or 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lines, proteasome inhibitors were coincubated with the antioxidant vitamin C (VitC), butylated hydroxyanisole (BHA), vitamin E (VitE) or ergothioneine (Erg). After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (b) IK cells were treated with 25 nmol/l bortezomib (top left), 1 μmol/l MG-132 (top right), 50 μmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of vitamin C. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. IK, ishikawa.

or caspase-3 activation. Out of all the combinations, we found that vitamin C inhibited caspase 3 activation in bortezomib-treated cells, but not in MG-132-treated ones. Such inhibition correlated with the inhibition of ubiquitin accumulation (Fig. 5a). These results further support the hypothesis that all the antioxidants that were found to prevent proteasome-induced cell death also blocked the ability of the proteasome inhibitor to block the proteasome. Results obtained with vitamin C on cell viability were confirmed by the MTT analysis of IK cells treated with bortezomib, MG-132, ALLN or epoxomicin (Fig. 5b).

Discussion

We have previously demonstrated that proteasome inhibitors are effective tools for inducing apoptosis of endometrial carcinoma cell lines and primary explants [13]. Here, we have demonstrated that different antioxidants can block the cytotoxicity induced by different proteasome inhibitors on endometrial carcinoma cells. Although tiron and vitamin C inhibit the cell death induced by the boronic acid-containing bortezomib, edaravone inhibits cell killing caused by the aldehyde-based proteasome inhibitors, MG-132 or ALLN. In contrast, edaravone does not have any effect on cell death triggered by bortezomib, but completely blocks cell death caused by treatment with MG-132 or ALLN. In addition, we have found no effects of any of the antioxidant substances that have been tested on epoxomicin-induced cell death. We have also seen that the antiapoptotic process is not caused by the modification of pathways downstream of the proteasome, but by interfering with the ability of the proteasome inhibitor to block the proteasome.

Recent findings have suggested that bortezomib and other proteasome inhibitors can increase the production of either endoplasmic reticulum stress or ROS, which is ultimately responsible for proteasome-inhibitor cytotoxicity [15,16,20]. For that reason, several works have approached the effects of antioxidants and radical scavengers in the process of apoptosis induced by proteasome inhibitors. Some studies have demonstrated that ROS scavengers such as glutathione (GSH)-reduced ethyl ester or *N*-acetylcysteine (NAC) block the features of bortezomib-induced apoptosis in mantle-cell lymphoma [17], whereas others have demonstrated that NAC or GSH do not affect apoptosis induced by bortezomib [18,19,21]. One of these free radical scavengers is tiron. Tiron has a potent antioxidant activity because of its ability to scavenge O_2^- radicals. Tiron has been shown to inhibit cell death induced by bortezomib in head and neck squamous cell carcinoma [16] and in pancreatic cancer cells [22]. Recent findings have suggested that ROS scavengers such as tiron can inhibit proteasome-induced cell death, whereas others like butylated hydroxyanisole do not. It has also been shown that antioxidants such as tiron or vitamin C can selectively interfere with and inhibit the function of bortezomib as a proteasome inhibitor. As a result, tiron or vitamin C can inhibit the cytotoxic action of bortezomib [18,19,21]. Both vitamin C and tiron have been shown to abrogate bortezomib activity via direct interaction and to form a biologically inactive complex with bortezomib [19]. In agreement with these reports, we have found that tiron and vitamin C inhibited bortezomib action in endometrial carcinoma cells, but have no effects on other proteasome inhibitors like the aldheyde-based MG-132 or ALLN, or the epoxyketone, epoxomicin. In addition to these data, we have evaluated the effects of edaravone on cytotoxicity induced by proteasome inhibitors. Edavarone is a free radical scavenger that primarily scavenges hydroxyl and peroxyl radicals and has the antioxidant ability to inhibit lipid peroxidation Edaravone has been proved to improve the clinical outcome of patients after acute myocardial infarction [23,24] and acute cerebrovascular injury [25,26]. Moreover, edaravone has been demonstrated to exhibit antitumourogenic activity [27,28]. Interestingly, our results demonstrate that although edaravone inhibits MG-132-induced or ALLN-induced cell death, it does not inhibit cell death caused by bortezomib or epoxomicin. Edaravone completely abolishes cytotoxicity, and decreases the number of apoptotic nuclei and the levels of caspase activation. We found, however, that edaravone inhibited the accumulation of ubiquitinated products, suggesting that it could block the proteasome-inhibiting action of MG-132. This possibility was further analysed by a proteasome-activity assay. In agreement with the results obtained with ubiquitin accumulation, we demonstrated that edaravone inhibited the action of MG-132. In summary, some antioxidants or ROS scavengers, such as tiron or vitamin C, might block the action of boronic acid-based proteasome inhibitors, such as bortezomib. In contrast, other antioxidants, such as edaravone, might block aldehyde-based proteasome inhibitors, without any effects on other types of proteasome inhibitors. Therefore, a careful study of the activity of the proteasome should be made when using antioxidants or free radical scavengers to block proteasome-inhibitor cytotoxicity.

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References

1 Hideshima T, Chauhan D, Richardson P, Mitsiades C, Mitsiades N, Hayashi T, *et al*. NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem* 2002; **277**:16639–16647.

2 Hideshima T, Chauhan D, Podar K, Schlossman RL, Richardson P, Anderson KC. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. *Semin Oncol* 2001; **28**:607–612.

3 Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, *et al*. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 2003; **348**:2609–2617.

4 Mitsiades N, Mitsiades CS, Richardson PG, Poulaki V, Tai YT, Chauhan D, *et al*. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. *Blood* 2003; **101**:2377–2380.

5 LeBlanc R, Catley LP, Hideshima T, Lentzsch S, Mitsiades CS, Mitsiades N, *et al*. Proteasome inhibitor PS-341 inhibits human myeloma cell growth *in vivo* and prolongs survival in a murine model. *Cancer Res* 2002; **62**:4996–5000.

6 Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annu Rev Med* 2006; **57**:33–47.

7 Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res* 1999; **5**:2638–2645.

8 Ling YH, Liebes L, Jiang JD, Holland JF, Elliott PJ, Adams J, *et al*. Mechanisms of proteasome inhibitor PS-341-induced G_2–M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clin Cancer Res* 2003; **9**:1145–1154.

9 Cusack JC Jr, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J, *et al*. Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. *Cancer Res* 2001; **61**:3535–3540.

10 Johnson TR, Stone K, Nikrad M, Yeh T, Zong WX, Thompson CB, *et al*. The proteasome inhibitor PS-341 overcomes trail resistance in bax and caspase 9-negative or bcl-xl overexpressing cells. *Oncogene* 2003; **22**:4953–4963.

11 Frankel A, Man S, Elliott P, Adams J, Kerbel RS. Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. *Clin Cancer Res* 2000; **6**:3719–3728.

12 Nawrocki ST, Carew JS, Pino MS, Highshaw RA, Dunner K Jr, Huang P, *et al*. Bortezomib sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis. *Cancer Res* 2005; **65**:11658–11666.

13 Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA, *et al*. Proteasome inhibitors induce death but activate NF-kappa B on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* 2006; **281**:22118–22130.

14 Myung J, Kim KB, Crews CM. The ubiquitin–proteasome pathway and proteasome inhibitors. *Med Res Rev* 2001; **21**:245–273.

15 Ling YH, Liebes L, Zou Y, Perez-Soler R. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. *J Biol Chem* 2003; **278**:33714–33723.

16 Fribley A, Zeng Q, Wang CY. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. *Mol Cell Biol* 2004; **24**:9695–9704.

17 Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of Ros and Noxa activation independent of p53 status. *Blood* 2006; **107**:257–264.

18 Fernandez Y, Miller TP, Denoyelle C, Esteban JA, Tang WH, Bengston AL, *et al*. Chemical blockage of the proteasome inhibitory function of bortezomib: impact on tumor cell death. *J Biol Chem* 2006; **281**: 1107–1118.

19 Zou W, Yue P, Lin N, He M, Zhou Z, Lonial S, *et al*. Vitamin C inactivates the proteasome inhibitor PS-341 in human cancer cells. *Clin Cancer Res* 2006; **12**:273–280.

20 Fribley A, Wang CY. Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress. *Cancer Biol Ther* 2006; **5**:745–748.

21 Catley L, Anderson KC. Velcade and vitamin C: too much of a good thing? *Clin Cancer Res* 2006; **12**:3–4.

22 Yeung BH, Huang DC, Sinicrope FA. PS-341 (bortezomib) induces lysosomal cathepsin B release and a caspase-2-dependent mitochondrial permeabilization and apoptosis in human pancreatic cancer cells. *J Biol Chem* 2006; **281**:11923–11932.

23 Tsujita K, Shimomura H, Kawano H, Hokamaki J, Fukuda M, Yamashita T, *et al*. Effects of edaravone on reperfusion injury in patients with acute myocardial infarction. *Am J Cardiol* 2004; **94**:481–484.

24 Tsujita K, Shimomura H, Kaikita K, Kawano H, Hokamaki J, Nagayoshi Y, *et al*. Long-term efficacy of edaravone in patients with acute myocardial infarction. *Circ J* 2006; **70**:832–837.

25 Dohi K, Satoh K, Mihara Y, Nakamura S, Miyake Y, Ohtaki H, *et al*. Alkoxyl radical-scavenging activity of edaravone in patients with traumatic brain injury. *J Neurotrauma* 2006; **23**:1591–1599.

26 Yoshida H, Yanai H, Namiki Y, Fukatsu-Sasaki K, Furutani N, Tada N. Neuroprotective effects of edaravone: a novel free radical scavenger in cerebrovascular injury. *CNS Drug Rev* 2006; **12**:9–20.

27 Kokura S, Yoshida N, Sakamoto N, Ishikawa T, Takagi T, Higashihara H, *et al*. The radical scavenger edaravone enhances the antitumor effects of CPT-11 in murine colon cancer by increasing apoptosis via inhibition of NF-kappa B. *Cancer Lett* 2005; **229**:223–233.

28 Suzuki R, Gopalrao RK, Maeda H, Rao P, Yamamoto M, Xing Y, *et al*. MCI-186 inhibits tumor growth through suppression of EGFR phosphorylation and cell cycle arrest. *Anticancer Res* 2005; **25**: 1131–1138.

Discussió

1. LES CÈL·LULES DE CÀNCER D'ENDOMETRI SÓN RESISTENTS A L'APOPTOSI INDUIDA PER TRAIL.

L'apoptosi és un procés clau en la regulació de l'homeòstasi cel·lular. Alteracions en l'apoptosi, per tant, poden jugar un paper important en el desenvolupament i progressió del càncer. La desregulació de proteïnes implicades en el control de l'apoptosi pot resultar en l'aparició de poblacions cel·lulars que siguin capaces d'evadir la mort apoptòtica [146]. La falta d'una resposta enfront a estímuls apoptòtics pot produir un avantatge en termes de supervivència i la posterior expansió de poblacions de cèl·lules neoplàsiques. Encara més, defectes en l'apoptosi poden conferir a les cèl·lules que els presentin resistència a la teràpia antitumoral.

La teràpia antitumoral convencional no és sempre efectiva i presenta desavantatges. Un d'aquests és la baixa capacitat selectiva ja que no distingeix entre cèl·lules tumorals i cèl·lules normals. En aquest sentit són moltes les aproximacions i els estudis realitzats encaminats a trobar i desenvolupar noves eines terapèutiques que presentin propietats més selectives i disminueixin els efectes secundaris derivats de la teràpia convencional.

TRAIL, un membre de la família del TNF, s'ha erigit com un agent amb propietats antitumorals de gran interès. Aquest interès es centra, a diferència d'altres membres de la mateixa família com el propi TNFα o FasL, en l'absència d'efectes secundaris derivats de la seva administració sistèmica i en la seva selectivitat enfront cèl·lules tumorals sense causar danys en les cèl·lules normals [190,196]. Tanmateix, s'han documentat nombrosos casos de tumors que exhibeixen mecanismes de resistència al tractament per TRAIL. En aquests tumors l'apoptosi es troba bloquejada com a resultat d'alteracions moleculars en proteïnes que regulen tant la via apoptòtica extrínseca com la intrínseca.

En aquest treball demostrem en primer terme que les cèl·lules canceroses de carcinoma d'endometri són resistents al tractament per TRAIL. Posteriorment demostrem que FLIP es troba freqüentment expressat en el carcinoma d'endometri i s'observa, per primer cop, que FLIP pot jugar un paper significatiu en la resistència de les cèl·lules de carcinoma d'endometri a l'apoptosi induïda per TRAIL.

La primera aproximació en l'ús de TRAIL com a agent antitumoral es realitzà en cultius de dues línies cel·lulars de carcinoma d'endometri. L'assaig de viabilitat

cel·lular demostrà que ambdues línies, Ishikawa i KLE, són resistents al tractament per TRAIL.

Tradicionalment, una de les estratègies per sensibilitzar cèl·lules a l'apoptosi induïda per lligands de mort ha estat l'ús d'inhibidors generals de la transcripció (actinomicina D) i de la traducció proteica (cicloheximida). Aquests inhibidors causarien la reducció en l'expressió de proteïnes de vida mitjana curta que podrien participar en la resistència a aquesta mort. De forma esperada, el cotractament de TRAIL amb l'inhibidor actinomicina D resulta en una reducció de la viabilitat cel·lular causada per una mort de característiques apoptòtiques.

Aquests resultats indiquen que aquestes cèl·lules possiblement expressen alguna proteïna en uns nivells suficients com per inhibir la mort induïda per TRAIL.

2. FLIP JUGA UN PAPER EN LA RESISTÈNCIA A TRAIL I ES TROBA FREQÜENTMENT EXPRESSAT EN EL CARCINOMA D'ENDOMETRI.

Una de les proteïnes directament implicades en la regulació de la senyalització per TRAIL és FLIP. En consonància amb els resultats obtinguts, els estudis tant per RT-PCR com per Western blot revelen que els nivells de FLIP cauen en resposta al tractament amb actinomicina D. Aquests resultats apunten a FLIP com a mecanisme de resistència i suporten els resultats obtinguts per altres investigadors en altres tipus de neoplàsies [221,424].

FLIP és la versió humana homòloga a la forma viral v-FLIP, que és expressada pels herpesvirus-γ i que s'ha demostrat que són capaços de bloquejar l'apoptosi induïda per FasL/CD95 a través de l'associació al complex DISC que es forma a nivell de receptor [206].

S'han observat alts nivells d'expressió de FLIP en diversos tumors com són el càncer de pròstata [425], limfoma de Hodgkin [426], càncer d'estómac [427] melanoma [428] o el carcinoma de bufeta [429]. També s'han apreciat nivells elevats de FLIP en el càncer d'ovari [430], un tipus de carcinoma molt similar al càncer d'endometri.

En alguns tipus de tumors, com el cas del carcinoma de bufeta, l'expressió de FLIP correlaciona amb estadis avançats de la malaltia [429]. De forma similar, en el nostre estudi l'expressió de FLIP mostrà diferències estadísticament significants

entre els estadis I i II del carcinoma d'endometri suggerint que la resistència a TRAIL podria estar relacionada amb la progressió tumoral.

D'una altra banda, l'expressió de FLIP no correlaciona amb el tipus histològic, suggerint que podria efectuar la seva funció antiapopòtica en ambdós tipus de carcinoma d'endometri: tipus I (endometrioide) i tipus II (no endometrioide).

Però quina és la rellevància real de FLIP com a possible mecanisme en la resistència al tractament per TRAIL? Aquesta qüestió s'adreçà altre cop in vitro utilitzant les línies cel·lulars Ishikawa i KLE. Per determinar que el descens en l'expressió de FLIP és la causant de la sensibilització a la mort per TRAIL, les cèl·lules de carcinoma d'endometri foren transfectades amb un plàsmid codificant per FLIP. Els assajos de transfecció-sobreexpressió resultaren en la prevenció de l'apoptosi induïda per TRAIL quan aquest era administrat en combinació amb l'actinomicina D. Encara més, la incubació d'aquestes cèl·lules amb un inhibidor de la caspasa-8 resultà en la completa inhibició de l'apoptosi. En resum, aquestes dades indiquen que FLIP és, com a mínim, una de les proteïnes implicades en la resistència a la mort per TRAIL.

L'evidència directa del paper que juguen els nivells endògens de FLIP en aquesta resistència foren adreçats mitjançant l'ús de RNAs d'interferència (siRNA) per tal de bloquejar selectivament l'expressió d'aquesta proteïna. El descens dels nivells endògens de FLIP com a conseqüència de l'ús dels siRNA resultà en un marcat descens de la viabilitat cel·lular quan les cèl·lules eren tractades amb TRAIL. Aquest descens es caracteritzà per una activació de la caspasa-8 i de la caspasa-3 indicant l'activació de la via apoptòtica extrínseca.

En resum, aquests resultats evidencien que FLIP s'expressa en el carcinoma d'endometri i que és responsable en part de la resistència a l'apoptosi induïda per TRAIL en aquest tipus de tumor.

3. CK2 MODULA LA RESPOSTA A TRAIL I FAS.

Un cop caracteritzada la resistència al tractament per TRAIL a través de FLIP, els nostre treball es centra en identificar el mecanisme que controla els nivells de FLIP i que, en darrer terme, modula aquesta resistència.

Entre els reguladors de l'apoptosi induïda pels receptors de mort, i més concretament de TRAIL i FasL, evidències recents apunten a la proteïna casein cinasa 2 (CK2) en diferents models com el carcinoma de pròstata [431,432], càncer d'esòfag [433], còlon [292,434] o rabdomiosarcoma [291].

CK2 és una proteïna ubiqua i altament conservada de composició tetramèrica formada per dues subunitats reguladores (CK2β) i dues subunitats catalítiques (CK2α o CK2α'). Increments en l'activitat associada a CK2 s'han associat amb creixement cel·lular i proliferació i molts tipus de tumors presenten elevada aquesta activitat [235,268,270].

Aquestes evidències, doncs, apunten a la CK2 com a una possible diana per la teràpia antitumoral [432,435].

En aquest estudi demostrem que CK2 és determinant en la resistència al tractament per TRAIL i FasL i que aquesta sensibilització correlaciona amb una reducció en els nivells endògens de FLIP. Encara més, el treball realitzat amb shRNA (***s***hort ***h***airpin ***RNA***) contra FADD i la caspasa-8 suggereix que per a que es doni aquesta sensibilització és necessària la correcta formació del DISC a nivell de receptor.

En primer lloc, la contribució de CK2 en la resistència al tractament per TRAIL fou adreçat fent ús de diversos inhibidors farmacològics de l'activitat de CK2. Els tres inhibidors utilitzats (DRB, apigenina i emodina) sensibilitzaren les cèl·lules de carcinoma d'endometri a l'apoptosi quan foren coadministrats amb TRAIL/FasL. Aquestes dades suggerien que CK2 podia tenir un paper en la modulació de la resposta d'aquestes cèl·lules enfront TRAIL.

Donat que les subunitats catalítiques, com a conseqüència de la seva alta homologia, poden presentar fenòmens de redundància funcional [235,268,270]; la contribució de CK2 en aquesta resistència la vam abordar inhibint de forma selectiva l'expressió de la subunitat reguladora CK2β.

Els nostres estudis indiquen que l'ús de shRNA contra CK2β té els mateixos efectes que els inhibidors farmacològics, suggerint que el bloqueig de l'expressió de la subunitat reguladora és suficient per truncar l'activitat de CK2.

4. CK2 CONTROLA LA RESPOSTA A TRAIL I FAS A TRAVÉS DE LA REGULACIÓ DE FLIP.

Com ja hem descrit prèviament, FLIP és un conegut regulador de la senyalització per receptors de mort i més concretament, de TRAIL i FasL. El següent pas, doncs, consisteix en establir una possible relació entre CK2 i FLIP en el cas que actuïn conjuntament en la modulació de la resposta a TRAIL i FasL. En aquest sentit, les evidències documentades al respecte són poques i controvertides. Per exemple, Shin i col·laboradors (2005) demostren que la sobreexpressió de FLIP no inhibeix la mort caspasa dependent en cèl·lules de càncer d'esòfag i còlon quan són cotractades amb TRAIL i DRB [433]. En contraposició a aquestes observacions, els nostres resultats demostren que la sobreexpressió de FLIP inhibeix completament l'activació de les caspases i l'apoptosi induïda per l'administració de TRAIL/FasL amb DRB, apigenina o shRNA contra CK2β.

D'altra banda, evidències recents suggereixen que FLIP juga un paper fonamental en la modulació per part de CK2 de la resposta enfront TRAIL [431]. Els autors demostren que en cèl·lules de carcinoma de pròstata el cotractament de TRAIL més l'inhibidor farmacològic de CK2 TBB resulta en un descens en l'expressió de FLIP i una conseqüent activació de la caspasa inductora 8. A més a més, els autors demostren que la sobreexpressió de CK2α restaura l'expressió de FLIP i, per tant, la resistència a TRAIL. En consonància amb aquestes observacions, els nostres estudis demostren que en les cèl·lules canceroses d'endometri tant la inhibició farmacològica com el bloqueig de l'expressió de CK2β es tradueix en un descens en els nivell de proteïna de FLIP.

Basant-nos en aquests resultats, el nostre treball es centrà en el mecanisme pel qual CK2 és capaç de regular els nivells de FLIP. Està ben establert que FLIP pot ésser regulat negativament de forma transcripcional en resposta a drogues antineoplàsiques com el 5-fluorouracil o l'oxaliplatina [436]. D'una altra banda també està ben documentat que a nivell de proteïna FLIP està subjecte al mecanisme convencional ubiquitina/proteasoma de degradació i recanvi de proteïnes [437-439]. Encara més, publicacions recents indiquen que diverses drogues antitumorals com l'inhibidor de la ciclooxigenasa-2 celecoxib [440] i els compostos flavonoids silibinina

[441] o flavopiridol [442] poden induir la degradació de FLIP a través del proteasoma i sensibilitzar així les cèl·lules tumorals a la mort per TRAIL.

Les nostres dades indiquen que CK2 regula els nivells de FLIP de forma transcripcional, tal com indiquen els assajos realitzats per real-time PCR. No obstant, CK2 també regula FLIP a nivell de proteïna ja que l'ús d'inhibidors del proteasoma com el MG-132 restaura els nivells de FLIP en cèl·lules tractades amb inhibidors farmacològics de l'activitat CK2. Per tant, en el nostre model CK2 manté els nivells de FLIP a través del control de la seva expressió i degradació.

Hem demostrat que la inhibició de CK2 en combinació amb l'administració de TRAIL o FasL activa la caspasa iniciadora 8 i executora 3 suggerint l'activació de la via extrínseca. No obstant també s'activen altres caspases com la -9 i la -2.

D'altra banda, a partir dels nostres estudis també es dedueix que la simple inhibició de la CK2 tant amb inhibidors com amb shRNA resulta en una activació de la caspasa-8 que s'atura en la fase inicial de la seva seqüència de tall; això és, es generen els fragments p43/p41. Evidències recents que podrien explicar aquest fet es basen en els estudis de les caspases que s'activen com a conseqüència de la inhibició de CK2. Aquest treball realitzat per Shin i col·laboradors (2005) demostra que d'aquesta inhibició en deriva l'activació de la caspasa-2. Estudis previs ja demostraren en el seu moment que la caspasa-2 s'activa en resposta a la inducció d'estrès per permeabilització mitocondrial [443] o en resposta a receptors de mort [444-446] però el seu rol com a caspasa iniciadora encara està per debatre. El treball posterior realitzat per Shin demostra que com a conseqüència de la inhibició de CK2 la caspasa-2 s'activa i processa la caspasa-8 en una forma (p43/p41) que s'acabarà d'activar definitivament en resposta a algun lligand de mort com, per exemple, TRAIL.

Contràriament a aquestes observacions, però, els nostres resultats demostren que en el nostre model la caspasa-2 només s'activa en resposta a TRAIL i és per tant dependent de lligand. A més a més, la inhibició de FADD i caspasa-8 amb shRNA o la sobreexpressió de FLIP bloqueja l'activació de la caspasa-2. Així doncs es dedueix que la caspasa-2 no pot ésser la causant d'aquesta activació inicial però incompleta de la caspasa-8.

Finalment, demostrem que aquestes dades es reprodueixen quan intentem extrapolar aquests resultats en cultius primaris de carcinoma d'endometri indicant que la CK2 és clau en la regulació de FLIP i la conseqüent resistència a TRAIL. Així doncs la CK2 pot representar una diana propicia per a l'ús de teràpia combinada en el càncer endometrial.

Donada la importància de CK2 en el nostre model hom es planteja les següents preguntes: és CK2 l'únic regulador d'una proteina tan crítica com FLIP? D'altra banda, quin és el mecanisme pel qual un proteina que és capaç de regular tal nombre de substractes modula una proteina tan específica com FLIP? És una regulació directa o potser CK2 forma part d'una via molt més complexa de regulació de la resistància a lligands de mort?

Aquestes i altres preguntes són qüestions a resoldre que encara tenim pendents per poder aprofundir en el coneixement de la regulació dels efectes de TRAIL i Fas. Un possible indici que podria respondre -en part- a totes aquestes preguntes ens el pot haver donat el laboratori de la Deborah K. Morrison. En un treball recent del seu laboratori Ritt i col·laboradors descriuen com CK2 és capaç de regular l'estat d'activació de B-Raf a través de la proteina "scaffold" KSR1[447]. Aquest treball descriu KSR1 com una proteïna capaç d'interaccionar alhora amb B-Raf i CK2. Fruit d'aquesta interacció, B-Raf és activada de manera CK2-dependent i senyalitza a través de la via MAPK.

Els membres de KSR constitueixen una nova família de proteïnes que presenten una semblança estructural amb proteïnes de la família Raf. Tots els membres contenen cinc dominis conservats[294,448] (Fig41): una regió única en les proteïnes KSR (CA1), una regió rica en prolines (CA2), un domini en estructura de dits de zinc ric en cisteïnes (CA3), una regió rica en residus serina/treonina (CA4) i finalment un possible domini cinasa (CA5).

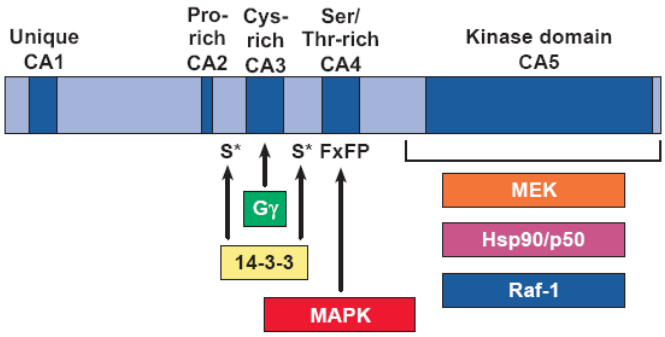

Figura 41: Representació esquemàtica de la KSR i dominis coneguts. (*Morrison, DK. Journal of Cell Science, 2001. 114:1609-12)*[448].

Resultats preliminars realitzats en el nostre laboratori postulen un possible rol per KSR1 i B-Raf, juntament amb CK2, en la resistència a l'apoptosi induida per TRAIL i Fas. Treballs futurs nostres determinaran l'abast i la rellevància d'aquesta nova via en referància a TRAIL i Fas.

5. CK2β ES TROBA SOBREEXPRESSADA EN EL CÀNCER D'ENDOMETRI RESPECTE AL TEIXIT NO TUMORAL.

Treballs realitzats amb anterioritat demostren mitjançant extractes de teixits i tècniques immunohistoquímiques que l'expressió de CK2 es troba anormalment elevada en múltiples classes de tumors [268]. En alguns tipus de tumors, com el de cap i coll, els nivells de CK2 correlacionen amb grau, estadi i pronòstic.

Quina significat pren la CK2 en relació al desenvolupament i progressió del carcinoma d'endometri? Treballs posteriors en el nostre laboratori utilitzant el sistema TMA (*t*issue *m*icro*a*rray) demostren que la CK2β es troba freqüentment expressada en el càncer d'endometri. S'ha observat un increment significatiu en la immunotinció citoplasmàtica i nuclear de CK2β en el carcinoma d'endometri respecte al teixit normal no tumoral. Concretament dels 88 carcinomes avaluats el 100% dels casos presentà tinció positiva nuclear i el 87,5% tinció positiva citoplasmàtica, sempre en ambdós casos significativament superiors al mostrat pel teixit normal. No obstant, en contraposició a altres treballs que demostren una

immunotinció superior en el front invasiu tumoral, nosaltres no hi trobem una correlació positiva, així com tampoc es trobaren diferències en quant a tipus histològic del tumor (tipus I versus tipus II) i en quant a tumors d'estadi II i III.

6. CK2β JUGA UN PAPER EN LA REGULACIÓ DE LA PROLIFERACIÓ CEL·LULAR I CREIXEMENT INDEPENDENT DE SUBSTRACT.

Nivells elevats de CK2 s'han associat tradicionalment a increments en la taxa de proliferació cel·lular. Això es basa en la importància de CK2 en algunes fases del cicle cel·lular com les transicions G1/S i G2/M [449,450]. Recentment s'ha observat que la subunitat reguladora CK2β és capaç de controlar la transició G2/M a través de la inhibició per fosforilació en Wee1, un conegut inhibidor de CDK1 [451]. Els mecanismes, però, pels quals la CK2 és capaç de regular la proliferació cel·lular són múltiples i diversos en funció del model estudiat degut a l'ampli espectre de substractes que té la CK2. Per exemple, la CK2 és capaç de regular per fosforilació l'activitat de factors de transcripció implicats en proliferació com c-myc, c-myb o AP-1. D'altra banda, la CK2 també pot fosforilar la subunitat de NF-κB p65 incrementant la seva activitat transcripcional [281] o regular també l'activitat NF-κB a través de la fosforilació de la proteïna inhibitòria IκBα [282-285].

En aquest sentit, l'expressió de CK2 fou correlacionada positivament amb la immunotinció per Ki67, un conegut marcador de proliferació cel·lular. Contràriament a l'esperat no es trobà correlació estadística amb la ciclina D1 mentre que en la línia cel·lular de carcinoma d'endometri Ishikawa el bloqueig de l'expressió de CK2β resulta en un marcat descens en el nivells de ciclina D1. S'ha de tenir en compte, però, que la ciclina D1 també és diana d'altres vies de senyalització que sovint es troben alterades en el carcinoma d'endometri com la inestabilitat de microsatèl.lits, mutacions en CTNNB1 o NF-κB [356,452,453].

De forma interessant, en aquest estudi demostrem que existeix un relació entre CK2β i les vies PI3K i Wnt, dues vies freqüentment mutades en el carcinoma d'endometri. CK2β mostrà una marcatge que correlacionava positivament amb Akt i quasi negativament amb PTEN de forma significativa. En referència a la relació entre CK2 i la via PI3K/Akt són múltiples els treballs realitzats al respecte. Per exemple,

s'ha documentat que CK2 és capaç de fosforilar directament Akt in vitro/in vivo en la posició Ser129 i que la inhibició farmacològica de CK2 resulta en un descens en l'activitat de Akt [454]. D'altra banda, PTEN també constitueix una diana de fosforilació de CK2 [455] i s'ha observat que la fosforilació en la regió carboxil-terminal de PTEN per CK2 disminueix l'estabilitat de PTEN i la seva conseqüent degradació a través del proteasoma [456,457].

D'altra banda en aquest estudi també es demostra una correlació significativa entre la immunoexpressió nuclear de CK2β i la tinció per β-catenina. Aquests resultats suporten les observacions realitzades per altres investigadors que descriuen com CK2 és capaç de regular positivament la via Wnt [286,458] a través de la fosforilació en β-catenina i augmentant la seva activitat transcripcional. Tanmateix, animals transgènics per CK2α que desenvolupen tumors de mama presenten la via Wnt/β-catenina hiperactivada [273].

Tots aquests resultats demostren un estret vincle entre la proteïna CK2 i la proliferació cel·lular que també hem estudiat in vitro utilitzant la línia cel·lular de carcinoma d'endometri Ishikawa. En aquestes cèl·lules, la inhibició de l'expressió de CK2β es tradueix en un descens en la proliferació cel·lular tal com indiquen els estudis de comptatge de cèl·lules. A més a més, els anàlisis per Western-blot indiquen que el bloqueig de CK2β resulta en el descens de marcadors típics de proliferació cel·lular com són p-ERK1/2 i la ciclina D1.

Finalment, també es va realitzar un anàlisi d'incorporació de bromodeoxiuridina (BrdU). En concordamça amb els resultats obtinguts prèviament observem com les cèl·lules amb menor expressió de CK2β presenten una menor incorporació d'aquest anàleg de la timina. Aquesta dada sugereix que aquelles cèl·lules amb uns nivells inferiors de CK2β presenten o bé un cicle de divisió més espaiat o bé deixen de ciclar i s'acumulen en alguna fase del cicle.

D'altra banda i en concordança amb els resultats obtingut prèviament, l'anàlisi per TMA mostra de forma clara una correlació directa entre l'expressió de CK2 i FLIP. Aquests resultats suportarien els nostres resultats previs on demostrem que els nivells de FLIP estarien controlats per CK2.

Finalment, una de les propietats més ben conegudes de les cèl·lules canceroses és la seva capacitat de créixer independent de l'adhesió a un substract.

Consistentment, les cèl·lules Ishikawa són capaces de créixer en medi que conté agar semisòlid. No obstant, en condicions on la CK2β es troba inhibida per shRNA aquestes cèl·lules es veuen impedides per créixer en aquestes condicions suggerint que la CK2 regula tant la proliferació cel·lular com el creixement independent de substract.

Finalment, i per demostrar que aquest descens en termes de proliferació cel·lular no es deu a una resposta apoptòtica que en realitat ens estaria enmascarant els efectes observats vam realitzar un assaig de mort cel·lular a través de l'incorporació de iodur de propidi. Aquesta tècnica ens mostra un prefil general de l'estat de les diferents fases del cicle cel·lular i a demés ens mostra la fase SubG1, un marcador clàssic de pèrdua de material genètic i, per tant, un indicador de mort cel·lular. El bloqueig de l'expressió de CK2β es tradueix en un increment de la fase SubG1 d'un 4,8% aproximadament que difícilment podria explicar la caiguda en proliferació cel·lular apreciada amb el comptatge de cèl·lules i incorporació de BrdU. Tanmateix, també es realirzà una tinció per Hoechst i un assaig d'activació de caspases. Ambdós resultats sugereixen que el descens en la proliferació no és degut a possibles efectes citotòxics derivats del bloqueig de CK2β.

7. EL TRACTAMENT AMB SORAFENIB INDUEIX MORT APOPTÒTICA EN CÈL·LULES DE CARCINOMA D'ENDOMETRI I SENSIBILITZA AL TRACTAMENT PER TRAIL I FAS PER DIFERENTS VIES.

Recentment s'ha aprovat l'ús del sorafenib per al tractament del carcinoma de ronyó avançat. El sorafenib és considerat un inhibidor d'ampli espectre de proteïnes amb activitat cinasa en residus tirosina. Tanmateix, s'ha pogut demostrar que el sorafenib també exerceix els seus efectes en altres tipus de proteïnes cinases, i de fet cal remarcar que el sorafenib sorgeix com a conseqüència de la recerca envers inhibidors de Raf-1.

Els mecanismes pel quals el sorafenib exerceix els seus efectes no es coneixen en profunditat però ja s'han pogut descriure alguns dels seus efectes com poden ser disminució de proteïnes de la familia de Bcl-2 com la pròpia Bcl-2, Bcl-xL o Mcl-1[459-462], descens dels nivells de c-IAP2 o de FLIP[367,368].

Aquest darrer ha propiciat diversos estudis detallant els potencials efectes beneficosos que podria arribar a tenir una possible teràpia combinada del sorafenib en combinació amb TRAIL. Aquest, no obstant, no és un fet extraordinari i segueix un raonament certament lògic si tenim en compte el paper clau que pot jugar FLIP en la senyalització per receptors de mort.

Els nostres estudis evidencien un clar efecte nociu del sorafenib sobre les linies cèl·lulars de carcinoma d'endometri i sobre cultius primaris. Pel que fa al cas de les línies cel·lulars, però, els efectes són divergents en funció de la linia que estem estudiant. Un exemple són les cèl·lules Ishikawa que mostren una davallada de la viabilitat cel·lular i activació de les caspases al cap de 24 hores d'estimulació amb sorafenib. D'una altra banda observem que les cèl·lules KLE mostren una resistència més pronunciada i pràcticament remanen inalterades passades 48 hores de tractament. Així doncs, podem apreciar que existeixen nivells de sensibilitat diferents al tractament amb sorafenib.

L'estudi de possibles proteïnes diana afectades pel sorafenib ens mostra com, d'acord amb altres models, el tractament amb l'inhibidor es tradueix en un descens dels nivells de Mcl-1 i FLIP que es reverteixen quan el tractament amb sorafenib es fa sobre cèl·lules preestimulades amb l'inhibidor del proteasoma MG-132.

Tal i com succeeix amb els patrons de sensibilitat, la cinètica de disminució de FLIP i Mcl-1 segueix comportaments diferents. Mentre que en el cas de FLIP el descens es dóna ja a les tres hores de tractament en cèl·lules Ishikawa i 24 hores en cèl·lules KLE; els nivells de Mcl-1 cauen al cap de poques hores en cèl·lules Ishikawa però es mantenen pràcticament inalterats en cèl·lules KLE.

Podria explicar aquesta diferència la sensibilitat variable entre línies cel·lulars?, sens dubte aquesta és una qüestió que no ha passat desapercebuda i que es tractarà de forma inmediata en futurs treballs.

D'altra banda resulta necessari esbrinar el significat dels canvis en Mcl-1 i FLIP. Per abordar aquesta qüestió es va sobreexpressar tant Mcl-1 com FLIP. L'exposició a sorafenib demostra com aquelles cèl·lules que sobreexpressaven Mcl-1 esdevenien resistents a la toxicitat induida pel sorafenib mentre que la sobreexpressió de FLIP en cap cas comportava una major capacitat de supervivència envers el tractament.

Així doncs, un cop coneguda la rellevància de Mcl-1 en el mecanisme citotòxic del sorafenib, quin és el paper de FLIP?

Donada la importància que té FLIP en la resistència a la mort induida per TRAIL i Fas en les cèl.lules de càncer d'endometri vam decidir estudiar si, tal com succeeix en altres models, el sorafenib és capaç de vèncer aquesta resistència donat els seus efectes sobre FLIP. Els nostre treball demostra com el sorafenib, a través de la disminució de FLIP, és capaç de sensibilitzar les nostres línies cel·lulars i cultius primaris a la mort per TRAIL i Fas i encara més, la combinació dels dos agents accelera de forma dràstica el temps de respota apoptòtica.

Per determinar el paper singular que juga FLIP en aquest context es sobreexpressà aquesta proteina i s'observà com es restablien les condicions inicials de resistència. Per contra, la sobreexpressió de Mcl-1 no repercutia en canvis apreciables en termes de sensibilització-resistència, sugerint que, a diferència de FLIP, Mcl-1 no opera en el mecanisme de sensibilització a TRAIL i Fas per part del sorafenib.

En resum, aquests resultats en mostren com el sorafenib és capaç d'induir per si mateix mort cel·lular i alhora sensibilitzar les nostres cèl·lules canceroses al tractament per lligands de mort a través de mecanismes diferents.

8. LA SENSIBILITZACIÓ DEL SORAFENIB A TRAIL ÉS INDEPENDENT DE L'ACTIVITAT CINASA DE B-RAF O MEK1/2.

S'accepta de forma general que els mecanismes efectors del sorafenib, tot i que no completament coneguts, passen en bona mesura per una inhibició de la via de les MAPK. En el nostre cas, el tractament amb sorafenib ens indueix un marcat descens en l'estat de forforilació basal de ERK1/2 tant en línies cel·lulars com en cultius primaris de carcinoma d'endometri.

Diversos autors han demostrat la rellevància de via MAPK en els mecanismes de tumorogènesi i, més concretament, en la resistència a lligands de mort. Tanmateix, de la mateixa manera com s'ha descrit com la inhibició farmacològica de la via MAPK sensibilitza cèl·lules tumorals a TRAIL[295-299], altres autors han documentat que aquesta via no té implicacions en aquest fenòmen[463] o, encara de forma més

controvertida, com l'activitat de ERK2 és necessària per la inducció d'apoptosi per part de TRAIL[464].

Quin paper juga aquesta via en la sensibilització a TRAIL i Fas?. Per tal de solucionar aquesta qüestió vam intervenir la via MAPK fent ús de diferents mètodes. Inicialment es va procedir a la sobreexpressió d'una forma mutada cinasa-inactiva de B-Raf: B-Raf^{K483M}. Les nostres observacions demostren que la sobreexpressió d'aquesta forma no és capaç de sensibilitzar els nostres cultius a TRAIL i Fas.

D'altra banda, per tal d'inhibir la via a un altre nivell, vam preestimular les nostres cèl·lules amb l'inhibidor farmacològic de MEK1/2 U0126. Les nostres dades sugereixen que aquest inhibidor tampoc és capaç de véncer la resistència de les cèl·lules de càncer d'endometri al tractament per TRAIL i Fas. Cal posar de relleu el fet que ni en condicions de sobreexpressió de B-Raf^{K483M} ni fent ús del U0126 els nivells de FLIP no es veuen alterats.

Així doncs, aquestes dades indiquen que la inhibició de la via de les MAPK exercida pel sorafenib no és el mecanisme d'acció pel qual sensibilitza les nostres cèl·lules a la mort per TRAIL i Fas. En referència a aquest fet cal esmentar que ja s'han documentat caigudes en els nivells de Bcl-2 o Bcl-xL causades pel sorafenib que no venen donades per una inhibició de la via MAPK[462]. Certament el mecanisme podria ésser degut a la inhibició d'una altra proteina/proteïnes diana. Tanmateix, no s'han de descartar possibles mecanismes d'inhibició cinasa-independents. En aquest sentit s'han documentat funcions per Raf-1 independents de la seva activitat cinasa, concretament en el control de les desicions vida-mort cel·lulars. Futurs treballs determinaran si el sorafenib és capaç d'inhibir a més de l'activitat cinasa de determinades proteïnes la seva activitat cinasa-independent.

9. ELS INHIBIDORS DEL PROTEASOMA CAUSEN LA MORT DE LES CÈL·LULES DE CARCINOMA D'ENDOMETRI EN CULTIU.

La importància de NF-κB en el carcinoma d'endometri ja ha estat prèviament descrita i explorada [356]. Basant-nos en aquests estudis ens vam disposar a estudiar la rellevància d'aquesta via de senyalització i la possible aplicació terapèutica com a resultat del seu bloqueig.

Un dels agents antitumorals més prometedors per la seva capacitat inhibitòria de l'activitat NF-κB és el bortezomib (PS-341 o Velcade), un compost que pertany a la família coneguda com a inhibidors del proteasoma.

El bortezomib, així com d'altres inhibidors del proteasoma, indueixen l'aturada en la proliferació cel·lular i l'apoptosi en diverses classes de tumors [360]. Actualment és usat com a agent quimioterapèutic per al tractament del mieloma múltiple refractari [465-469] i s'estan estudiant les seves possibles aplicacions en tumors sòlids.

En múltiples tipus cel·lulars tumorals la inhibició del proteasoma causa mort cel·lular a través del bloqueig de la via NF-κB. NF-κB representa una família de factors de transcripció implicats en la regulació de gens que participen en respostes inflamatòries i que codifiquen per citoquines, receptors de citoquines i molècules d'adhesió [324,470,471]. D'altra banda NF-κB també s'ha relacionat amb la carcinogènesi a través de la regulació de gens implicats en apoptosi, cicle cel·lular, diferenciació, invasió i migració cel·lular [324,330].

En aquesta part del nostre treball demostrem per primer cop que el bortezomib i altres tres inhibidors del proteasoma (MG-132, epoxomicina i ALLN) indueixen mort apoptòtica en cèl·lules de carcinoma d'endometri.

Aquesta mort de característiques apoptòtiques es caracteritzà per presentar activació de caspases i morfologia nuclear apoptòtica. No obstant, en contraposició a la pràctica totalitat de les observacions realitzades fins ara aquesta mort no ve donada per un bloqueig de l'activitat NF-κB ja que, en aquest model, els inhibidors del proteasoma incrementen enlloc d'inhibir l'activitat NF-κB.

10. ELS INHIBIDORS DEL PROTEASOMA INDUEIXEN ACTIVITAT NF-KB EN LINIES CEL·LULARS I CULTIUS PRIMARIS DE CARCINOMA D'ENDOMETRI.

A partir d'aquí un dels principals objectius fou el de caracteritzar el mecanisme d'activació de NF-κB. A diferència de les dades publicades fins al moment, el bortezomib i els altres inhibidors del proteasoma enlloc d'estabilitzar la proteïna repressora de NF-κB IκB-α el que induïen era la seva fosforilació i conseqüent reducció. Cal posar de relleu, però, que algunes observacions prèvies suggerien que el inhibidor del proteasoma MG-132 era capaç d'activar NF-κB en línies cel·lulars de carcinoma de còlon [472]. No obstant, els autors d'aquest treball no especifiquen quin és l'efecte del MG-132 en termes de viabilitat cel·lular. En concordança amb aquest treball, però, nosaltres també observem un descens en els nivell de IκB-α, fosforilació de les proteïnes cinases IKK i un increment en l'activitat transcripcional de NF-κB.

La fosforilació i degradació de IκB-α és secundada per un increment en l'activitat NF-κB. A, els assajos per EMSA (**e**lectrophoretic **m**otility **s**hift **a**ssay) indiquen que els inhibidors del proteasoma indueixen la formació de complexes DNA-NF-κB de propietats similars a les observades després d'estimular les nostres cèl·lules amb TNF.

TNF és un conegut activador de la via canònica de NF-κB que resulta en la translocació nuclear de p65. L'ús d'un anticòs anti-p65 per induir un supershift en el complex DNA-NF-κB ens indica que, efectivament, p65 juga un paper en aquesta activació. A, els extractes nuclears i citoplasmàtics mostren que p65 es transloca a nucli en resposta al tractament pels inhibidors del proteasoma. De forma complementària, l'ús de shRNA contra p65 redueix l'activitat transcripcional induïda per aquests mentre que el shRNA contra c-Rel no.

D'altra banda, són cada cop més les evidències que indiquen que fenòmens de fosforilació en NF-κB actuen com a mecanismes imprescindibles en la regulació de NF-κB [473]. En aquest sentit trobem que el tractament amb els inhibidors del proteasoma indueix la fosforilació de p65 en el residu Ser536. S'ha observat que aquesta fosforilació, crítica per l'activitat transcripcional de p65, pot ésser executada per la cinasa IKKα en resposta a estímuls com TNFα [474] i limfotoxina-β [475] o per

IKKβ en resposta al lipopolisacàrid [475]. També s'ha descrit que aquesta fosforilació la poden dur a terme altres proteïnes cinases com TBK1, Akt o IKKε [473,476]. En resum, aquests resultats apunten clarament a p65 com una de les subunitats que participen en l'activació de NF-κB.

De forma general, les proteïnes cinases IKK es consideren proteïnes clau en l'activació de NF-κB en resposta a múltiples estímuls [324,330]. Per contra, altres autors han descrit que l'activació de NF-κB pot donar-se de forma independent a les proteïnes IKK [477].

Per estudiar el possible paper de les proteïnes cinases IKK vam transfectar les línies cel·lulars de càncer endometrioide Ishikawa i HEC-1A amb les formes dominant-negativa de IKKα i IKKβ o amb shRNA i vàrem analitzar l'activitat luciferasa NF-κB dependent en resposta al bortezomib i al MG-132. Els experiments mostren de forma clara que l'activació de NF-κB i fosforilació de p65 en el residu Ser536 requereix tant de IKKα com de IKKβ.

D'altra banda, els assajos luciferasa per determinar i quantificar l'activitat NF-κB demostren que les cèl·lules transfectades amb el superrepressor de IκB-α no presenten incrementada la seva activitat NF-κB en resposta als inhibidors del proteasoma. No obstant, l'ús de shRNA contra IKKα i IKKβ no impedeix el descens en els nivells de IκB-α suggerint que la degradació d'aquesta és independent les proteïnes IKK i que, per tant, un segon mecanisme hi està implicat.

Amb tot, aquests resultats indiquen que l'activitat NF-κB induïda pels inhibidors del proteasoma està estrictament regulada i que requereix tant de la degradació de IκB-α com de la fosforilació de p65 IKK dependent.

Donat que el bortezomib s'usa per tractar pacient amb mieloma múltiple i tenint en compte els bons resultats observats en tumors sòlids vam decidir tractar explants de carcinoma d'endometri amb bortezomib i MG-132. Ambdós inhibidors induïren l'activació de la caspasa executora 3 suggerint una mort de tipus apoptòtica. D'altra banda i corroborant els resultats obtinguts amb les línies, els inhibidors també causaren una davallada en els nivells de IκB-α i la fosforilació en p65.

11. ELS INHIBIDORS DEL PROTEASOMA ACTIVEN MECANISMES D' ESTRÉS A RETICLE I MITOCÒNDRIA.

Durant els últims anys s'han descrit mecanismes alternatius d'acció dels inhibidors del proteasoma de forma independent al seu efecte en NF-κB. Entre ells s'han descrit efectes d'estrès en reticle endoplasmàtic [410-413], increments en l'expressió de receptors de mort [419,420] o de proteïnes de la família BH3-*only* com Noxa [416-418] o Bik [414,415].

En aquest sentit les nostres dades indiquen que el tractament amb inhibidors del proteasoma podria causar estrés a reticle endoplasmàtic donat l'increment en els nivells de missatger per proteïnes marcadores d'aquest estrés com són GADD153/CHOP i HOX-1 (***h***eme ***ox***ygenase-***1***). Per una altra banda els inhibidors del proteasoma no causen un increment en els nivells proteics de Puma o Noxa però sí de Bik suggerint que el tractament podria revertir en una hipotètica desestabilització de la mitocòndria.

En resum, podem concloure en funció d'aquests resultats que els inhibidors del proteasoma causen la mort per apoptosi de les cèl·lules del carcinoma d'endometri però, de forma inesperada, indueixen l'activació de NF-κB. Com a resultat, el tractament podria resultar en el increment de l'expressió de gens implicats en proliferació o angiogènesi. És per aquesta raó que l'ús dels inhibidors del proteasoma com a inhibidors de NF-κB hauria d'ésser analitzada de forma acurada en funció del tipus cel·lular a tractar.

12. ELS ANTIOXIDANTS COM EL TIRON O LA VITAMINA C NO REPRESENTEN UN BON MÈTODE D'ESTUDI DELS MECANISMES D'ACCIÓ DEL BORTEZOMIB.

Evidències recents suggereixen que un altre mecanisme d'acció citotòxica dels inhibidors del proteasoma és a través de la generació d'espècies reactives d'oxigen (ROS, ***r***eactive ***o***xygen ***s***pecies) en diferents neoplàsies com el NSCLC (***n***on-***s***mall ***c***ell ***l***ung ***c***arcinoma) [421], en cèl·lules del limfoma del mantell [417] o el carcinoma escamós de cap i coll [413]. Per aquesta raó, diversos treballs han abordat l'estudi dels efectes de les espècies reactives d'oxigen fent ús d'una gran varietat d'antioxidants.

Un dels antioxidants més utilitzats ha estat, sens dubte, el Tiron. S'ha descrit que el Tiron és capaç de bloquejar la mort apoptòtica induïda per ROS en cèl·lules de carcinoma colorectal [478], càncer de pàncrees [479] o càncer de pròstata [480] . També s'ha observat que és capaç d'inhibir la producció intracel·lular de O_2^- en models experimentals de diabetis [481-483] així com en patologies associades a estrés oxidatiu i isquèmia in vivo [484,485].

Encara més, el Tiron fou utilitzat com un inhibidor de ROS en cèl·lules cancerígenes tractades amb bortezomib [413,421]. En aquests treballs l'ús del Tiron inhibia la producció de ROS, bloquejava l'activació de les caspases, l'estrès a reticle endoplasmàtic i l'alteració en el potencial de membrana mitocondrial amb la conseqüent alliberació de citocrom C a citoplasma.

Era d'esperar, doncs, que aquelles cèl·lules tumorals tractades amb Tiron i bortezomib presentessin l'acumulament normal de productes ubiquitinitzats en resposta a la inhibició del proteasoma. Tanmateix i de forma sorprenent un control tant rutinari com aquest no es dugué mai a terme i no és fins al 2006 quan Fernández i col·laboradors observen que aquest acumulament no té lloc en resposta al tractament [422]. En aquest treball els autors demostren que el Tiron, enlloc d'actuar com un inhibidor de ROS, quan és coadministrat amb el bortezomib reacciona amb aquest formant un complex biològicament inactiu. D'aquesta manera el bortezomib no pot exercir la seva funció sobre el proteasoma.

Aquests resultats posen en dubte, doncs, les conclusions errònies que han derivat de la interpretació de la funció del Tiron com a antioxidant en resposta al bortezomib. Tot i així, sorprèn que s'hagin publicat treballs respecte a l'ús del Tiron com a antioxidant en cèl·lules tractades amb bortezomib posteriorment als estudis de Fernández i col·laboradors [412,486]. Aquestes observacions foren posteriorment ampliades en un altre treball on demostren que la vitamina C (o àcid ascòrbic), un altre compost amb propietats antioxidants, també bloqueja la funció del inhibidor bortezomib [423].

En el nostre laboratori hem pogut reproduir els resultats obtinguts prèviament amb el Tiron i la vitamina C i hem demostrat que no exerceixen el mateix efecte en altres tipus d'inhibidors com el MG-132, ALLN (ambdós de la família dels pèptids aldehids) o la epoxomicina (de la família de les epoxicetones). A hem avaluat els

efectes d'un altre compost antioxidant conegut com a edavarona en la citotoxicitat induïda pels inhibidors del proteasoma.

13. L'EDAVARONA INHIBEIX LA FUNCIÓ INHIBITÒRIA DELS INHIBIDORS MG-132 I ALLN.

L'edavarona és un antioxidant que ha estat utilitzat en pacients que han sofert infart de miocardi [487,488] o danys cerebrovasculars [489,490].

De forma interessant els nostres resultats indiquen que l'edavarona és capaç d'inhibir la mort per apoptosi induïda per MG-132 i ALLN mentre que és incapaç de fer-ho amb inhibidors d'altres famílies com el bortezomib i la epoxomicina. La inhibició de la mort es caracteritzà per un descens en el nombre de nuclis apoptòtics i en la completa inhibició de l'activació de les caspases.

Per contra, l'edavarona impedeix l'acumulació de productes ubiquitinitzats pel tractament amb MG-132 i ALLN suggerint que podria estar bloquejant l'acció d'aquests. Aquesta possibilitat s'adreçà realitzant un assaig d'activitat del proteasoma d'on es conclou que, en efecte, l'edavarona inhibeix la funció dels inhibidors del proteasoma de la família dels pèptids aldehids (MG-132 i ALLN) però no d'inhibidors d'altres famílies com el bortezomib o la epoxomicina.

En resum, les dades de les que disposem indiquen que l'activitat dels inhibidors del proteasoma es pot veure greument afectada quan s'estudien els seus efectes fent ús d'antioxidants i que, per tant, l'acumulació pertinent de productes ubiquitinitzats és una bona eina per determinar el correcte funcionament d'aquests inhibidors.

Conclusions

Primera: Les cèl·lules de carcinoma d'endometri són resistents a TRAIL i Fas. Aquesta resistència es deu en primer terme a l'acció de la proteïna inhibitòria FLIP.

Segona: FLIP es troba freqüentment expressat en carcinoma d'endometri i la seva expressió correlaciona amb l'estadiatge però no amb el tipus histològic, grau o índex apoptòtic.

Tercera: El bloqueig dels nivells endògens de FLIP sensibilitza les cèl·lules canceroses d'endometri a la mort per TRAIL i Fas. Aquesta mort presenta característiques morfològiques típiques de l'apoptosi que s'acompanya amb l'activació de caspases.

Quarta: Els inhibidors farmacològics de l'activitat de CK2 així com l'ús de shRNA contra la seva subunitat reguladora CK2β sensibilitza les cèl·lules de càncer d'endometri a la mort per TRAIL i Fas.
Aquesta sensibilització es caracteritza per un descens dels nivells endògens de FLIP i requereix un correcte funcionament dels components del DISC.

Cinquena: CK2β modula la resistència a TRAIL i Fas a través del seu control sobre FLIP. Aquest control es dóna a nivell transcripcional i a nivell d'estabilitat proteica.

Sisena: L'expressió de CK2β es veu incrementada en el carcinoma d'endometri respecte l'endometri normal però no correlaciona amb estadiatge grau o tipus histològic.

Setena: L'expressió de CK2β correlaciona amb marcadors de proliferació com Ki67, Akt o β-catenina i amb FLIP i participa en el control de la regulació del creixement independent d'ancoratge en cèl·lules de càncer d'endometri.

Vuitena: El sorafenib indueix mort cel·lular i sensibilitza a l'apoptosi induida per TRAIL i Fas en línies cel·lulars i cultius primaris de carcinoma d'endometri independentment de l'activació de la via MAPK.

Novena: El sorafenib indueix un descens dels nivells proteics de FLIP i Mcl-1 que són revertits quan s'inhibeix el proteasoma. La disminució de Mcl-1 està implicada en els efectes citotòxics del sorafenib mentre que la disminució de FLIP correlaciona amb la sensibilització al tractament amb TRAIL i Fas

Desena: Els inhibidors del proteasoma indueixen mort de tipus apoptòtic en cultius de línies cel·lulars de carcinoma d'endometri.

Onzena: Els inhibidors del proteasoma activen la via NF-κB en línies cel·lulars i cultius primaris de càncer d'endometri a través de les proteïnes cinases IKKα i IKKβ i de la degradació de IκB-α.

Dotzena: Els antioxidants Tiron i vitamina C bloquegen la funció inhibitòria del bortezomib mentre que la edavarona ho fa en els inhibidors MG-132 i ALLN.

Annex

PUBLICACIONS

1. **Llobet D**, Eritja N, Encinas M, Yeramian A, Santacana S, González-Tallada FJ, Matias-Guiu X, Dolcet X. KSR1/CK2/B-Raf regulate TRAIL sensitivity in endometrial cancer cells. [manuscrit en preparació].

2. **Llobet D**, Eritja N, Yeramian A, Pallarés J, Sorolla A, Santacana M, González-Tallada FJ, Matias-Guiu X, Dolcet X. Sorafenib induces apoptosis and sensitizes endometrial cancer cells to TRAIL by different molecular mechanisms. [sotmès a *Journal of Cellular and Molecular Medicine*].

3. Zapata JM, **Llobet D,** Krajewska M, Lefebvre S, Kress CL, Reed JC. Lymphocyte-specific TRAF3-transgenic mice have enhanced humoral responses and develop plasmacytosis, autoimmunity, inflammation, and cancer. *Blood*. 2009. [en premsa].

4. Pallarés J, Santacana M, Puente S, López S, Yeramian A, Eritja N, Sorolla A, **Llobet D**, Dolcet X, Matias-Guiu X. A review of the aplications of tissue microarray technology in understanding the molecular features of endometrial carcinoma. *Analytical and Quantitative Cytology and Histology*. 2009. [en premsa].

5. **Llobet D**, Pallarés J, Yeramian A, Santacana M, Eritja N, Velasco A, Dolcet X, Matias-Guiu X. Molecular pathology of endometrial carcinoma; practical aspects from the diagnostic and therapeutical view points. *Journal of Clinical Pathology*. 2009. [en premsa].

6. Pallarés J*, **Llobet D***, Santacana M, Eritja N, Velasco A, Cuevas D, López S, Palomar-Asenjo V, Yeramian A, Dolcet X, Matias-Guiu X. CK2β is expressed in endometrial carcinoma, and has a role in apoptosis resistance and cell proliferation. *American Journal of Pathology*. 174:287-96. 2009.

7. Ortega E, Martí RM, Yeramian A, Sorolla A, Dolcet X, **Llobet D**, Abal L, Santacana M, Pallarés J, Llombart-Cussac A, Matias-Guiu X. Targeted therapies in gynecologic cancers and melanoma. *Seminars in Diagnostic Pathology*. 25:262-273. 2008.

8. Gallel P, Pallarés J, Dolcet X, **Llobet D**, Eritja N, Santacana M, Yeramian A, Palomar-Asenjo V, Lagarda H, Mauricio D, Encinas M, Matias-Guiu X. Nuclear factor–kB activation is associated with somatic and germ line RET mutations in medullary thyroid carcinoma. *Human Pathology*. 39:994-1001. 2008.

9. Sorolla A, Yeramian A, Dolcet X, Pérez de Santos AM, **Llobet D**, Schoenenberger JA, Casanova JM, Soria X, Egido R, Llombart A, Vilella R, Matias-Guiu X, Martí RM. Effect of proteasome inhibitors on proliferation and apoptosis of human cutaneous melanoma-derived cell lines. *British Journal of Dermatology*, 158:496-504. 2008.

10. **Llobet D**, Eritja N, Sorolla A, Yeramian A, Schoenenberger JA, Llombart-Cussac A, Martí RM, Matias-Guiu X, Dolcet X. Antioxidants block proteasome inhibitors function in endometrial carcinoma cells. *Anti-Cancer Drugs*. 19:115-124. 2008.

11. **Llobet D**, Eritja N, Encinas M, Llecha N, Yeramian A, Pallarés J, Sorolla A, Gonzalez-Tallada FJ, Matias-Guiu X, Dolcet X. CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells. *Oncogene*. 27:2513-24. 2008.

12. Velasco A, Bussaglia E, Pallarés J, Dolcet X, **Llobet D**, Encinas M, Llecha N, Palacios J, Prat J, Matias-Guiu X. PIK3CA gene mutations in endometrial carcinoma: correlation with PTEN and K-RAS alterations. *Human Pathology*. 37:1465-72. 2006.

13. Dolcet X*, **Llobet D***, Encinas M, Pallarés J, Cabero A, Schoenenberger JA, Comella JX, Matias-Guiu X. Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *Journal of Biological Chemistry*. 281:22118-30. 2006.

14. Pallarés J, Martínez-Guitarte JL, Dolcet X, **Llobet D**, Rue M, Palacios J, Prat J, Matias-Guiu X. Survivin exression in endometrial carcinoma. A tissue microarray study with correlation with PTEN and STAT3. *International Journal of Gynecological Pathology*. 24:247-53. 2005.

15. Dolcet X, **Llobet D**, Pallarés J, Comella JX, Matias-Guiu X. Flip is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Laboratory Investigation*. 85:885-94. 2005.

16. Dolcet X, **Llobet D**, Pallarés J, Matias-Guiu X. NF-KappaB in development and progression of cancer. *Archiv Virchows*. 446:475-82. 2005.

17. Pallarés J, Bussaglia E, Martínez-Guitarte JL, Dolcet X, **Llobet D**, Rue M, Sanchez-Verde L, Palacios J, Prat J, Matias-Guiu X. Immunohistochemical analysis of PTEN in endometrial carcinoma: a tissue microarray study with a comparison of four commercial antibodies in correlation with molecular abnormalities. *Modern Pathology*. 18:719-27. 2005.

18. Pallarés J, Martínez-Guitarte JL, Dolcet X, **Llobet D**, Rue M, Palacios J, Prat J, Matias-Guiu X. Abnormalities in the NF-kappaB family and related proteins in endometrial carcinoma. *Journal of Pathology*. 204:569-77. 2004.

COMUNICACIONS A CONGRESSOS

1. López S, Puente S, Pallarés J, **Llobet D**, Santacana M, Eritja N, Palomar-Asenjo V, Yeramian A, Dolcet X, Matias-Guiu X. Estudi de l'expressié de CK2β en el càncer d'endometri mitjançant un array de teixits. Implicació en la resistencia a l'apoptosi i proliferació cel·lular. VIIIè Congrés de l'Associació Catalana d'Anatomia Patològica. Girona, Catalunya, Spain. 2008.

2. Pallarés J, Dolcet X, **Llobet D**, Eritja N, Matias-Guiu X. Protein Kinase CK2 plays a role in apoptosis resistance in endometrial carcinoma by regulatinf FLIP. USCAP Annual Meeting. Denver, Colorado, USA. 2008.

3. Martí RM, Sorolla A, Yeramian A, Dolcet X, Pérez de Santos AM, **Llobet D**, Casanova JMA., Soria X, Vilella R, Matias-Guiu X. Effect of proteasome inhibitors on proliferation and apoptosis of melanoma cell lines. First World Meeting of Interdisciplinary Melanoma/Skin Cancer Centers. Barcelona, Catalunya, Spain. 2007.

4. Pallarés J, Velasco A, Dolcet X, **Llobet D**, Encinas M, Puente S, Palomar V, Matias-Guiu X. Reduced expression of RASSF1A and spry-2 in endometrial carcinoma. USCAP Annual Meeting. San Diego, CA, USA. 2007.

5. Gallel P, Pallarés J, Dolcet X, **Llobet D**, Encinas M, Palomar V, Mauricio D, Matias-Guiu X. NF-Kappa B activation is associated with somatic and germline RET mutations in medullary thyroid. USCAP Annual Meeting. San Diego, CA, USA. 2007.

6. Dolcet X, **Llobet D**, Pallarés J, Rué M, Matias-Guiu X. c-FLIP expression may have a role in the suppression of apoptosis in endometrial carcinoma. USCAP Annual Meeting. San Antonio, TX, USA.2005.

7. Pallarés J, Dolcet X, **Llobet D**, Rué M, Matias-Guiu X. Apoptosis-related proteins expression in endometrial carcinoma. A tissue microarray study. USCAP Annual Meeting. San Antonio, TX, USA. 2005.

Bibliografia

1. Stevens, A.L., J. *Histología Humana*, (Hancourt Brace, 1998).
2. Bokhman, J.V. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* **15**, 10-7 (1983).
3. Prat, J., Gallardo, A., Cuatrecasas, M. & Catasus, L. Endometrial carcinoma: pathology and genetics. *Pathology* **39**, 72-87 (2007).
4. Matias-Guiu, X. et al. Molecular pathology of endometrial hyperplasia and carcinoma. *Hum Pathol* **32**, 569-77 (2001).
5. Kerr, J.F., Wyllie, A.H. & Currie, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-57 (1972).
6. Majno, G. & Joris, I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* **146**, 3-15 (1995).
7. Hart, S.P., Haslett, C. & Dransfield, I. Recognition of apoptotic cells by phagocytes. *Experientia* **52**, 950-6 (1996).
8. Zimmermann, K.C., Bonzon, C. & Green, D.R. The machinery of programmed cell death. *Pharmacol Ther* **92**, 57-70 (2001).
9. Ellis, H.M. & Horvitz, H.R. Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**, 817-29 (1986).
10. Joza, N., Kroemer, G. & Penninger, J.M. Genetic analysis of the mammalian cell death machinery. *Trends Genet* **18**, 142-9 (2002).
11. Vogt, C. Sdothum: Jent and Gassman. **130**(1842).
12. Lockshin, R.A. & Williams, C.M. Programmed cell death. IV. The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. *J Insect Physiol* **11**, 803-9 (1965).
13. Hengartner, M.O. & Horvitz, H.R. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76**, 665-76 (1994).
14. Nicholson, D.W. & Thornberry, N.A. Caspases: killer proteases. *Trends Biochem Sci* **22**, 299-306 (1997).
15. Shi, Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* **9**, 459-70 (2002).
16. Thornberry, N.A. et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* **356**, 768-74 (1992).
17. Cerretti, D.P. et al. Molecular cloning of the interleukin-1 beta converting enzyme. *Science* **256**, 97-100 (1992).
18. Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. & Horvitz, H.R. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75**, 641-52 (1993).
19. Miura, M., Zhu, H., Rotello, R., Hartwieg, E.A. & Yuan, J. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell* **75**, 653-60 (1993).
20. Gagliardini, V. et al. Prevention of vertebrate neuronal death by the crmA gene. *Science* **263**, 826-8 (1994).
21. Tewari, M. & Dixit, V.M. Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. *J Biol Chem* **270**, 3255-60 (1995).
22. Clem, R.J., Fechheimer, M. & Miller, L.K. Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* **254**, 1388-90 (1991).

23. Cohen, G.M. Caspases: the executioners of apoptosis. *Biochem J* **326 (Pt 1)**, 1-16 (1997).

24. Nicholson, D.W. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* **6**, 1028-42 (1999).

25. Degterev, A., Boyce, M. & Yuan, J. A decade of caspases. *Oncogene* **22**, 8543-67 (2003).

26. Riedl, S.J. & Shi, Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**, 897-907 (2004).

27. Boldin, M.P., Goncharov, T.M., Goltsev, Y.V. & Wallach, D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**, 803-15 (1996).

28. Muzio, M. et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* **85**, 817-27 (1996).

29. Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. & Croce, C.M. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226**, 1097-9 (1984).

30. Vaux, D.L., Cory, S. & Adams, J.M. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**, 440-2 (1988).

31. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. & Korsmeyer, S.J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-6 (1990).

32. Reed, J.C. et al. BCL2-mediated tumorigenicity of a human T-lymphoid cell line: synergy with MYC and inhibition by BCL2 antisense. *Proc Natl Acad Sci U S A* **87**, 3660-4 (1990).

33. Reed, J.C. et al. Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. *Cancer Res* **50**, 6565-70 (1990).

34. Kitada, S., Miyashita, T., Tanaka, S. & Reed, J.C. Investigations of antisense oligonucleotides targeted against bcl-2 RNAs. *Antisense Res Dev* **3**, 157-69 (1993).

35. LeBrun, D.P., Warnke, R.A. & Cleary, M.L. Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. *Am J Pathol* **142**, 743-53 (1993).

36. Lu, Q.L., Poulsom, R., Wong, L. & Hanby, A.M. Bcl-2 expression in adult and embryonic non-haematopoietic tissues. *J Pathol* **169**, 431-7 (1993).

37. Cory, S. & Adams, J.M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647-56 (2002).

38. Huang, D.C., Cory, S. & Strasser, A. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* **14**, 405-14 (1997).

39. Wilson-Annan, J. et al. Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. *J Cell Biol* **162**, 877-87 (2003).

40. McDonnell, T.J. et al. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57**, 79-88 (1989).

41. Strasser, A., Harris, A.W. & Cory, S. bcl-2 transgene inhibits T cell death and

perturbs thymic self-censorship. *Cell* **67**, 889-99 (1991).

42. Strasser, A. et al. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci U S A* **88**, 8661-5 (1991).
43. Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O. & Korsmeyer, S.J. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67**, 879-88 (1991).
44. Ogilvy, S. et al. Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc Natl Acad Sci U S A* **96**, 14943-8 (1999).
45. Veis, D.J., Sorenson, C.M., Shutter, J.R. & Korsmeyer, S.J. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75**, 229-40 (1993).
46. Motoyama, N. et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* **267**, 1506-10 (1995).
47. Print, C.G. et al. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci U S A* **95**, 12424-31 (1998).
48. Ross, A.J. et al. Testicular degeneration in Bclw-deficient mice. *Nat Genet* **18**, 251-6 (1998).
49. Hamasaki, A. et al. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *J Exp Med* **188**, 1985-92 (1998).
50. Rinkenberger, J.L., Horning, S., Klocke, B., Roth, K. & Korsmeyer, S.J. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev* **14**, 23-7 (2000).
51. Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R. & Thompson, C.B. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* **15**, 1481-6 (2001).
52. Cheng, E.H. et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* **8**, 705-11 (2001).
53. Puthalakath, H. et al. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* **293**, 1829-32 (2001).
54. Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M. & Strasser, A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* **3**, 287-96 (1999).
55. Zha, J., Harada, H., Yang, E., Jockel, J. & Korsmeyer, S.J. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-28 (1996).
56. Li, H., Zhu, H., Xu, C.J. & Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501 (1998).
57. Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**, 481-90 (1998).
58. Imaizumi, K. et al. Molecular cloning of a novel polypeptide, DP5, induced during

programmed neuronal death. *J Biol Chem* **272**, 18842-8 (1997).

59. Oda, E. et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053-8 (2000).
60. Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W. & Vogelstein, B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* **7**, 673-82 (2001).
61. Nakano, K. & Vousden, K.H. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**, 683-94 (2001).
62. Lindsten, T. et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* **6**, 1389-99 (2000).
63. Wei, M.C. et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-30 (2001).
64. Gross, A., McDonnell, J.M. & Korsmeyer, S.J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**, 1899-911 (1999).
65. Martinou, J.C. & Green, D.R. Breaking the mitochondrial barrier. *Nat Rev Mol Cell Biol* **2**, 63-7 (2001).
66. Hsu, Y.T. & Youle, R.J. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *J Biol Chem* **273**, 10777-83 (1998).
67. Mikhailov, V. et al. Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. *J Biol Chem* **276**, 18361-74 (2001).
68. Nechushtan, A., Smith, C.L., Hsu, Y.T. & Youle, R.J. Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J* **18**, 2330-41 (1999).
69. Puthalakath, H. & Strasser, A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* **9**, 505-12 (2002).
70. Khosravi-Far, R. & Esposti, M.D. Death receptor signals to mitochondria. *Cancer Biol Ther* **3**, 1051-7 (2004).
71. Gupta, S. Molecular mechanisms of apoptosis in the cells of the immune system in human aging. *Immunol Rev* **205**, 114-29 (2005).
72. Chen, F. et al. Translocation of C. elegans CED-4 to nuclear membranes during programmed cell death. *Science* **287**, 1485-9 (2000).
73. del Peso, L., Gonzalez, V.M., Inohara, N., Ellis, R.E. & Nunez, G. Disruption of the CED-9.CED-4 complex by EGL-1 is a critical step for programmed cell death in Caenorhabditis elegans. *J Biol Chem* **275**, 27205-11 (2000).
74. Degterev, A. & Yuan, J. Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* **9**, 378-90 (2008).
75. Yang, X., Chang, H.Y. & Baltimore, D. Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. *Science* **281**, 1355-7 (1998).
76. Liu, X., Kim, C.N., Yang, J., Jemmerson, R. & Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147-57 (1996).
77. Li, P. et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-89 (1997).
78. Zou, H., Henzel, W.J., Liu, X., Lutschg, A. & Wang, X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent

activation of caspase-3. *Cell* **90**, 405-13 (1997).

79. Hausmann, G. et al. Pro-apoptotic apoptosis protease-activating factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-x(L). *J Cell Biol* **149**, 623-34 (2000).
80. Moriishi, K., Huang, D.C., Cory, S. & Adams, J.M. Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc Natl Acad Sci U S A* **96**, 9683-8 (1999).
81. Hu, Y., Ding, L., Spencer, D.M. & Nunez, G. WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. *J Biol Chem* **273**, 33489-94 (1998).
82. Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T. & Alnemri, E.S. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* **1**, 949-57 (1998).
83. Acehan, D. et al. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* **9**, 423-32 (2002).
84. Wang, S. & El-Deiry, W.S. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* **22**, 8628-33 (2003).
85. Wang, X. The expanding role of mitochondria in apoptosis. *Genes Dev* **15**, 2922-33 (2001).
86. Du, C., Fang, M., Li, Y., Li, L. & Wang, X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33-42 (2000).
87. Verhagen, A.M. et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43-53 (2000).
88. Susin, S.A. et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-6 (1999).
89. Li, L.Y., Luo, X. & Wang, X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95-9 (2001).
90. Antonsson, B. et al. Inhibition of Bax channel-forming activity by Bcl-2. *Science* **277**, 370-2 (1997).
91. Pavlov, E.V. et al. A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. *J Cell Biol* **155**, 725-31 (2001).
92. Tsujimoto, Y. & Shimizu, S. VDAC regulation by the Bcl-2 family of proteins. *Cell Death Differ* **7**, 1174-81 (2000).
93. Zamzami, N. & Kroemer, G. The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol* **2**, 67-71 (2001).
94. Miyashita, T. & Reed, J.C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-9 (1995).
95. Vousden, K.H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604 (2002).
96. Wu, Y., Mehew, J.W., Heckman, C.A., Arcinas, M. & Boxer, L.M. Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene* **20**, 240-51 (2001).
97. Cecconi, F., Alvarez-Bolado, G., Meyer, B.I., Roth, K.A. & Gruss, P. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727-37 (1998).
98. Yoshida, H. et al. Apaf1 is required for mitochondrial pathways of apoptosis and

brain development. *Cell* **94**, 739-50 (1998).

99. Hakem, R. et al. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* **94**, 339-52 (1998).

100. Kuida, K. et al. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325-37 (1998).

101. Honarpour, N. et al. Embryonic neuronal death due to neurotrophin and neurotransmitter deprivation occurs independent of Apaf-1. *Neuroscience* **106**, 263-74 (2001).

102. Hara, H. et al. The apoptotic protease-activating factor 1-mediated pathway of apoptosis is dispensable for negative selection of thymocytes. *J Immunol* **168**, 2288-95 (2002).

103. Marsden, V.S. et al. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* **419**, 634-7 (2002).

104. Ashkenazi, A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* **2**, 420-30 (2002).

105. O'Malley, W.E., Achinstein, B. & Shear, M.J. Action of Bacterial Polysaccharide on Tumors. Iii. Repeated Response of Sarcoma 37, in Tolerant Mice, to Serratia Marcescens Endotoxin. *Cancer Res* **23**, 890-5 (1963).

106. Carswell, E.A. et al. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**, 3666-70 (1975).

107. Pennica, D. et al. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* **312**, 724-9 (1984).

108. Gray, P.W. et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature* **312**, 721-4 (1984).

109. Browning, J.L. et al. Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* **72**, 847-56 (1993).

110. Ashkenazi, A. & Dixit, V.M. Death receptors: signaling and modulation. *Science* **281**, 1305-8 (1998).

111. Locksley, R.M., Killeen, N. & Lenardo, M.J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501 (2001).

112. Simonet, W.S. et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309-19 (1997).

113. Emery, J.G. et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* **273**, 14363-7 (1998).

114. Pitti, R.M. et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* **396**, 699-703 (1998).

115. Yu, K.Y. et al. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J Biol Chem* **274**, 13733-6 (1999).

116. Kischkel, F.C. et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14**, 5579-88 (1995).

117. Igney, F.H. & Krammer, P.H. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* **2**, 277-88 (2002).

118. Strasser, A., Harris, A.W., Huang, D.C., Krammer, P.H. & Cory, S. Bcl-2 and

Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J* **14**, 6136-47 (1995).

119. Huang, D.C. et al. Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x(L). *Proc Natl Acad Sci U S A* **96**, 14871-6 (1999).
120. Newton, K., Harris, A.W., Bath, M.L., Smith, K.G. & Strasser, A. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J* **17**, 706-18 (1998).
121. Bouillet, P. et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286**, 1735-8 (1999).
122. Gross, A. et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* **274**, 1156-63 (1999).
123. Scaffidi, C. et al. Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17**, 1675-87 (1998).
124. Johnstone, R.W., Frew, A.J. & Smyth, M.J. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* **8**, 782-98 (2008).
125. Markee, J.E. Menstruation in intraocular endometrial transplants in the rhesus monkey. *Contrib. Embryol. Carnegie Inst. Wash.* **28**, 219–308 (1940).
126. Bartelmez, G.W. The form and the functions of the uterine blood vessels in the rhesus monkey. *Contrib. Embryol. Carnegie Inst. Wash.* **36**, 153–182 (1957).
127. Hopwood, D. & Levison, D.A. Atrophy and apoptosis in the cyclical human endometrium. *J Pathol* **119**, 159-66 (1976).
128. Kokawa, K., Shikone, T. & Nakano, R. Apoptosis in the human uterine endometrium during the menstrual cycle. *J Clin Endocrinol Metab* **81**, 4144-7 (1996).
129. Shikone, T. et al. Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. *J Clin Endocrinol Metab* **81**, 2376-80 (1996).
130. Tao, X.J. et al. Differential expression of members of the bcl-2 gene family in proliferative and secretory human endometrium: glandular epithelial cell apoptosis is associated with increased expression of bax. *J Clin Endocrinol Metab* **82**, 2738-46 (1997).
131. Vaskivuo, T.E. et al. Apoptosis and apoptosis-related proteins in human endometrium. *Mol Cell Endocrinol* **165**, 75-83 (2000).
132. Yamashita, H., Otsuki, Y., Matsumoto, K., Ueki, K. & Ueki, M. Fas ligand, Fas antigen and Bcl-2 expression in human endometrium during the menstrual cycle. *Mol Hum Reprod* **5**, 358-64 (1999).
133. Otsuki, Y. et al. Cyclic bcl-2 gene expression in human uterine endometrium during menstrual cycle. *Lancet* **344**, 28-9 (1994).
134. Gompel, A. et al. Bcl-2 expression in normal endometrium during the menstrual cycle. *Am J Pathol* **144**, 1195-202 (1994).
135. Tao, X.J., Sayegh, R.A., Tilly, J.L. & Isaacson, K.B. Elevated expression of the proapoptotic BCL-2 family member, BAK, in the human endometrium coincident with apoptosis during the

secretory phase of the cycle. *Fertil Steril* **70**, 338-43 (1998).

136. Rotello, R.J., Lieberman, R.C., Lepoff, R.B. & Gerschenson, L.E. Characterization of uterine epithelium apoptotic cell death kinetics and regulation by progesterone and RU 486. *Am J Pathol* **140**, 449-56 (1992).

137. Koh, E.A., Illingworth, P.J., Duncan, W.C. & Critchley, H.O. Immunolocalization of bcl-2 protein in human endometrium in the menstrual cycle and simulated early pregnancy. *Hum Reprod* **10**, 1557-62 (1995).

138. Garcia-Velasco, J.A., Mulayim, N., Kayisli, U.A. & Arici, A. Elevated soluble Fas ligand levels may suggest a role for apoptosis in women with endometriosis. *Fertil Steril* **78**, 855-9 (2002).

139. Song, J., Rutherford, T., Naftolin, F., Brown, S. & Mor, G. Hormonal regulation of apoptosis and the Fas and Fas ligand system in human endometrial cells. *Mol Hum Reprod* **8**, 447-55 (2002).

140. Otsuki, Y. Apoptosis in human endometrium: apoptotic detection methods and signaling. *Med Electron Microsc* **34**, 166-73 (2001).

141. Watanabe, H. et al. Bcl-2 and Fas expression in eutopic and ectopic human endometrium during the menstrual cycle in relation to endometrial cell apoptosis. *Am J Obstet Gynecol* **176**, 360-8 (1997).

142. Peng, X., Maruo, T., Matsuo, H., Takekida, S. & Deguchi, J. Serum deprivation-induced apoptosis in cultured porcine granulosa cells is characterized by increased expression of p53 protein, Fas antigen and Fas ligand and by decreased expression of PCNA. *Endocr J* **45**, 247-53 (1998).

143. Ghobrial, I.M., Witzig, T.E. & Adjei, A.A. Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin* **55**, 178-94 (2005).

144. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

145. Lowe, S.W., Cepero, E. & Evan, G. Intrinsic tumour suppression. *Nature* **432**, 307-15 (2004).

146. Kaufmann, S.H. & Vaux, D.L. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* **22**, 7414-30 (2003).

147. Ashkenazi, A. & Herbst, R.S. To kill a tumor cell: the potential of proapoptotic receptor agonists. *J Clin Invest* **118**, 1979-90 (2008).

148. Mollinedo, F. & Gajate, C. Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **8**, 413-50 (2003).

149. Pommier, Y., Sordet, O., Antony, S., Hayward, R.L. & Kohn, K.W. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* **23**, 2934-49 (2004).

150. Hollstein, M. et al. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* **22**, 3551-5 (1994).

151. Lee, J.M. & Bernstein, A. Apoptosis, cancer and the p53 tumour suppressor gene. *Cancer Metastasis Rev* **14**, 149-61 (1995).

152. Reed, J.C. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol* **3**, 388-98 (2006).

153. Fesik, S.W. Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer* **5**, 876-85 (2005).

154. Fischer, U. & Schulze-Osthoff, K. Apoptosis-based therapies and drug

targets. *Cell Death Differ* **12 Suppl 1**, 942-61 (2005).

155. Fischer, U. & Schulze-Osthoff, K. New approaches and therapeutics targeting apoptosis in disease. *Pharmacol Rev* **57**, 187-215 (2005).
156. Wiley, S.R. et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3**, 673-82 (1995).
157. Pan, G. et al. The receptor for the cytotoxic ligand TRAIL. *Science* **276**, 111-3 (1997).
158. Pan, G. et al. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**, 815-8 (1997).
159. Sheridan, J.P. et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**, 818-21 (1997).
160. Walczak, H. et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *Embo J* **16**, 5386-97 (1997).
161. Wu, G.S. et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* **17**, 141-3 (1997).
162. Degli-Esposti, M.A. et al. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7**, 813-20 (1997).
163. Marsters, S.A. et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* **7**, 1003-6 (1997).
164. Truneh, A. et al. Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* **275**, 23319-25 (2000).
165. Holen, I., Croucher, P.I., Hamdy, F.C. & Eaton, C.L. Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. *Cancer Res* **62**, 1619-23 (2002).
166. LeBlanc, H.N. & Ashkenazi, A. Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10**, 66-75 (2003).
167. Falschlehner, C., Emmerich, C.H., Gerlach, B. & Walczak, H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* **39**, 1462-75 (2007).
168. Schneider, P. et al. Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Biol Chem* **278**, 5444-54 (2003).
169. Cretney, E. et al. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* **168**, 1356-61 (2002).
170. Sedger, L.M. et al. Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. *Eur J Immunol* **32**, 2246-54 (2002).
171. Takeda, K. et al. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* **195**, 161-9 (2002).
172. Johnsen, A.C. et al. Regulation of APO-2 ligand/trail expression in NK cells-involvement in NK cell-mediated cytotoxicity. *Cytokine* **11**, 664-72 (1999).
173. Kayagaki, N. et al. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol* **163**, 1906-13 (1999).

174. Griffith, T.S. et al. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med* **189**, 1343-54 (1999).

175. Kayagaki, N. et al. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. *J Immunol* **162**, 2639-47 (1999).

176. Mariani, S.M. & Krammer, P.H. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. *Eur J Immunol* **28**, 1492-8 (1998).

177. Jeremias, I., Herr, I., Boehler, T. & Debatin, K.M. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *Eur J Immunol* **28**, 143-52 (1998).

178. Thomas, W.D. & Hersey, P. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J Immunol* **161**, 2195-200 (1998).

179. Martinez-Lorenzo, M.J. et al. Involvement of APO2 ligand/TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. *Eur J Immunol* **28**, 2714-25 (1998).

180. Kayagaki, N. et al. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs. *J Exp Med* **189**, 1451-60 (1999).

181. Chen, Q. et al. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. *Blood* **98**, 2183-92 (2001).

182. Gong, B. & Almasan, A. Genomic organization and transcriptional regulation of human Apo2/TRAIL gene. *Biochem Biophys Res Commun* **278**, 747-52 (2000).

183. Clarke, P. et al. Reovirus-induced apoptosis is mediated by TRAIL. *J Virol* **74**, 8135-9 (2000).

184. Secchiero, P. et al. Human herpesvirus 7 induces the functional up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) coupled to TRAIL-R1 down-modulation in CD4(+) T cells. *Blood* **98**, 2474-81 (2001).

185. Sato, K. et al. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol* **31**, 3138-46 (2001).

186. Monleon, I. et al. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J Immunol* **167**, 6736-44 (2001).

187. Mariani, S.M. & Krammer, P.H. Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol* **28**, 973-82 (1998).

188. Beutler, B. & Cerami, A. Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* **320**, 584-8 (1986).

189. Lejeune, F.J., Lienard, D., Matter, M. & Ruegg, C. Efficiency of recombinant human TNF in human cancer therapy. *Cancer Immun* **6**, 6 (2006).

190. Walczak, H. et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5**, 157-63 (1999).

191. Ashkenazi, A. & Dixit, V.M. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* **11**, 255-60 (1999).

192. Kelley, S.K. et al. Preclinical studies to predict the disposition of Apo2L/tumor

necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* **299**, 31-8 (2001).

193. Mitsiades, C.S. et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood* **98**, 795-804 (2001).
194. Roth, W. et al. Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. *Biochem Biophys Res Commun* **265**, 479-83 (1999).
195. Pollack, I.F., Erff, M. & Ashkenazi, A. Direct stimulation of apoptotic signaling by soluble Apo2l/tumor necrosis factor-related apoptosis-inducing ligand leads to selective killing of glioma cells. *Clin Cancer Res* **7**, 1362-9 (2001).
196. Ashkenazi, A. et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**, 155-62 (1999).
197. Gliniak, B. & Le, T. Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11. *Cancer Res* **59**, 6153-8 (1999).
198. Chinnaiyan, A.M. et al. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci U S A* **97**, 1754-9 (2000).
199. Duiker, E.W. et al. The clinical trail of TRAIL. *Eur J Cancer* **42**, 2233-40 (2006).
200. Tolcher, A.W. et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* **25**, 1390-5 (2007).
201. Thome, M. et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**, 517-21 (1997).
202. Goltsev, Y.V. et al. CASH, a novel caspase homologue with death effector domains. *J Biol Chem* **272**, 19641-4 (1997).
203. Han, D.K. et al. MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. *Proc Natl Acad Sci U S A* **94**, 11333-8 (1997).
204. Hu, S., Vincenz, C., Ni, J., Gentz, R. & Dixit, V.M. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem* **272**, 17255-7 (1997).
205. Inohara, N., Koseki, T., Hu, Y., Chen, S. & Nunez, G. CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci U S A* **94**, 10717-22 (1997).
206. Irmler, M. et al. Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190-5 (1997).
207. Shu, H.B., Halpin, D.R. & Goeddel, D.V. Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* **6**, 751-63 (1997).
208. Srinivasula, S.M. et al. FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis. *J Biol Chem* **272**, 18542-5 (1997).
209. Rasper, D.M. et al. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ* **5**, 271-88 (1998).

210. Scaffidi, C., Schmitz, I., Krammer, P.H. & Peter, M.E. The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* **274**, 1541-8 (1999).

211. Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H. & Kirchhoff, S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *Journal of Biological Chemistry* **276**, 20633-20640 (2001).

212. Siegel, R.M. et al. Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. *J Cell Biol* **141**, 1243-53 (1998).

213. Yeh, W.C. et al. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* **12**, 633-42 (2000).

214. Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. & Dixit, V.M. An induced proximity model for caspase-8 activation. *J Biol Chem* **273**, 2926-30 (1998).

215. Micheau, O. et al. The long form of FLIP is an activator of caspase-8 at the fas death-inducing signaling complex. *Journal of Biological Chemistry* **277**, 45162-45171 (2002).

216. Kataoka, T. et al. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol* **10**, 640-8 (2000).

217. Chaudhary, P.M. et al. Activation of the NF-kappaB pathway by caspase 8 and its homologs. *Oncogene* **19**, 4451-60 (2000).

218. Kataoka, T. & Tschopp, J. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappa B signaling pathway. *Molecular and Cellular Biology* **24**, 2627-2636 (2004).

219. Dohrman, A. et al. Cellular FLIP (long form) regulates CD8+ T cell activation through caspase-8-dependent NF-kappa B activation. *J Immunol* **174**, 5270-8 (2005).

220. Hyer, M.L., Samuel, T. & Reed, J.C. The FLIP-side of Fas signaling. *Clin Cancer Res* **12**, 5929-31 (2006).

221. Griffith, T.S., Chin, W.A., Jackson, G.C., Lynch, D.H. & Kubin, M.Z. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* **161**, 2833-40 (1998).

222. Mueller, C.M. & Scott, D.W. Distinct molecular mechanisms of Fas resistance in murine B lymphoma cells. *J Immunol* **165**, 1854-62 (2000).

223. Djerbi, M. et al. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* **190**, 1025-32 (1999).

224. Medema, J.P., de Jong, J., van Hall, T., Melief, C.J. & Offringa, R. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. *J Exp Med* **190**, 1033-8 (1999).

225. Fulda, S., Meyer, E. & Debatin, K.M. Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer Res* **60**, 3947-56 (2000).

226. Santiago, B., Galindo, M., Palao, G. & Pablos, J.L. Intracellular regulation of Fas-induced apoptosis in human fibroblasts by extracellular factors and cycloheximide. *J Immunol* **172**, 560-6 (2004).

227. Mathas, S. et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med* **199**, 1041-52 (2004).

228. Poulaki, V. et al. Regulation of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in thyroid carcinoma cells. *Am J Pathol* **161**, 643-54 (2002).

229. Lozeman, F.J. et al. Isolation and characterization of human cDNA clones encoding the alpha and the alpha' subunits of casein kinase II. *Biochemistry* **29**, 8436-47 (1990).

230. Litchfield, D.W. et al. Subunit structure of casein kinase II from bovine testis. Demonstration that the alpha and alpha' subunits are distinct polypeptides. *J Biol Chem* **265**, 7638-44 (1990).

231. Maridor, G., Park, W., Krek, W. & Nigg, E.A. Casein kinase II. cDNA sequences, developmental expression, and tissue distribution of mRNAs for alpha, alpha', and beta subunits of the chicken enzyme. *J Biol Chem* **266**, 2362-8 (1991).

232. Glover, C.V., 3rd. On the physiological role of casein kinase II in Saccharomyces cerevisiae. *Prog Nucleic Acid Res Mol Biol* **59**, 95-133 (1998).

233. Xu, X., Rich, E.S., Jr. & Seldin, D.C. Murine protein kinase CK2 alpha': cDNA and genomic cloning and chromosomal mapping. *Genomics* **48**, 79-86 (1998).

234. Litchfield, D.W. et al. Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners. *Mol Cell Biochem* **227**, 21-9 (2001).

235. Litchfield, D.W. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* **369**, 1-15 (2003).

236. Pinna, L.A. & Meggio, F. Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation. *Prog Cell Cycle Res* **3**, 77-97 (1997).

237. Gietz, R.D., Graham, K.C. & Litchfield, D.W. Interactions between the subunits of casein kinase II. *J Biol Chem* **270**, 13017-21 (1995).

238. Graham, K.C. & Litchfield, D.W. The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. *J Biol Chem* **275**, 5003-10 (2000).

239. Canton, D.A., Zhang, C. & Litchfield, D.W. Assembly of protein kinase CK2: investigation of complex formation between catalytic and regulatory subunits using a zinc-finger-deficient mutant of CK2beta. *Biochem J* **358**, 87-94 (2001).

240. Boldyreff, B., Mietens, U. & Issinger, O.G. Structure of protein kinase CK2: dimerization of the human beta-subunit. *FEBS Lett* **379**, 153-6 (1996).

241. Chantalat, L. et al. Crystal structure of the human protein kinase CK2 regulatory subunit reveals its zinc finger-mediated dimerization. *Embo J* **18**, 2930-40 (1999).

242. Niefind, K., Guerra, B., Ermakowa, I. & Issinger, O.G. Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. *Embo J* **20**, 5320-31 (2001).

243. Hilgard, P., Huang, T., Wolkoff, A.W. & Stockert, R.J. Translated Alu sequence determines nuclear localization of a novel catalytic subunit of casein kinase 2. *Am J Physiol Cell Physiol* **283**, C472-83 (2002).

244. Yu, I.J., Spector, D.L., Bae, Y.S. & Marshak, D.R. Immunocytochemical localization of casein kinase II during

interphase and mitosis. *J Cell Biol* **114**, 1217-32 (1991).

245. Faust, M. & Montenarh, M. Subcellular localization of protein kinase CK2. A key to its function? *Cell Tissue Res* **301**, 329-40 (2000).

246. Penner, C.G., Wang, Z. & Litchfield, D.W. Expression and localization of epitope-tagged protein kinase CK2. *J Cell Biochem* **64**, 525-37 (1997).

247. Olsten, M.E., Weber, J.E. & Litchfield, D.W. CK2 interacting proteins: emerging paradigms for CK2 regulation? *Mol Cell Biochem* **274**, 115-24 (2005).

248. Olsten, M.E. & Litchfield, D.W. Order or chaos? An evaluation of the regulation of protein kinase CK2. *Biochem Cell Biol* **82**, 681-93 (2004).

249. Olsten, M.E., Canton, D.A., Zhang, C., Walton, P.A. & Litchfield, D.W. The Pleckstrin homology domain of CK2 interacting protein-1 is required for interactions and recruitment of protein kinase CK2 to the plasma membrane. *J Biol Chem* **279**, 42114-27 (2004).

250. Miyata, Y. & Yahara, I. Interaction between casein kinase II and the 90-kDa stress protein, HSP90. *Biochemistry* **34**, 8123-9 (1995).

251. Messenger, M.M. et al. Interactions between protein kinase CK2 and Pin1. Evidence for phosphorylation-dependent interactions. *J Biol Chem* **277**, 23054-64 (2002).

252. Heriche, J.K. et al. Regulation of protein phosphatase 2A by direct interaction with casein kinase 2alpha. *Science* **276**, 952-5 (1997).

253. Jakobi, R., Voss, H. & Pyerin, W. Human phosvitin/casein kinase type II. Molecular cloning and sequencing of full-length cDNA encoding subunit beta. *Eur J Biochem* **183**, 227-33 (1989).

254. Heller-Harrison, R.A., Meisner, H. & Czech, M.P. Cloning and characterization of a cDNA encoding the beta subunit of human casein kinase II. *Biochemistry* **28**, 9053-8 (1989).

255. Glotzer, M., Murray, A.W. & Kirschner, M.W. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132-8 (1991).

256. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu Rev Biochem* **67**, 425-79 (1998).

257. Zhang, C., Vilk, G., Canton, D.A. & Litchfield, D.W. Phosphorylation regulates the stability of the regulatory CK2beta subunit. *Oncogene* **21**, 3754-64 (2002).

258. King, R.W., Glotzer, M. & Kirschner, M.W. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol Biol Cell* **7**, 1343-57 (1996).

259. Marin, O., Meggio, F., Sarno, S. & Pinna, L.A. Physical dissection of the structural elements responsible for regulatory properties and intersubunit interactions of protein kinase CK2 beta-subunit. *Biochemistry* **36**, 7192-8 (1997).

260. Sarno, S. et al. Cooperative modulation of protein kinase CK2 by separate domains of its regulatory beta-subunit. *Biochemistry* **39**, 12324-9 (2000).

261. Bibby, A.C. & Litchfield, D.W. The multiple personalities of the regulatory subunit of protein kinase CK2: CK2 dependent and CK2 independent roles reveal a secret identity for CK2beta. *Int J Biol Sci* **1**, 67-79 (2005).

262. Pinna, L.A. A historical view of protein kinase CK2. *Cell Mol Biol Res* **40**, 383-90 (1994).

263. Issinger, O.G. Casein kinases: pleiotropic mediators of cellular regulation. *Pharmacol Ther* **59**, 1-30 (1993).

264. Mestres, P., Boldyreff, B., Ebensperger, C., Hameister, H. & Issinger, O.G. Expression of casein kinase 2 during mouse embryogenesis. *Acta Anat (Basel)* **149**, 13-20 (1994).

265. Perez, M., Grande, J. & Itarte, E. Casein kinase 2 activity increases in the prereplicative phase of liver regeneration. *FEBS Lett* **238**, 273-6 (1988).

266. Pancetti, F., Bosser, R., Itarte, E. & Bachs, O. Changes in the activity of nuclear protein kinase CK2 during rat liver regeneration. *Biochem Biophys Res Commun* **218**, 35-9 (1996).

267. Munstermann, U. et al. Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. *Eur J Biochem* **189**, 251-7 (1990).

268. Tawfic, S. et al. Protein kinase CK2 signal in neoplasia. *Histol Histopathol* **16**, 573-82 (2001).

269. Guerra, B. & Issinger, O.G. Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* **20**, 391-408 (1999).

270. Ahmed, K., Gerber, D.A. & Cochet, C. Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* **12**, 226-30 (2002).

271. Faust, R.A., Tawfic, S., Davis, A.T., Bubash, L.A. & Ahmed, K. Antisense oligonucleotides against protein kinase CK2-alpha inhibit growth of squamous cell carcinoma of the head and neck in vitro. *Head Neck* **22**, 341-6 (2000).

272. Stalter, G. et al. Asymmetric expression of protein kinase CK2 subunits in human kidney tumors. *Biochem Biophys Res Commun* **202**, 141-7 (1994).

273. Landesman-Bollag, E. et al. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**, 3247-57 (2001).

274. Daya-Makin, M. et al. Activation of a tumor-associated protein kinase (p40TAK) and casein kinase 2 in human squamous cell carcinomas and adenocarcinomas of the lung. *Cancer Res* **54**, 2262-8 (1994).

275. Yenice, S. et al. Nuclear casein kinase 2 (CK-2) activity in human normal, benign hyperplastic, and cancerous prostate. *Prostate* **24**, 11-6 (1994).

276. Seldin, D.C. & Leder, P. Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* **267**, 894-7 (1995).

277. Kelliher, M.A., Seldin, D.C. & Leder, P. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIalpha. *EMBO J* **15**, 5160-6 (1996).

278. Landesman-Bollag, E., Channavajhala, P.L., Cardiff, R.D. & Seldin, D.C. p53 deficiency and misexpression of protein kinase CK2alpha collaborate in the development of thymic lymphomas in mice. *Oncogene* **16**, 2965-74 (1998).

279. Orlandini, M. et al. Protein kinase CK2alpha' is induced by serum as a delayed early gene and cooperates with Ha-ras in fibroblast transformation. *J Biol Chem* **273**, 21291-7 (1998).

280. Allende, J.E. & Allende, C.C. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *Faseb J* **9**, 313-23 (1995).

281. Bird, T.A., Schooley, K., Dower, S.K., Hagen, H. & Virca, G.D. Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the

p65 subunit. *J Biol Chem* **272**, 32606-12 (1997).

282. Janosch, P. et al. Characterization of IkappaB kinases. IkappaB-alpha is not phosphorylated by Raf-1 or protein kinase C isozymes, but is a casein kinase II substrate. *J Biol Chem* **271**, 13868-74 (1996).

283. Lin, R., Beauparlant, P., Makris, C., Meloche, S. & Hiscott, J. Phosphorylation of IkappaBalpha in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol Cell Biol* **16**, 1401-9 (1996).

284. Pando, M.P. & Verma, I.M. Signal-dependent and -independent degradation of free and NF-kappa B-bound IkappaBalpha. *J Biol Chem* **275**, 21278-86 (2000).

285. Schwarz, E.M., Van Antwerp, D. & Verma, I.M. Constitutive phosphorylation of IkappaBalpha by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IkappaBalpha. *Mol Cell Biol* **16**, 3554-9 (1996).

286. Song, D.H., Sussman, D.J. & Seldin, D.C. Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. *J Biol Chem* **275**, 23790-7 (2000).

287. Desagher, S. et al. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell* **8**, 601-11 (2001).

288. Krippner-Heidenreich, A. et al. Targeting of the transcription factor Max during apoptosis: phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic acid residue in position P1. *Biochem J* **358**, 705-15 (2001).

289. Ruzzene, M., Penzo, D. & Pinna, L.A. Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem J* **364**, 41-7 (2002).

290. Yin, X., Gu, S. & Jiang, J.X. The development-associated cleavage of lens connexin 45.6 by caspase-3-like protease is regulated by casein kinase II-mediated phosphorylation. *J Biol Chem* **276**, 34567-72 (2001).

291. Izeradjene, K., Douglas, L., Delaney, A. & Houghton, J.A. Influence of casein kinase II in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human rhabdomyosarcoma cells. *Clin Cancer Res* **10**, 6650-60 (2004).

292. Izeradjene, K., Douglas, L., Delaney, A. & Houghton, J.A. Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. *Oncogene* **24**, 2050-8 (2005).

293. Duncan, J.S. & Litchfield, D.W. Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta* **1784**, 33-47 (2008).

294. Kolch, W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* **6**, 827-37 (2005).

295. Grosse-Wilde, A. et al. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* **118**, 100-10 (2008).

296. Zhang, X.D., Borrow, J.M., Zhang, X.Y., Nguyen, T. & Hersey, P. Activation of ERK1/2 protects melanoma cells from

TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene* **22**, 2869-81 (2003).

297. Tran, S.E., Holmstrom, T.H., Ahonen, M., Kahari, V.M. & Eriksson, J.E. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* **276**, 16484-90 (2001).

298. Lee, T.J., Lee, J.T., Park, J.W. & Kwon, T.K. Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun* **351**, 1024-30 (2006).

299. Ortiz-Ferron, G. et al. The mitogen-activated protein kinase pathway can inhibit TRAIL-induced apoptosis by prohibiting association of truncated Bid with mitochondria. *Cell Death Differ* **13**, 1857-65 (2006).

300. Rapp, U.R. et al. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc Natl Acad Sci U S A* **80**, 4218-22 (1983).

301. Jansen, H.W., Ruckert, B., Lurz, R. & Bister, K. Two unrelated cell-derived sequences in the genome of avian leukemia and carcinoma inducing retrovirus MH2. *Embo J* **2**, 1969-75 (1983).

302. Moelling, K., Heimann, B., Beimling, P., Rapp, U.R. & Sander, T. Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins. *Nature* **312**, 558-61 (1984).

303. Avruch, J., Zhang, X.F. & Kyriakis, J.M. Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem Sci* **19**, 279-83 (1994).

304. Leicht, D.T. et al. Raf kinases: function, regulation and role in human cancer. *Biochim Biophys Acta* **1773**, 1196-212 (2007).

305. Garnett, M.J. & Marais, R. Guilty as charged: B-RAF is a human oncogene. *Cancer Cell* **6**, 313-9 (2004).

306. Davies, H. et al. Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-54 (2002).

307. Mercer, K.E. & Pritchard, C.A. Raf proteins and cancer: B-Raf is identified as a mutational target. *Biochim Biophys Acta* **1653**, 25-40 (2003).

308. Mercer, K. et al. Expression of endogenous oncogenic V600EB-raf induces proliferation and developmental defects in mice and transformation of primary fibroblasts. *Cancer Res* **65**, 11493-500 (2005).

309. Emuss, V., Garnett, M., Mason, C. & Marais, R. Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF. *Cancer Res* **65**, 9719-26 (2005).

310. Wellbrock, C. et al. V599EB-RAF is an oncogene in melanocytes. *Cancer Res* **64**, 2338-42 (2004).

311. Thomas, N.E. BRAF somatic mutations in malignant melanoma and melanocytic naevi. *Melanoma Res* **16**, 97-103 (2006).

312. Chan, T.L., Zhao, W., Leung, S.Y. & Yuen, S.T. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. *Cancer Res* **63**, 4878-81 (2003).

313. Lee, J.W. et al. Mutational analysis of the ARAF gene in human cancers. *Apmis* **113**, 54-7 (2005).

314. Fransen, K. et al. Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. *Carcinogenesis* **25**, 527-33 (2004).

315. Tran, N.H., Wu, X. & Frost, J.A. B-Raf and Raf-1 are regulated by distinct autoregulatory mechanisms. *J Biol Chem* **280**, 16244-53 (2005).

316. Cunningham, C.C. et al. A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin Cancer Res* **6**, 1626-31 (2000).

317. Oza, A.M. et al. Phase II study of CGP 69846A (ISIS 5132) in recurrent epithelial ovarian cancer: an NCIC clinical trials group study (NCIC IND.116). *Gynecol Oncol* **89**, 129-33 (2003).

318. Cripps, M.C. et al. Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada clinical trials group study. *Clin Cancer Res* **8**, 2188-92 (2002).

319. Coudert, B. et al. Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer. A European Organization for Research and Treatment of Cancer (EORTC) Early Clinical Studies Group report. *Eur J Cancer* **37**, 2194-8 (2001).

320. Tolcher, A.W. et al. A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clin Cancer Res* **8**, 2530-5 (2002).

321. Baldwin, A.S., Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**, 649-83 (1996).

322. Ghosh, S., May, M.J. & Kopp, E.B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**, 225-60 (1998).

323. Karin, M. & Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**, 621-63 (2000).

324. Karin, M., Cao, Y., Greten, F.R. & Li, Z.W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**, 301-10 (2002).

325. Bonizzi, G. & Karin, M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**, 280-8 (2004).

326. Hayden, M.S. & Ghosh, S. Signaling to NF-kappaB. *Genes Dev* **18**, 2195-224 (2004).

327. Greten, F.R. & Karin, M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett* **206**, 193-9 (2004).

328. Solan, N.J., Miyoshi, H., Carmona, E.M., Bren, G.D. & Paya, C.V. RelB cellular regulation and transcriptional activity are regulated by p100. *J Biol Chem* **277**, 1405-18 (2002).

329. Senftleben, U. et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**, 1495-9 (2001).

330. Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. NF-kB in development and progression of human cancer. *Virchows Arch* **446**, 475-82 (2005).

331. Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K. & Tschopp, J. NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* **21**, 5299-305 (2001).

332. Kreuz, S., Siegmund, D., Scheurich, P. & Wajant, H. NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor

signaling. *Mol Cell Biol* **21**, 3964-73 (2001).

333. Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V. & Baldwin, A.S., Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281**, 1680-3 (1998).

334. Deveraux, Q.L. et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J* **17**, 2215-23 (1998).

335. Takahashi, R. et al. A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* **273**, 7787-90 (1998).

336. Lee, J.U. et al. Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. *Eur J Cancer* **35**, 1374-80 (1999).

337. Chen, Q.M. & Tu, V.C. Apoptosis and heart failure: mechanisms and therapeutic implications. *Am J Cardiovasc Drugs* **2**, 43-57 (2002).

338. Vasudevan, K.M., Gurumurthy, S. & Rangnekar, V.M. Suppression of PTEN expression by NF-kappa B prevents apoptosis. *Mol Cell Biol* **24**, 1007-21 (2004).

339. Kim, K.M. & Lee, Y.J. Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the P13K-Akt pathway. *Oncogene* **24**, 355-66 (2005).

340. Datta, S.R., Brunet, A. & Greenberg, M.E. Cellular survival: a play in three Akts. *Genes Dev* **13**, 2905-27 (1999).

341. Hinz, M. et al. NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol* **19**, 2690-8 (1999).

342. Hinz, M. et al. Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. *Blood* **97**, 2798-807 (2001).

343. Hsia, C.Y., Cheng, S., Owyang, A.M., Dowdy, S.F. & Liou, H.C. c-Rel regulation of the cell cycle in primary mouse B lymphocytes. *Int Immunol* **14**, 905-16 (2002).

344. Chen, F., Castranova, V. & Shi, X. New insights into the role of nuclear factor-kappaB in cell growth regulation. *Am J Pathol* **159**, 387-97 (2001).

345. Duyao, M.P. et al. Transactivation of the c-myc promoter by human T cell leukemia virus type 1 tax is mediated by NF kappa B. *J Biol Chem* **267**, 16288-91 (1992).

346. La Rosa, F.A., Pierce, J.W. & Sonenshein, G.E. Differential regulation of the c-myc oncogene promoter by the NF-kappa B rel family of transcription factors. *Mol Cell Biol* **14**, 1039-44 (1994).

347. Thornburg, N.J., Pathmanathan, R. & Raab-Traub, N. Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. *Cancer Res* **63**, 8293-301 (2003).

348. Kucharczak, J., Simmons, M.J., Fan, Y. & Gelinas, C. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* **22**, 8961-82 (2003).

349. Baldwin, A.S. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *J Clin Invest* **107**, 241-6 (2001).

350. Rayet, B. & Gelinas, C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **18**, 6938-47 (1999).

351. Cabannes, E., Khan, G., Aillet, F., Jarrett, R.F. & Hay, R.T. Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IkappaBalpha. *Oncogene* **18**, 3063-70 (1999).

352. Mukhopadhyay, T., Roth, J.A. & Maxwell, S.A. Altered expression of the p50 subunit of the NF-kappa B transcription factor complex in non-small cell lung carcinoma. *Oncogene* **11**, 999-1003 (1995).

353. Sovak, M.A. et al. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J Clin Invest* **100**, 2952-60 (1997).

354. Nair, A., Venkatraman, M., Maliekal, T.T., Nair, B. & Karunagaran, D. NF-kappaB is constitutively activated in high-grade squamous intraepithelial lesions and squamous cell carcinomas of the human uterine cervix. *Oncogene* **22**, 50-8 (2003).

355. Sasaki, N. et al. Nuclear factor-kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. *Clin Cancer Res* **7**, 4136-42 (2001).

356. Pallares, J. et al. Abnormalities in the NF-kappaB family and related proteins in endometrial carcinoma. *J Pathol* **204**, 569-77 (2004).

357. Hall, M.A. & Cleveland, J.L. Clearing the TRAIL for Cancer Therapy. *Cancer Cell* **12**, 4-6 (2007).

358. Koschny, R., Walczak, H. & Ganten, T.M. The promise of TRAIL--potential and risks of a novel anticancer therapy. *J Mol Med* **85**, 923-35 (2007).

359. Frew, A.J. et al. Combination therapy of established cancer using a histone deacetylase inhibitor and a TRAIL receptor agonist. *Proc Natl Acad Sci U S A* **105**, 11317-22 (2008).

360. Adams, J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* **4**, 349-60 (2004).

361. Hyer, M.L. et al. Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer. *Cancer Res* **68**, 2927-33 (2008).

362. Hyer, M.L. et al. Synthetic triterpenoids cooperate with tumor necrosis factor-related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. *Cancer Res* **65**, 4799-808 (2005).

363. Kress, C.L. et al. Triterpenoids display single agent anti-tumor activity in a transgenic mouse model of chronic lymphocytic leukemia and small B cell lymphoma. *PLoS ONE* **2**, e559 (2007).

364. Force, T., Krause, D.S. & Van Etten, R.A. Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* **7**, 332-44 (2007).

365. Wilhelm, S. et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* **5**, 835-44 (2006).

366. Meng, X.W. et al. Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *J Biol Chem* **282**, 29831-46 (2007).

367. Ricci, M.S. et al. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* **12**, 66-80 (2007).

368. Rosato, R.R., Almenara, J.A., Coe, S. & Grant, S. The multikinase inhibitor

sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res* **67**, 9490-500 (2007).

369. Ciechanover, A. The ubiquitin-proteasome proteolytic pathway. *Cell* **79**, 13-21 (1994).
370. Hough, R., Pratt, G. & Rechsteiner, M. Purification of two high molecular weight proteases from rabbit reticulocyte lysate. *J Biol Chem* **262**, 8303-13 (1987).
371. Waxman, L., Fagan, J.M. & Goldberg, A.L. Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *J Biol Chem* **262**, 2451-7 (1987).
372. Brooks, P. et al. Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem J* **346 Pt 1**, 155-61 (2000).
373. Peters, J.M., Cejka, Z., Harris, J.R., Kleinschmidt, J.A. & Baumeister, W. Structural features of the 26 S proteasome complex. *J Mol Biol* **234**, 932-7 (1993).
374. Dahlmann, B. et al. The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. *FEBS Lett* **251**, 125-31 (1989).
375. Zwickl, P. et al. Primary structure of the Thermoplasma proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase. *Biochemistry* **31**, 964-72 (1992).
376. Zwickl, P., Lottspeich, F., Dahlmann, B. & Baumeister, W. Cloning and sequencing of the gene encoding the large (alpha-) subunit of the proteasome from Thermoplasma acidophilum. *FEBS Lett* **278**, 217-21 (1991).
377. Wojcik, C. Regulation of apoptosis by the ubiquitin and proteasome pathway. *J Cell Mol Med* **6**, 25-48 (2002).
378. Wolf, D.H. & Hilt, W. The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochim Biophys Acta* **1695**, 19-31 (2004).
379. Lowe, J. et al. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science* **268**, 533-9 (1995).
380. Baumeister, W., Walz, J., Zuhl, F. & Seemuller, E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-80 (1998).
381. Seemuller, E. et al. Proteasome from Thermoplasma acidophilum: a threonine protease. *Science* **268**, 579-82 (1995).
382. Volker, C. & Lupas, A.N. Molecular evolution of proteasomes. *Curr Top Microbiol Immunol* **268**, 1-22 (2002).
383. Heinemeyer, W., Trondle, N., Albrecht, G. & Wolf, D.H. PRE5 and PRE6, the last missing genes encoding 20S proteasome subunits from yeast? Indication for a set of 14 different subunits in the eukaryotic proteasome core. *Biochemistry* **33**, 12229-37 (1994).
384. Ciechanover, A. The ubiquitin-proteasome pathway: on protein death and cell life. *Embo J* **17**, 7151-60 (1998).
385. DeMartino, G.N. & Slaughter, C.A. The proteasome, a novel protease regulated by multiple mechanisms. *J Biol Chem* **274**, 22123-6 (1999).
386. Kisselev, A.F., Akopian, T.N., Castillo, V. & Goldberg, A.L. Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. *Mol Cell* **4**, 395-402 (1999).
387. Nussbaum, A.K. et al. Cleavage motifs of the yeast 20S proteasome beta subunits

deduced from digests of enolase 1. *Proc Natl Acad Sci U S A* **95**, 12504-9 (1998).

388. Groll, M. et al. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* **386**, 463-71 (1997).

389. Rock, K.L. & Goldberg, A.L. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu Rev Immunol* **17**, 739-79 (1999).

390. Pamer, E. & Cresswell, P. Mechanisms of MHC class I--restricted antigen processing. *Annu Rev Immunol* **16**, 323-58 (1998).

391. Glickman, M.H. et al. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**, 615-23 (1998).

392. Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W. & Goldberg, A.L. ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol Cell* **11**, 69-78 (2003).

393. Drexler, H.C. Activation of the cell death program by inhibition of proteasome function. *Proc Natl Acad Sci U S A* **94**, 855-60 (1997).

394. Masdehors, P. et al. Increased sensitivity of CLL-derived lymphocytes to apoptotic death activation by the proteasome-specific inhibitor lactacystin. *Br J Haematol* **105**, 752-7 (1999).

395. Drexler, H.C., Risau, W. & Konerding, M.A. Inhibition of proteasome function induces programmed cell death in proliferating endothelial cells. *Faseb J* **14**, 65-77 (2000).

396. Richardson, P.G. et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* **348**, 2609-17 (2003).

397. Adams, J., Palombella, V.J. & Elliott, P.J. Proteasome inhibition: a new strategy in cancer treatment. *Invest New Drugs* **18**, 109-21 (2000).

398. Joazeiro, C.A., Anderson, K.C. & Hunter, T. Proteasome inhibitor drugs on the rise. *Cancer Res* **66**, 7840-2 (2006).

399. Feling, R.H. et al. Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus salinospora. *Angew Chem Int Ed Engl* **42**, 355-7 (2003).

400. Chauhan, D. et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. *Cancer Cell* **8**, 407-19 (2005).

401. Kuhn, D.J. et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood* **110**, 3281-90 (2007).

402. Demo, S.D. et al. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res* **67**, 6383-91 (2007).

403. Stapnes, C. et al. The proteasome inhibitors bortezomib and PR-171 have antiproliferative and proapoptotic effects on primary human acute myeloid leukaemia cells. *Br J Haematol* **136**, 814-28 (2007).

404. Orlowski, R.Z. & Kuhn, D.J. Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clin Cancer Res* **14**, 1649-57 (2008).

405. Wojcik, C., Schroeter, D., Stoehr, M., Wilk, S. & Paweletz, N. An inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (20S

proteasome) induces arrest in G2-phase and metaphase in HeLa cells. *Eur J Cell Biol* **70**, 172-8 (1996).

406. Machiels, B.M. et al. Detailed analysis of cell cycle kinetics upon proteasome inhibition. *Cytometry* **28**, 243-52 (1997).
407. Murray, A.W. Recycling the cell cycle: cyclins revisited. *Cell* **116**, 221-34 (2004).
408. Chene, P. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nat Rev Cancer* **3**, 102-9 (2003).
409. Momand, J., Wu, H.H. & Dasgupta, G. MDM2--master regulator of the p53 tumor suppressor protein. *Gene* **242**, 15-29 (2000).
410. Nawrocki, S.T. et al. Bortezomib sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis. *Cancer Res* **65**, 11658-66 (2005).
411. Nawrocki, S.T. et al. Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. *Cancer Res* **65**, 11510-9 (2005).
412. Fribley, A. & Wang, C.Y. Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress. *Cancer Biol Ther* **5**, 745-8 (2006).
413. Fribley, A., Zeng, Q. & Wang, C.Y. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. *Mol Cell Biol* **24**, 9695-704 (2004).
414. Zhu, H. et al. Bik/NBK accumulation correlates with apoptosis-induction by bortezomib (PS-341, Velcade) and other proteasome inhibitors. *Oncogene* **24**, 4993-9 (2005).
415. Zhu, H. et al. Proteasome inhibitors-mediated TRAIL resensitization and Bik accumulation. *Cancer Biol Ther* **4**, 781-6 (2005).
416. Qin, J.Z. et al. Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res* **65**, 6282-93 (2005).
417. Perez-Galan, P. et al. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood* **107**, 257-64 (2006).
418. Fernandez, Y. et al. Differential regulation of noxa in normal melanocytes and melanoma cells by proteasome inhibition: therapeutic implications. *Cancer Res* **65**, 6294-304 (2005).
419. Liu, X. et al. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Res* **67**, 4981-8 (2007).
420. Yoshida, T. et al. Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. *Cancer Res* **65**, 5662-7 (2005).
421. Ling, Y.H., Liebes, L., Zou, Y. & Perez-Soler, R. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. *J Biol Chem* **278**, 33714-23 (2003).
422. Fernandez, Y. et al. Chemical blockage of the proteasome inhibitory function of bortezomib: impact on tumor cell death. *J Biol Chem* **281**, 1107-18 (2006).

423. Zou, W. et al. Vitamin C inactivates the proteasome inhibitor PS-341 in human cancer cells. *Clin Cancer Res* **12**, 273-80 (2006).

424. Okano, H. et al. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. *Lab Invest* **83**, 1033-43 (2003).

425. Zhang, X. et al. Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* **64**, 7086-91 (2004).

426. Dutton, A. et al. Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci U S A* **101**, 6611-6 (2004).

427. Lee, S.H. et al. Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. *Apmis* **111**, 309-14 (2003).

428. Zeise, E., Weichenthal, M., Schwarz, T. & Kulms, D. Resistance of human melanoma cells against the death ligand TRAIL is reversed by ultraviolet-B radiation via downregulation of FLIP. *J Invest Dermatol* **123**, 746-54 (2004).

429. Korkolopoulou, P. et al. c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. *Urology* **63**, 1198-204 (2004).

430. Abedini, M.R., Qiu, Q., Yan, X. & Tsang, B.K. Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells in vitro. *Oncogene* **23**, 6997-7004 (2004).

431. Wang, G., Ahmad, K.A. & Ahmed, K. Role of protein kinase CK2 in the regulation of tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis in prostate cancer cells. *Cancer Res* **66**, 2242-9 (2006).

432. Wang, G., Unger, G., Ahmad, K.A., Slaton, J.W. & Ahmed, K. Downregulation of CK2 induces apoptosis in cancer cells--a potential approach to cancer therapy. *Mol Cell Biochem* **274**, 77-84 (2005).

433. Shin, S. et al. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. *Embo J* **24**, 3532-42 (2005).

434. Ravi, R. & Bedi, A. Sensitization of tumor cells to Apo2 ligand/TRAIL-induced apoptosis by inhibition of casein kinase II. *Cancer Research* **62**, 4180-4185 (2002).

435. Ahmad, K.A., Wang, G., Slaton, J., Unger, G. & Ahmed, K. Targeting CK2 for cancer therapy. *Anticancer Drugs* **16**, 1037-43 (2005).

436. Galligan, L. et al. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* **4**, 2026-36 (2005).

437. Kim, Y., Suh, N., Sporn, M. & Reed, J.C. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* **277**, 22320-9 (2002).

438. Poukkula, M. et al. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* **280**, 27345-55 (2005).

439. Chang, L. et al. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124**, 601-13 (2006).

440. Liu, X., Yue, P., Schonthal, A.H., Khuri, F.R. & Sun, S.Y. Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* **66**, 11115-9 (2006).

441. Son, Y.G. et al. Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* **67**, 8274-84 (2007).

442. Palacios, C., Yerbes, R. & Lopez-Rivas, A. Flavopiridol Induces Cellular FLICE-Inhibitory Protein Degradation by the Proteasome and Promotes TRAIL-Induced Early Signaling and Apoptosis in Breast Tumor Cells. *Cancer Res* **66**, 8858-69 (2006).

443. Lassus, P., Opitz-Araya, X. & Lazebnik, Y. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297**, 1352-4 (2002).

444. Droin, N. et al. Involvement of caspase-2 long isoform in Fas-mediated cell death of human leukemic cells. *Blood* **97**, 1835-44 (2001).

445. Wagner, K.W., Engels, I.H. & Deveraux, Q.L. Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J Biol Chem* **279**, 35047-52 (2004).

446. Lavrik, I.N., Golks, A., Baumann, S. & Krammer, P.H. Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. *Blood* **108**, 559-65 (2006).

447. Ritt, D.A. et al. CK2 Is a component of the KSR1 scaffold complex that contributes to Raf kinase activation. *Curr Biol* **17**, 179-84 (2007).

448. Morrison, D.K. KSR: a MAPK scaffold of the Ras pathway? *J Cell Sci* **114**, 1609-12 (2001).

449. Pepperkok, R., Lorenz, P., Ansorge, W. & Pyerin, W. Casein kinase II is required for transition of G0/G1, early G1, and G1/S phases of the cell cycle. *J Biol Chem* **269**, 6986-91 (1994).

450. Lorenz, P., Pepperkok, R., Ansorge, W. & Pyerin, W. Cell biological studies with monoclonal and polyclonal antibodies against human casein kinase II subunit beta demonstrate participation of the kinase in mitogenic signaling. *J Biol Chem* **268**, 2733-9 (1993).

451. Yde, C.W., Olsen, B.B., Meek, D., Watanabe, N. & Guerra, B. The regulatory beta-subunit of protein kinase CK2 regulates cell-cycle progression at the onset of mitosis. *Oncogene* **27**, 4986-97 (2008).

452. Machin, P. et al. CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. *Hum Pathol* **33**, 206-12 (2002).

453. Moreno-Bueno, G. et al. Cyclin D1 gene (CCND1) mutations in endometrial cancer. *Oncogene* **22**, 6115-8 (2003).

454. Di Maira, G. et al. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ* **12**, 668-77 (2005).

455. Miller, S.J., Lou, D.Y., Seldin, D.C., Lane, W.S. & Neel, B.G. Direct identification of PTEN phosphorylation sites. *FEBS Lett* **528**, 145-53 (2002).

456. Torres, J. & Pulido, R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem* **276**, 993-8 (2001).

457. Arevalo, M.A. & Rodriguez-Tebar, A. Activation of casein kinase II and

inhibition of phosphatase and tensin homologue deleted on chromosome 10 phosphatase by nerve growth factor/p75NTR inhibit glycogen synthase kinase-3beta and stimulate axonal growth. *Mol Biol Cell* **17**, 3369-77 (2006).

458. Song, D.H. et al. CK2 phosphorylation of the armadillo repeat region of beta-catenin potentiates Wnt signaling. *J Biol Chem* **278**, 24018-25 (2003).

459. Rahmani, M., Davis, E.M., Bauer, C., Dent, P. & Grant, S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem* **280**, 35217-27 (2005).

460. Ding, Q. et al. Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Res* **68**, 6109-17 (2008).

461. Yu, C. et al. The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* **24**, 6861-9 (2005).

462. Panka, D.J., Wang, W., Atkins, M.B. & Mier, J.W. The Raf inhibitor BAY 43-9006 (Sorafenib) induces caspase-independent apoptosis in melanoma cells. *Cancer Res* **66**, 1611-9 (2006).

463. Vaculova, A., Hofmanova, J., Soucek, K. & Kozubik, A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. *FEBS Lett* **580**, 6565-9 (2006).

464. Frese, S. et al. PG490-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis requires activation of ERK2. *Oncogene* **22**, 5427-35 (2003).

465. Hideshima, T. et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* **61**, 3071-6 (2001).

466. Hideshima, T. et al. NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem* **277**, 16639-47 (2002).

467. LeBlanc, R. et al. Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. *Cancer Res* **62**, 4996-5000 (2002).

468. Mitsiades, N. et al. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. *Blood* **101**, 2377-80 (2003).

469. Orlowski, R.Z. et al. Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. *J Clin Oncol* **20**, 4420-7 (2002).

470. Karin, M. & Lin, A. NF-kappaB at the crossroads of life and death. *Nat Immunol* **3**, 221-7 (2002).

471. Aggarwal, B.B. Nuclear factor-kappaB: the enemy within. *Cancer Cell* **6**, 203-8 (2004).

472. Nemeth, Z.H. et al. Proteasome inhibitors induce inhibitory kappa B (I kappa B) kinase activation, I kappa B alpha degradation, and nuclear factor kappa B activation in HT-29 cells. *Mol Pharmacol* **65**, 342-9 (2004).

473. Viatour, P., Merville, M.P., Bours, V. & Chariot, A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci* **30**, 43-52 (2005).

474. O'Mahony, A.M., Montano, M., Van Beneden, K., Chen, L.F. & Greene, W.C. Human T-cell lymphotropic virus type 1 tax induction of biologically Active NF-kappaB requires IkappaB kinase-1-mediated phosphorylation of RelA/p65. *J Biol Chem* **279**, 18137-45 (2004).

475. Mattioli, I. et al. Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. *J Immunol* **172**, 6336-44 (2004).

476. Buss, H. et al. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. *J Biol Chem* **279**, 55633-43 (2004).

477. Tergaonkar, V., Bottero, V., Ikawa, M., Li, Q. & Verma, I.M. IkappaB kinase-independent IkappaBalpha degradation pathway: functional NF-kappaB activity and implications for cancer therapy. *Mol Cell Biol* **23**, 8070-83 (2003).

478. Shen, S.C., Ko, C.H., Tseng, S.W., Tsai, S.H. & Chen, Y.C. Structurally related antitumor effects of flavanones in vitro and in vivo: involvement of caspase 3 activation, p21 gene expression, and reactive oxygen species production. *Toxicol Appl Pharmacol* **197**, 84-95 (2004).

479. Vaquero, E.C., Edderkaoui, M., Pandol, S.J., Gukovsky, I. & Gukovskaya, A.S. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. *J Biol Chem* **279**, 34643-54 (2004).

480. Lebedeva, I.V. et al. Melanoma differentiation associated gene-7, mda-7/interleukin-24, induces apoptosis in prostate cancer cells by promoting mitochondrial dysfunction and inducing reactive oxygen species. *Cancer Res* **63**, 8138-44 (2003).

481. Ceolotto, G. et al. Insulin generates free radicals by an NAD(P)H, phosphatidylinositol 3'-kinase-dependent mechanism in human skin fibroblasts ex vivo. *Diabetes* **53**, 1344-51 (2004).

482. Takahashi, Y., Negoro, M. & Wakabayashi, I. Decreased modulation by lipopolysaccharide of aortic smooth muscle contractility in streptozotocin-induced hyperglycemic rats. *J Cardiovasc Pharmacol* **41**, 162-70 (2003).

483. Kajstura, J. et al. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. *Diabetes* **50**, 1414-24 (2001).

484. Palmer, B.S., Klawitter, P.F., Reiser, P.J. & Angelos, M.G. Degradation of rat cardiac troponin I during ischemia independent of reperfusion. *Am J Physiol Heart Circ Physiol* **287**, H1269-75 (2004).

485. Novalija, E., Kevin, L.G., Eells, J.T., Henry, M.M. & Stowe, D.F. Anesthetic preconditioning improves adenosine triphosphate synthesis and reduces reactive oxygen species formation in mitochondria after ischemia by a redox dependent mechanism. *Anesthesiology* **98**, 1155-63 (2003).

486. Yeung, B.H., Huang, D.C. & Sinicrope, F.A. PS-341 (bortezomib) induces lysosomal cathepsin B release and a

caspase-2-dependent mitochondrial permeabilization and apoptosis in human pancreatic cancer cells. *J Biol Chem* **281**, 11923-32 (2006).

487. Tsujita, K. et al. Effects of edaravone on reperfusion injury in patients with acute myocardial infarction. *Am J Cardiol* **94**, 481-4 (2004).

488. Tsujita, K. et al. Long-term efficacy of edaravone in patients with acute myocardial infarction. *Circ J* **70**, 832-7 (2006).

489. Dohi, K. et al. Alkoxyl radical-scavenging activity of edaravone in patients with traumatic brain injury. *J Neurotrauma* **23**, 1591-9 (2006).

490. Yoshida, H. et al. Neuroprotective effects of edaravone: a novel free radical scavenger in cerebrovascular injury. *CNS Drug Rev* **12**, 9-20 (2006).