International Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XIX cycle - 2003–2007
Coordinator: Prof. Giancarlo Vecchio

"Molecular and cellular effects of the combined treatment with Camptothecin and Roscovitine in human lung and colon adenocarcinoma cells"

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“Molecular and cellular effects of the combined treatment with Camptothecin and Roscovitine in human lung and colon adenocarcinoma cells”

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Mauro C, Crescenzi E, **De Mattia R**, Pacifico F, Mellone S, Salzano S, de Luca C, D'Adamio L, Palumbo G, Formisano S, Vito P, Leonardi A.

"Central role of the scaffold protein tumor necrosis factor receptor-associated factor 2 in regulating endoplasmic reticulum stress-induced apoptosis." J Biol Chem. 2006; 281(5):2631-8

ABSTRACT

Researchers have achieved an experimental breakthrough in the simultaneous use of two or more agents for treating cancer, known as combination therapy. Many times the combination of chemotherapeutic drugs is useful because the two compounds generally act in different phases of the cell cycle and with different mechanisms of action. Usually one of them is a so called "standard" chemotherapic acting on cell cycle progression and the other is a "new generation" drug with a specific molecular target.

My work concerns this specific field of study. It contributed to characterize the cellular and molecular response of four tumor cell lines to combination of two chemotherapic compounds: Camptothecin that is a well known topoisomerase I inhibitor and Roscovitine, a CDK inhibitor recently used for cancer treatment. In vitro treatment with two drugs alone or in combination revealed that Roscovitine, is able to protect cells from Camptothecin-induced DNA damage and to produce a sort of chemoresistance. The human cell lines used in this work A549 ($p53^{+/+}$) and H1299 ($p53^{-/-}$) derived from Non Small Cells Lung Cancer and HCT116 ($p53^{+/+}$ o $p53^{-/-}$) from colon cancer.

Roscovitine and Camptothecin have different effects on cellular cycle. Cells treated with Roscovitine partially accumulate in the G2 phase, Camptothecin, instead, leads to different effects according to p53 status: lines $p53^{+/+}$ arrest in S phase, lines $p53^{-/-}$ arrest in G2 phase. The combined treatment determines ulterior effects: in $p53^{+/+}$ cells we observe an increase in G1 phase; in $p53^{-/-}$ we find a block in S phase. Clonogenic assays performed 10 days after the treatment suggest that cells incubated with the two drugs together generate more colonies than which incubated with Camptothecin alone. After the combined treatment, we found a consistent decrease in the number of senescent cells (40%~) (flatten morphology and ß-galattosidase positivity) compared with that obtained after single treatment with Camptothecin. According to this observation, must be noted that we can see a minor activation of p53(less phosphorylation in ser 15) in p53 positive cells and a decrease in p21 expression in p53 negative cells. Further results indicate that the main target of Roscovitine in our model is CDK2 and that the response to the DNA damage involves PCNA and Cdc6 proteins. Roscovitine seems to be able to activate more effectively DNA damage sensors previously induced by Camptothecin. This activation may be responsible of an anticipated cell cycle block, a more effective reply to the damage, a prolonged surviving and a minor induction of cellular senescence.

1. INTRODUCTION

1.1 The cell cycle control proteins

The cell cycle is controlled by numerous mechanisms ensuring correct cell division and DNA replication. The transition from one cell cycle phase to another occurs in an logical and organized way and is regulated by different proteins. In a

mitosis-competent cell, the accurate entry and proper progression through the cell cycle are monitored by a series of checkpoint controls. Key regulatory proteins of these restriction points are the cyclin-dependent kinases (CDK), a family of serine/threonine kinases that are activated at specific phases of the cell cycle (Fig.1). Until now, nine CDK have been identified. Five of them are active during the cell cycle i.e. entering G_1 phase (CDK3) (Ren 2004 Cell), during G_1 phase (CDK4, CDK6 and CDK2), S phase (CDK2), G_2 phase and M phase (CDK1). CDK7 acts as CDK activating kinase with its her catalytic subunity cyclin H (Fisher and Morgan 1994). CDK8 (Donner 2007) and CDK9 (Marshall 2006) seem to be transcriptional co-regulators. CDK proteins levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Cyclin proteins levels rise and fall during the cell cycle and, in this way, the function of CDK is periodically activated. (Evans 1983). Different cyclins are required at different phases of cell cycle. The three D type cyclins bind to CDK4 and to CDK6 and are essential for entry in G_1 (Sherr 1994). Another G_1 cyclin is cyclin E which associates to CDK2 to regulate progression from G_1 into S phase (Ohtsubo 1995). Cyclin A binds with CDK2 and this complex is required during S phase (Girard 1991). In late G2 and early M cyclin A complexes with CDK1 to promote entry into M phase. Mitosis is further regulated by cyclin B in complex with CDK1. (King 1994).

In addiction to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. These phosphorylations induce conformational changes and enhance the binding of cyclins (Jeffrey 1995). Dephosphorylation on these sites by the enzyme Cdc25 is necessary for progression through the cell cycle (Lew 1996). CDK activity can be also negatively controlled by inhibitory proteins called CDK inhibitors (CKI) which bind to CDK alone or to the CDK-cyclin complex. Two distinct families of CDK inhibitors have been identified, the INK4 family and the Cip/Kip family (Hirai 1995). The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), p19 (INK4d), which specifically inactivate G1 CDK (CDK4 and CDK6). This family of proteins form stable complex with CDK before cyclin binding, preventing association with cyclin D (Carnero 1998). The second family of inhibitors, the Cip/Kip includes p21 (Waf1, Cip1), p27 (Cip2), p57 (Kip2). These inhibitors inactivate cyclin/CDK complexes (Polyak 1994). CDK' s targets proteins are key proteins for cell cycle progression. Among them the most important are the retinoblastoma tumor suppressor gene (pRb), CDK's own regulators WEE1 and Cdc25, and cytoskeletal proteins such as nuclear lamins, microtubules and vimentin which are required for correct mitosis (Heald e McKeon 1990;)

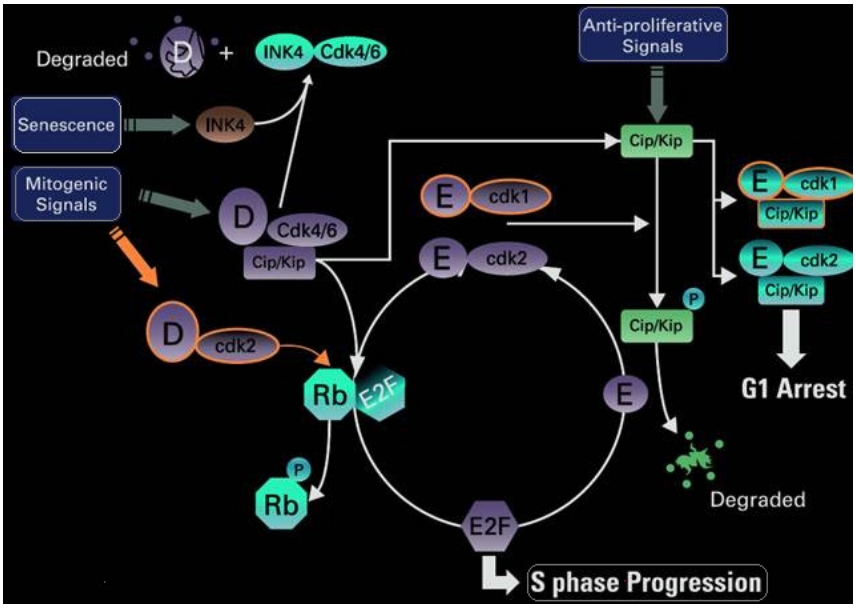


Fig. 1 Schematic representation of major CDK/cyclins complexes and CDK inhibitors involved in the progression of the cell cycle. These protein complexes determine cell cycle progression or block according to the balance between anti-proliferative and mitotic signals.

1.2 Cell cycle checkpoints

The cell cycle proceeds by a defined sequence of events where late events depend upon completion of early events. The aim of the dependency of events is to distribute complete and accurate replicas of the genome to daughter cells. To monitor this dependency, cells are equipped with the checkpoints that are set at various stages of the cell cycle (Fig.2). The first of this control points is the so called "restriction point". This is defined as a point of no return in G_1 following which the cell is committed to enter the cell cycle (Kaufmann 1995). This checkpoint depends on external conditions such as serum concentration in the medium of culture. If the environment is not indicated for division, cell enter a phase of quiescence called G_0 in which it remains until the conditions become favourable to division. But the most important control that cell makes before duplication is DNA integrity. When cells have DNA damages that have to be repaired, then activate *DNA damage checkpoint* that arrests cell cycle. According to the cell cycle stages, DNA damage checkpoints are classified into at least three checkpoints: G_1/S (G1) checkpoint, intra-S checkpoint, and G2/M checkpoint. Upon perturbation of DNA replication by drugs that interfere with DNA synthesis, DNA lesions, or obstacles on DNA, cells activate *DNA replication checkpoint* that arrests cell cycle at G_2/M transition until DNA replication is complete. There are other two checkpoints called *Spindle checkpoint* and *Morphogenesis checkpoint*. The spindle checkpoint arrests cell cycle at M phase until all chromosomes are aligned on spindle. This checkpoint is very important for equal distribution of chromosomes. Morphogenesis checkpoint detects abnormality in cytoskeleton and arrests cell cycle at G2/M transition. At the G1/S checkpoint cell cycle arrest is mainly p53 dependent and the DNA damage lead to a rapid induction of the

protein. The G_2/M checkpoint is, instead, p53 independent because the arrest of the cycle is obtained by maintaining CDK1 in its inhibited form throught inhibitory phosphorylation or by sequestration outside the nucleus (Stark 2004).

The mechanisms of S phase DNA damage checkpoint are less understood but some studies demonstrated suppression of both the initiation and elongation phases of DNA replication. (Painter 1986; Paulovich & Hartwell 1995) The intra-S-phase checkpoint, in fact differs from the G1/S and G2/M checkpoints since its activation don't lead to a cell cycle block but only to a delay. This checkpoint, in fact, has to recognize and deal with replication intermediates and stalled replication forks. Also, in the S phase, another checkpoint that prevents transmission of unreplicated DNA is active. This process inhibits mitosis while DNA replication is ongoing or blocked. The signal for this checkpoint is represented by unreplicated DNA rather than DNA damage.

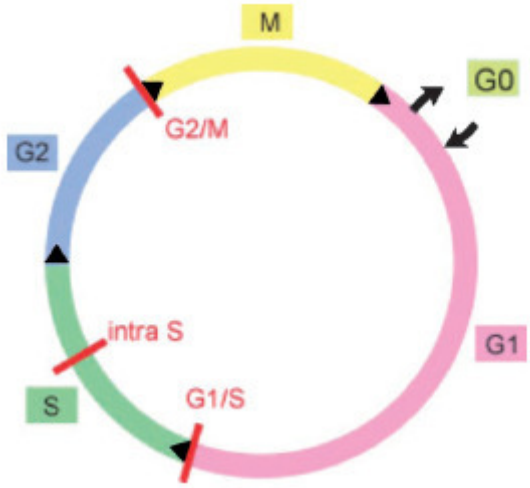


Fig. 2 Schematic representation of the cell cycle. DNA damage triggers activation of these cell cycle checkpoints, which can lead to an arrest at the G1/S, intra-S, or G2/M phase (indicated in red). During cell cycle arrest, the DNA damage can be repaired.

1.3 The DNA damage

A wide diversity of lesions (Fig.3) caused by environmental agents such as ultraviolet (UV) radiation in sunlight, ionizing radiation, and numerous genotoxic chemicals can arise in the DNA. In addition, the genome is also attached by internal products of normal cellular metabolism, such as reactive oxygen species (ROS; i.e., superoxide anions, hydroxyl radicals, and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation. These agents can cause a variety of damages in the DNA.

The DNA damage response during any phase of the cell cycle has the same pattern. After the detection of DNA damage by sensor proteins, signal transducer proteins transduce the signal to effector proteins (Fig.3). These effector proteins launch a cascade of events that causes cell cycle arrest, apoptosis, DNA repair, and/or activation of damage induced transcription programs. The most important players in the early response to DNA damage consists of two kinases

called Ataxia Telangiectasia (AT) Mutated (ATM) and ATM and Rad3 Related (ATR). Ataxia telangiectasia is an autosomal recessive disorder caused by mutated ATM, characterized by immunodeficiency, neurological disorders, and high cancer susceptibility. ATR was identified later on basis of sequence and functional homology to ATM.

These kinases phosphorylate p53 at serine 15 in response to DNA damage, resulting in the stabilization of the protein and subsequently amplifying the downstream p53 cascade and obtaining p21 blocking the cell cycle (Siliciano et al. 1997). The ATM and ATR proteins belong to the phosphatidylinositol 3-kinase-like (PIKK) family of serine/threonine protein kinases. This family also includes DNA protein kinase (DNA-PK). DNA-PK has an important role in G1/S checkpoint and seems to be a DNA double strand break repair enzyme. (Durocher & Jackson 2001).

ATM appears to be the primary player in response to ionizing radiation. Recent results suggest that the Rad50/Mre11/Nijmegen Breakage Syndrome 1 (NBS1) complex and Rad17 protein function as the DSBs sensor for ATM (Williams 2007). ATR, instead, is more important in response to UV radiation and replication inhibitors such as hydroxyurea (HU).

Other candidate DNA damage sensors are three proteins Rad9, Hus1, and Rad1 that form a ring structure (the so called "9–1–1" complex) that can encircle the damaged DNA and is expected to form a scaffold for downstream checkpoint and repair proteins (Jaco 2006).

All these sensors proteins are able to activate different downstream effectors such as the two checkpoint kinases Chk1 and Chk2.

Chk1 and Chk2 are, like ATM and ATR, serine/threonine protein kinases and phosphorylate targets that eventually result in the cell cycle arrest. The Double Strands Breaks (DSBs) signal sensed by ATM is transduced by Chk2, and the UV damage signal sensed by ATR is transduced by Chk1, although there is some overlap and redundancy between the functions of these two proteins. Chk1 and Chk2 transfer the signal of DNA damage to the phosphotyrosine phosphatases and cell division cycle proteins Cdc25A, Cdc25B, and Cdc25C. Phosphorylation of Cdc25A–C by Chk1 or Chk2 inactivates Cdc25A–C, whereas unphosphorylated Cdc25A–C promotes the G1/S and G2/M transition by dephosphorylating the cyclin dependent kinases (CDKs) directly involved in cell cycle transition.

The exact pathway of cell cycle arrest depends on the kind of damage. DNA DSBs lead to phosphorylation of ATM that subsequently phosphorylates Chk2. Single-strand gaps result in the activation of Rad17 or the 9–1–1 complex, and ATR, which leads to phosphorylation of Chk1. Subsequent phosphorylation of Cdc25A by Chk1 or Chk2 causes inactivation of this protein by nuclear exclusion and ubiquitin-mediated proteolytic degradation, leading to G1 arrest. ATM and ATR also phosphorylate p53, which leads to stabilization and accumulation of the p53protein and promotes its transcription factor activity. Two pathways mediate, instead, the intra-S-phase checkpoint. Firstly, the ATM/ATR–Chk2/Chk1–Cdc25A–CDK2 pathway is more or less similar to the G1/S checkpoint. In the S phase, this pathway delays replication (by blocking the loading of Cdc45 onto

chromatin that in turn attracts DNA polymerase-a into prereplication complexes) and, as a consequence, extends the DNA replication time, allowing DNA repair to take place. The second pathway involves Nbs1 which is phosphorylated by ATM together with Chk2, leading to a cascade involving also Mre11- and Rad50-like initial DSBs recognition, which plays a role not only in cell cycle arrest but also in activating the repair processes. When cells encounter DNA damage in G2, the G2/M checkpoint stops the cell cycle in order to prevent the cell from entering mitosis. As in the G1/S checkpoint, the kind of DNA damage determines the pathway that will be activated: ATM–Chk2–Cdc25 for DSBs and ATR–Chk1–Cdc25 for DNA lesions such as those created by UV light. Besides down-regulating Cdc25A, both Chk1 and Chk2 upregulate WEE1 by phosphorylation, which together control Cdc2/CyclinB activity. This latter complex promotes G2/M transition under normal circumstances, and inactivation blocks the cell cycle when damage occurs in G2.

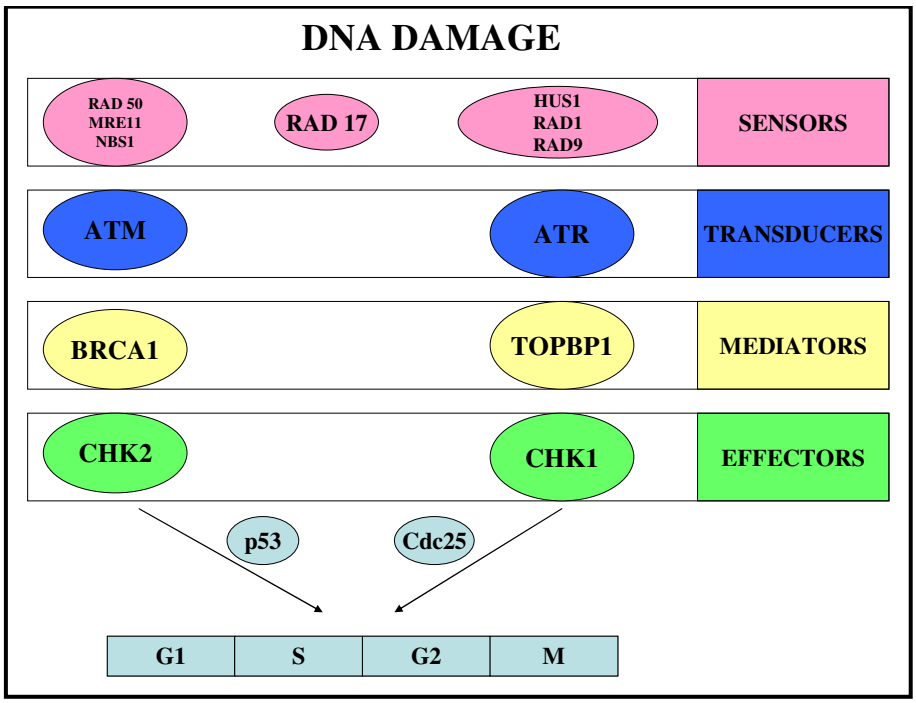


Fig. 3 Simplified representation of the DNA-damage-induced checkpoint response. After the detection of a given damage by sensor proteins, this signal is transduced to the effector proteins Chk1 and Chk2 via the transducer proteins ATR and ATM. Depending on the phase of the cell cycle in which the cell is, this can lead to activation of p53 and inactivation of CDC25, which eventually leads to cell cycle arrest.

1.4 DNA repair

The DNA damage has not only to be prevented, but also to be repaired when occurred. As there are many different lesions possible, different types of repair pathways have evolved. Important pathways in mammalian cells include base

excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and DSBs repair (Fig.4).

BER is the main guardian against damage due to cellular metabolism. Base damages are generated by ROS, ionizing radiation, and indirectly also by UV radiation (via generation of ROS) or can be the result of various chemicals like chemotherapeutic drugs (e.g., adriamycin, mitomycin C, and psoralen). In BER, the damaged base is removed by different DNA glycosylases (depending on the damage) and APE1 endonuclease. This results in an abasic site, from which both ends are trimmed by poly(ADP-ribose) polymerase and polynucleotide kinase to facilitate repair synthesis.

NER is the most important repair system to remove damage that distorts the normal architecture of the DNA helix and bulky DNA lesions that can be caused by UV radiation (thymidine dimers), chemicals, or ROS. Disruption of the DNA helix interferes with base pairing and hinders transcription and normal replication. The repair of damaged DNA involves at least 30 polypeptides within two different sub-pathways of NER known as transcription-coupled repair (TCR-NER) and global genome repair (GGR-NER). (Reardon and A. Sancar 2005). Repair is much more efficient in actively transcribed genes than in the overall genome, and the two pathways differ only in the initial DNA damage recognition step. NER includes a lot of different proteins but the most important of them are encoded by seven xeroderma pigmentosum (XP) complementation groups, XPA to XPG genes.

Replication errors by DNA polymerase- α can result in mismatched bases (A–G or C–T). The mismatch repair (MMR) process begins with the proteins Msh2–6 that recognize and bind to the mismatched base pairs Subsequently, the mismatched strands are cleaved, and the segment from the cleavage site to the mismatch is removed by an exonuclease. DNA polymerase-α fills in the single-strand gap.

The described repair systems are efficient only with damaging agents that cause single strand lesions but the most dangerous damages that occurs on DNA are DSBs.

DSBs are a very genotoxic type of DNA damage. Because both strands of the DNA double helix are broken, chromosomal fragmentation, translocation, and deletions can easily occur and rapid repair is crucial. DNA DSBs can be caused by ionizing radiation, ROS, and chemotherapeutic drugs and can arise during replication of a single-strand break.

In recent studies, it was shown that one of the first responses of eukaryotic cells to DSBs is the extensive phosphorylation of a member of the histone H2A family, H2AX, by the PI3-like kinases ATM, ATR and DNA-PK.

In order to repair DNA DSBs, two distinct pathways have evolved: Homologous Recombination (HR) and Non Homologous End Joining (NHEJ). The two main differences between these pathways are the requirement for extensive DNA homology on the sister chromatid in HR and the accuracy of repair. HR is mediated by the Rad52 group that includes the Rad51, Rad52, and Rad54 genes. After introduction of the DSB, Rad51, which is the central protein

in HR, searches the genome for an intact copy of the broken DNA on the sister chromatid. In this way the missing information on the broken strand is copied in, and the damage is repaired without loss of genetic information.

In NHEJ, on the contrary, there is no need for homology. The two ends of the broken double helix are directly ligated together by the DNA ligaseIV/Xrcc4 complex. Other proteins involved in this pathway are the Ku70/80 heterodimer, DNA-PKCS, and the Rad50/Mre11/NBS1 complex. NHEJ is less accurate and might give rise to deletions.

Although both DSB repair pathways are operational in mammals, their relative contribution differs depending on the stage of the cell cycle or the cell type. For HR to occur, there is a need for a sister chromatid, which is not produced until the S phase. For this reason, HR can only take place in dividing cells that are in the S or G2 phase. Cells in G0 and G1 or terminally differentiated cells mainly rely on NHEJ. A number of the previously mentioned DNA DSB repair proteins (for instance, Rad51, Rad54, and the MRN complex) and γ-H2AX relocate into nuclear foci after induction of DNA damage. These foci are believed to play an important role as DNA damage repair factories, harboring thousands of repair and cell cycle checkpoint proteins, although their exact role remains to be elucidated.

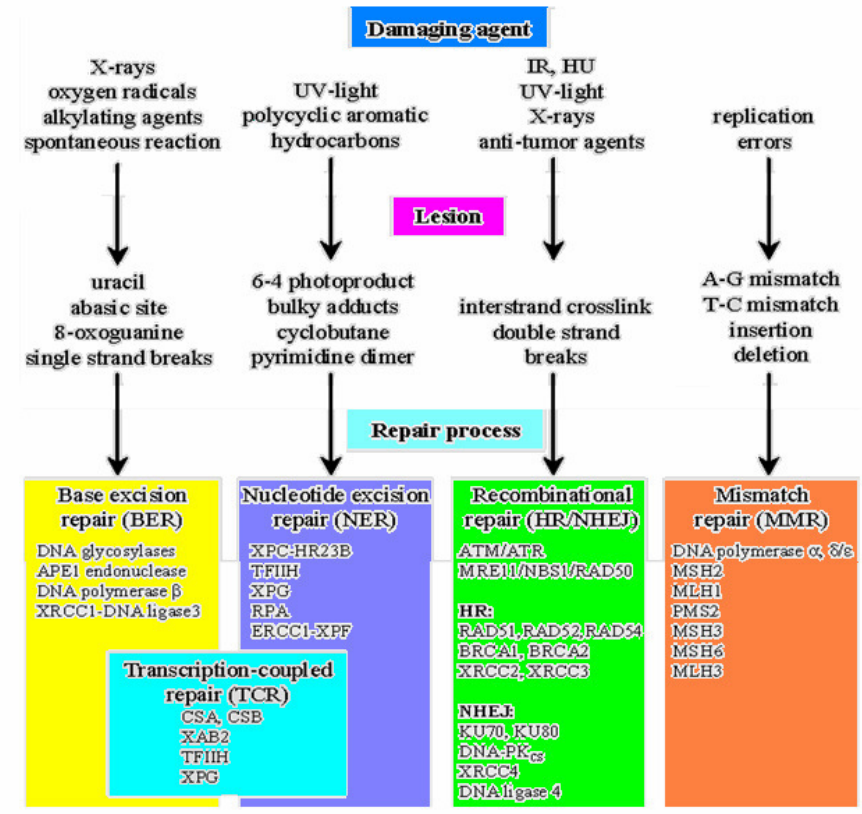


Fig. 4 Summary of the most common types of DNA lesions that can be caused by exogenous or endogenous damaging agents. They may affect a single strand or both strands of the DNA. The assumed repair pathway that operates on the various lesions is also indicated.

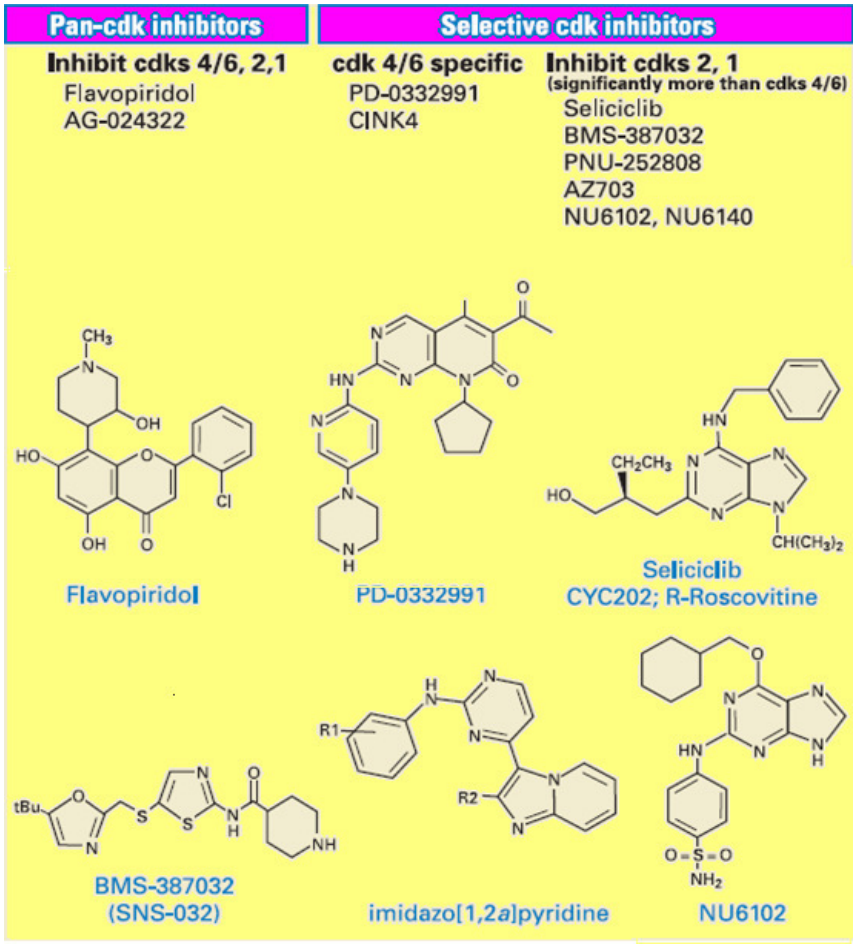
1.5. Chemotherapeutic Drug

1.5.1 CDK inhibitors

CDK inhibitors are a heterogeneous group of compounds that are able to inhibit CDKs involved in the cell cycle, transcription or neuronal functions. CDK inhibitors are chemically diverse, low-molecularweight (< 600 Da), hydrophobic heterocycles. CDK inhibitors compete with the ATP for the ATP binding site. No CDK inhibitor has been shown to compete with the target proteins of CDKs. The first CDK inhibitors were the natural products flavopiridol, butyrolactone, indirubin and staurosporine with its 7-hydroxy-derivative UCN-01. Later, purine and pyrimidine analogues were produced: olomoucine, R-Roscovitine, CGP74514A, BMS-387032, purvalanol B, the pyridopyrimidines (PD0183812 and PD0332991) and other chemical derivatives including the sulphonamide E7070.

According to their different specificity these inhibitors may be subdivided in three different groups: CDK2, 1 and 5 inhibitors; CDK4 and 6 specific inhibitors; pan CDK inhibitors (fig. 5). These compounds however are not so selective. In fact it was demonstrated that they inhibit not only CDKs but also other kinases, including the MAP kinases Erk1 and Erk2 (Schulze-Gahmen, 1994).

Flavopiridol (Flavo) is one of the best characterized CDK inhibitors. It is a semi-synthetic flavonoid derived from the natural alkaloid, rohitukine, originally isolated from leaves and stems of Amoora rohituka (Schang, 2005). Flavo was initially developed as an inhibitor of EGFR and protein kinase A (Sattler 2004). However, the compound was found to inhibit CDKs at far lower concentrations than those required for EGFR or protein kinase A inhibition. Then it was revealed that at nanomolar concentrations it inhibits CDK1, 2, 4, 9 and likely 6 (Schang, 2005; Senderowicz and Sausville, 2000). Flavo induces cell cycle arrest in G1 in vivo and in vitro, perhaps by inhibiting CDK1 and 2. It is cytotoxic to cells synthesizing DNA and causes apoptosis (Carlson, 1999).



From Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol. 2006

Fig. 5 Small-molecule cyclin-dependent kinase (cdk) inhibitors may be classified based on effects on the cell cycle cdks. Pan-cdk inhibitors, including flavopiridol inhibit cdks 4/6, 2, and 1. Other compounds are highly selective inhibitors of cdk4/6. Several other compounds inhibit cdk2 and cdk1 more selectively,

Another well-characterized CDK inhibitor of the first generation is UCN-01 (7-hydroxystaurosporine). UCN-01 is an alkaloid from Streptomyces bacteria, derived from staurosporine. Initially discovered to target CDK1 and CDK2, it is now known to have pan-CDK inhibitory activity, as well as promoting p53-independent apoptosis by targeting Chk1 and Chk2 (Wang et al., 1996). In *in vitro* assays UCN-01 causes cell cycle arrest and apoptosis (Akiyama et al., 1997).
The search for specific pharmacological CDK inhibitors resulted in the discovery of 6-aminopurines, semi-specific but not very potent CDK inhibitors (Shchemelinin et al.). Compounds containing a purine-like ring (purine-type CDK inhibitors) include Roscovitine, olomoucine, the purvalanols and related compounds. Purine-type CDK inhibhitors preferentially inhibit CDK1, 2, 5 and 7,

but not CDK4, 6, or 8 (Schang, 2005). Olomoucine (Olo) was the first specific and relatively potent CDK inhibitor discovered.

More potent but equally specific Roscovitine (Rosco) was then discovered (Schang, 2005). R-Roscovitine (CYC202 or Seliciclib) is an orally bioavailable purine analogue that competes for the ATP-binding site of CDK2/cyclin E, CDK4/cyclin D1, CDK7/cyclin H, CDK9/cyclin T1 (McClue et al., 2002). Rosco inhibits MDM2 expression and thus blocks p53 degradation.

Studies in the Lovo colorectal carcinoma cell line showed that Roscovitine induced cell death in all stages of the cell cycle. In xenograft studies, Roscovitine administered orally or intraperitoneally induced tumour growth delay (McClue et al., 2002). The anti-tumour efficacy of Roscovitine has been tested in a panel of 77 human tumour xenografts in order to find out which tumour types are sensitive to Rosco. A dose-dependent anti-tumour activity of CYC202 has been detected. CYC202 was most active in inhibiting the proliferation of colon, non-small-cell lung, breast and prostate human cancer xenografts (de Bono, 2006).

CDK inhibitors are apparently well tolerated, in animal experiments and human clinical trials against cancer.

Many other non-purine related CDK inhibitors have been designed, including other flavonoids, paullones, indirubines and aloisines. The development of novel CDK inhibitors still continues and new compounds are continuously added to this group (Schang, 2005).

A growing number of CDK inhibitors representing multiple chemical classes currently are in clinical trial. Trials using load/infusion schedules, designed to achieve and sustain micromolar levels, are more mature in hematologic malignancies and are just beginning evaluation in solid tumors. In solid tumor studies, flavopiridol, seliciclib, and BMS-387032 (SNS-032) have been the most extensively tested alone or in combination with other conventional chemotherapeutic drugs.

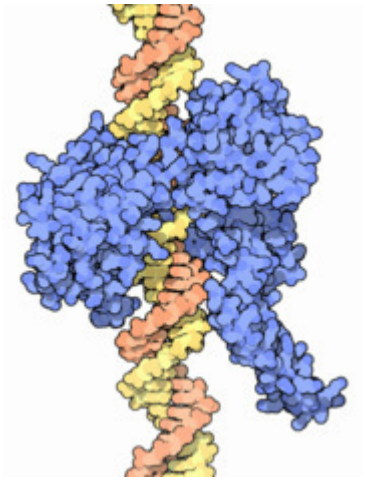
Flavopiridol and Seliciclib are currently used in phase II clinical trial for CLL, lymphoma, and multiple myeloma. For flavopiridol, novel drug schedules appear to be overcoming pharmacokinetic barriers. Initial trials, both in hematopoietic malignancies and solid tumors, used 24- to 72-hour continuous infusions to reflect preclinical observations that prolonged exposure enhanced apoptotic effects in vitro and repeated low-concentration drug treatment demonstrated antitumor activity in vivo (Senderowicz 1998).

Flavopiridol and Seliciclib are also used in combination with a lot of chemotherapeutic drugs. In particular combination with paclitaxel and docetaxel have been studied in phase I and II for breast and gastric carcinomas, combination with adriamycin and flavopiridol for osteosarcoma and combination with gemcitabine for non small cell lung cancer and colon cancer. (Bible 1997; Fornier 2007).

1.5.2 Topoisomerase I inhibitors

The double-helical configuration that DNA strands naturally reside in makes them difficult to separate, and yet they must be separated by helicase proteins if other

enzymes are to transcribe the sequences that encode proteins, or if chromosomes are to be replicated. Otherwise identical loops of DNA having different numbers of twists are topoisomers, and cannot be interconverted by any process that does not involve the breaking of DNA strands. Topoisomerases catalyze and guide the unknotting of DNA. Topoisomerases are divided into type I and II. Type I enzymes cleave only one strand of duplex DNA whereas type II enzymes cleave both strands. Type I topoisomerases (Fig.6A) are further subdivided into type IA and IB. Type IA enzymes cleave the DNA by forming a 5′-phosphotyrosyl covalent bond and relax DNA supercoiling by a strandpassing mechanism, whereas type IB enzymes form a covalent bond with the 3′ end of the DNA, and relax DNA by controlled rotation (Fig. 6B).



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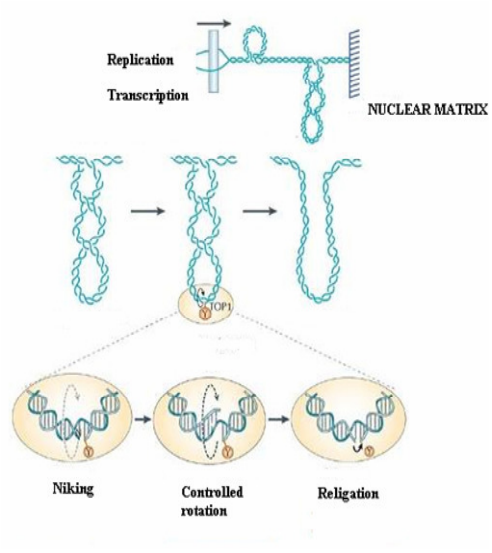


B

Fig. 6A Crystallographic representation of Topoisomerase I enzyme bound to DNA. The enzyme is able to cleave only one strand of DNA. **6B** Mechanism of action of Topoisomerase I enzyme. It cleaves the DNA by forming a 5′-phosphotyrosyl covalent bond and relax DNA supercoiling by a strandpassing mechanism, whereas type IB enzymes form a covalent bond with the 3′ end of the DNA, and relax DNA by controlled rotation.

Because of the size and mass of the replication and transcription complexes it is plausible that such complexes do not rotate freely around the DNA helix. In addition, because of the limited free rotation of the DNA domain flanking a given replication or transcription complex, DNA supercoiling is generated by DNA metabolism. Therefore, DNA tends to be overwound (positively supercoiled) upstream of replication or transcription forks and underwound (negatively supercoiled) downstream of these forks.
Such supercoiling tightens the DNA duplex and needs to be relaxed by topoisomerases (TOP1 and TOP2).

Mammalian TOP1, TOP1mt TOP2α and TOP2β can relax both positive and negative supercoiling. Therefore, TOP1 enzymes tend to be concentrated in supercoiled chromatin regions (particularly in association with transcription or replication complexes). DNA topoisomerases are particularly vulnerable to

topisomerase inhibitors during their cleavage intermediate step. TOP1 cleavage complexes (TOP1ccs) are normally so transient that they are not detectable, but it is these complexes that are specifically and reversibly trapped by pharmaceutical compounds. High levels of cellular TOP1ccs can accumulate owing to DNA modifications and finally to apoptosis.

For these reason Topoisomerase 1 is the target of some potent chemotherapeutic drugs. Camptothecin was the first of these compounds to be developed in the mid 1970s. It was first isolated from the bark of the Chinese tree, Camptotheca acuminate. It was discovered and developed by the US National Cancer Institute (NCI). TOP1 is the only cellular target of this drug. Camptothecin carboxylate was tested clinically and showed anticancer activity, but was discontinued because of its side effects (Wall et al.1995). It was not until after the discovery that TOP1 was the cellular target of Camptothecin that the water-soluble derivatives of Camptothecin — topotecan and irinotecan (also known as CPT-11) — were successfully developed.

Because TOP1 inhibitors bind reversibly to TOP1ccs, and TOP1 readily religates the cleaved DNA after drug removal, TOP1 inhibitors do not directly damage DNA. It is TOP1 itself that damages DNA in connection with DNA-helix-tracking processes — primarily replication and transcription. As TOP1 religation activity is slowed down by the drugs, replication and transcription complexes ‘catch up’ and ‘collide’ with the TOP1–DNA cleavage complexes, thereby generating irreversible TOP1 covalent complexes as the 5′ end of the nicked DNA template becomes misaligned with its substrate. As TOP1 religation activity is slowed down by the drugs, replication and transcription complexes ‘catch up’ and ‘collide’ with the TOP1–DNA cleavage complexes, thereby generating irreversible TOP1 covalent complexes as the 5′ end of the nicked DNA template becomes misaligned with its substrate. Replication-fork collision is the primary cytotoxic mechanism of TOP1 inhibitors in dividing cells (Fig.4). Indeed, cancer cells in culture tend to be resistant to Camptothecin when they are outside of S-phase (Horwitz 1973; O’Connor 1991) or when replication is arrested at the time of Camptothecin treatment (Holm 1989; Hsiang 1989). Moreover, like other cell-cycle-specific agents, Camptothecin is increasingly cytotoxic with increasing time of drug exposure. Camptothecin kills fewer than 50% of cells when they are exposed to the drug for less than 1 hour. This is different from TOP2 inhibitors, which can be highly cytotoxic (over 90%) even in the absence of active replication (Holm 1989).

As for most other anticancer agents, the prescription of TOP1 inhibitors is currently based on the cancer histology and tissue of origin. Irinotecan is approved for colon carcinomas, whereas topotecan is approved for ovarian cancers, although both drugs target TOP1 in a similar way. The different indications have been determined empirically over the course of clinical trials, rather than by the molecular characteristics of the tumours.

The search for non-Camptothecin TOP1 inhibitors was initiated immediately after the discovery that TOP1 was the cellular target of Camptothecins. The screening of chemical libraries and natural products with purified TOP1 and

isolated DNA substrates led to the discovery of various TOP1 inhibitors (), including the indolocarbazoles and phenanthroline derivatives. They seem to be more effective than the classic Camptothecins but several studies indicate that, although they are therapeutically effective, they are not curative as single agents. Several approaches need to be considered to improve the effectiveness of TOP1 inhibitors. First, the development of new inhibitors with activity against different cancers, improved pharmacokinetics and lower toxicity are needed. Second, further investigation of the molecular determinants of drug activity in model systems should lead to the development of molecular tools to classify tumours on the basis of whether they have a molecular network matching drug-specific pathways. Third, a rationale for the combination of TOP1 inhibitors with other drugs or biological treatments on the basis of the molecular network of individual tumours is needed. And fourth, reliable, sensitive and non-invasive biomarkers are required to follow the early response or lack of response to TOP1 inhibitors in combination with other treatments so that therapies can be rapidly and effectively adapted.

2. AIM OF THE WORK

Previous studies in our lab (Crescenzi et al. 2005) had reported that Roscovitine was able to modulate DNA repair and senescence. In particular it was demonstrated that Roscovitine reinforces doxorubicin (topoisomerase II inhibitor) - dependent G1 checkpoint in A549 (lung) and HEC1B (endometrium) leading to decreased frequency of double strands breaks and enhanced clonogenic survival. However Roscovitine dramatically sensitized other tumour cell lines, such as HCT116 (colon) and H1299 (lung) to doxorubicin. Roscovitine, negatively affecting DNA repair processes, appear to have the potential to inhibit recovery of damaged tumor cells after doxorubicin. However in some tumor cells, the cell cycle inhibitory effect of Roscovitine prevails over the DNA repair inhibitory effect favouring the clonogenic growth. These observations prompted us to investigate the effects of Roscovitine in combination with another chemotherapic compound namely Camptothecin. This drug is also a Topoisomerase inhibitor but is selective for topoisomerase I. Since Topoisomerase I specifically binds single strand DNA, Camptothecin at variance with Doxorubicin is particular active in S phase of the cell cycle. This work is then aimed to the clarification of the effects of Roscovitine in the modulation of the intra S checkpoint (that the specific target of Camptothecin). To this purpose we used two lung cancer cell lines: A549 $^{+/+}$ (p53 $^{+/+}$) and H1299 (p53 $^{-/-}$), a colon cell line (HCT116 p53$^{+/+}$) and a subclone of this one obtained from homologous recombination (HCT116 p53 $^{-/-}$).

3. MATERIALS AND METHODS

3.1 Cell cultures.

The NCI-H1299 human non–small cell lung cancer cell line was obtained from American Type Culture Collection (Rockville, MD). They were cultured in RPMI 1640, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4,500 mg/L glucose, 1,500 mg/L sodium bicarbonate, 100 Mg/mL streptomycin, 100 units/mL penicillin, and 10% FCS. The NCI-H1299 cells are $p53^{-/-}$.

The NCI-A549 human non–small cell lung cancer cell line was obtained from American Type Culture Collection (Rockville, MD). They were cultured in Ham's F12K, 2 mmol/L L-glutamine, 1,500 mg/L sodium bicarbonate, 100 units/mL penicillin and 10% FCS. All media and cell culture reagents were purchased from Life Technologies (San Giuliano Milanese, Italy). The NCI-A549 cells are $p53^{+/+}$.

The NCI-HCT116 cells human colon cancer cell line was obtained from American Type Culture Collection (Rockville, MD). They were cultured in McCoy's 5A, 2 mmol/L L-glutamine, 100 units/mL penicillin and 10% FCS.

Cell culture media and reagents were purchased by Invitrogen (San Giuliano Milanese, Milan, Italy).

3.2 Drugs treatments.

Camptothecin (Calbiochem, San Diego, CA) supplied as powder was dissolved in sterile water to a final 20 mM stock solution. Roscovitine (Calbiochem) was dissolved in DMSO to a final 5 mg/mL stock solution. Cells were incubated with Camptothecin (0.5 μM), Roscovitine (10μM) or both for 48hours, then washed in PBS 1X solution and analyzed (cell cycle, viability and clonogenic assay, protein extraction).

3.3 Colony forming efficiency assay.

Cells were plated in triplicate at 2 X 104 per well in a 6-well plate. After 16 hours, cells were treated with Camptothecin (0.5 μM or Camptothecin plus Roscovitine (10 μmol/L), or DMSO (vehicle). Cell number was assessed using a hemocytometer. After 8 to 10 days, colonies (>50 cells) were stained with 1% methylene blue in 50% ethanol.

3.4 Flow Cytometry.

Dishes (10 cm) containing 4 x 105 H1299/A549/HCT116 cells were incubated for 24 hours at 37 ° C in 7 mL complete medium (controls) or in medium supplemented with Camptothecin alone or associated with Roscovitine for 48 hours. Cells were washed twice with PBS 1X and then detached from the dishes by trypsinization, suspended in serum rich medium, centrifuged, washed twice with 1 mL PBS, and resuspended for storage (20°C) in 95% ethanol. Before analysis, fixed cells were washed twice, centrifuged, and resuspended in 1 mL

PBS containing 1 µg RNase and 100 µg propidium iodide (Crescenzi et al 2004). Samples were stored in the dark for 20 minutes at room temperature before final readings. The cellular orange fluorescence of propidium iodide was detected in a linear scale using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) equipped with an excitation laser line at 488 nm. About 30,000 events (i.e., fluorescence readings, corresponding to not less than 20,000 cells) were recorded for each sample. The cell cycle was examined after monotherapy and combined treatment at the indicated times. Data were analyzed with ModFit/LT (Verity Software, Topsham, ME).

3.5 Immunofluorescence microscopy

Cells were grown on to gelatin-treated glass coverslips in 60 mm dishes, and were allowed to adhere for 48 h. Cells were fixed with ice-cold methanol and permeabilized with 0.2% Tween 20 in TBS. Cells were blocked with 10% FBS in TBS-T buffer [Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20] for 15 min. Bcl-2 was detected by incubating the cells with anti-PCNA monoclonal antibody at a dilution of 1:200 for 1 h. Cells were washed with TBS-T and then incubatedwith a 1:200 dilution of fluorescein-tagged goat anti-mouse secondary antibody (Santa Cruz Biotechnology). After washes with TBS-T, the coverslips were mounted on to a microscope slide using a 90% solution of glycerol in TBS and analysed with a Zeiss Axioplan2microscope.

3.6 Flow cytometry for γ–H2AX or phospo (ser/thr) ATM/ATR substrates.

Cells were fixed with ethanol and routinely kept at -20°C overnight. Cells were washed twice with TBS and permeabilized with TBS, 4% fetal bovine serum, 0.1% Triton X-100 for 10 minutes on ice. Cells were washed with TBS and incubated with anti-γ–H2AX monoclonal antibody (JBV301) (Upstate Biotechnology Milton Keynes, United Kingdom) or monoclonal anti- phospo (ser/thr) ATM/ATR substrates antibody (2851) (Cell Signaling technology INC) in a 1:200 dilution in TBS, 4% fetal bovine serum, for 2 hours. Cells were washed twice with TBS-0.1% Tween 20 and incubated with 1:200 dilution of fluorescein-tagged goat anti-mouse secondary antibody. After washes with TBS-0.1% Tween 20, cells were resuspended in TBS and analyzed using a using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). Data were analyzed with ModFit/LT (Verity Software, Topsham, ME).

3.7 Western Blot Analysis

Total cell protein preparations were obtained by lysing cells in 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1% NP40, 0.1% Triton, 2 mmol/L EDTA, 10 Ag/mL aprotinin, and 100 Ag/mL phenylmethylsulfonyl fluoride. Protein concentration was routinely measured with the Bio-Rad protein assay (Bradford M. 1976). Polyacrylamide gels (10%–12%-15%) were prepared essentially as described by Laemmli (Laemmli 1971). Molecular weight standards were from New England Biolabs (Beverly, MA).Proteins separated on the polyacrylamide

gels were blotted onto nitrocellulose filters (Hybond-C pure, Amersham Italia, Milan, Italy). Filters were washed and stained with specific primary antibodies and then with secondary antisera conjugated with horseradish peroxidase (Bio-Rad; diluted 1:2,000). Filters were developed using an electrochemiluminescent Western blotting detection reagent (Amersham Italia). The anti Bax (N-20), p21CIP1 (C-19), CDK2 (M2), total p53 (DO-1), Cdc25A (F-6), PCNA (PC-10), Cdc6 (180.2), chk1 (FL-476), chk2 (H30C), actin (I-19) antibodies were purchased from Santa Cruz Biotechnology; anti-pRb (554136) was from BD Phar-Mingen (Franklin Lakes, NJ); antibodies specific for the phosphorylated state of ser345 in chk1, thr68 in chk2 and for ATM/ATR phosphorylated substrates were from Cell Signaling Technology (Danvers USA); anti-γ-H2AX (JBW301) was from Upstate Biotechnology.(Milton Keynes, United Kingdom);

3.8 Thymidine Incorporation

Thymidine incorporation experiments were performed in 24-well plates. In brief, cells (3 X 10^4 cells/well) were incubated at 37°C in 2 ml complete medium (controls) or medium supplement with Camptothecin (0.5 μM), Roscovitine (10μM), or both. All samples were run in triplicate. After 4 h or 48h incubation, each well was washed with 1 ml warm medium. the medium was replaced (all samples) with fresh culture medium containing labelled [3H]thymidine (0.5 ACi/ml; Amersham, Buckinghamshire,UK). After 4 h at 37°C, incorporation was blocked by extensive washing with warm serum-free medium. NaOH (0.1 M) was added to all wells (1 ml) and the plates were left at 37°C for 1 h under constant agitation. 10 mL of solutions were used to measure protein concentration by routinely measure with the Bio-Rad protein assay (Bradford M. 1976). and the remainder was used to measure the incorporated thymidine with the semiautomatic Harvester 96 (Skatron Instruments, Lier, Norway). Thymidine counts were expressed as a fraction of counts found in controls.

3.9 Fractioned proteins extraction

To isolate chromatin-bound proteins cells were cultured in 100-mm plates, washed three times with ice-cold PBS, collected in 1 ml of PBS by scraping, and pelleted by quick spinning at 1,000 rpm for 1 to 2 min. Soluble proteins were then extracted with ice-cold 0.1% Triton X-100 in CSK buffer for 20 min at 4°C. The insoluble, chromatin bound fraction was then pelleted by low-speed centrifugation at 3,000 rpm for 5 min at 4°C. These pellets were then reextracted by incubating in CSK buffer and collected by centrifugation at 3,000 rpm for 10 min at 4°C. The final pellet fraction (containing chromatin-bound proteins) or total cell pellets were solubilized in radioimmunoprecipitation assay (RIPA) buffer and equal protein was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

3.10 Senescence-associated β-galactosidase activity.

Staining for SA-β-gal was done as described previously (Dimri 1995). Routinely, cells were treated with Camptothecin or Camptothecin plus Roscovitine for 48hours, then washed exensively, trypsinized and plated in triplicate dishes at 1 x 10^5 in 60-mm dishes. The assay was performed 8 to 10 days after.

3.11 Statistical analysis

All the data were expressed as mean+/- SD. Significance was assessed by Student τ-test for comparison between two means. P values of less than 0.05 were considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 Roscovitine increases cell recovery after drug release from Camptothecin

Camptothecin is a widely used chemotherapeutic compound and its effect is due to accumulation of DNA DSBs. Deficiencies in both the checkpoint and DNA-repair pathways determine cellular sensitivity to TOP1 inhibitors. The identification of such defects in tumours should address guide the rational use of TOP1 inhibitors. Targeting checkpoint and repair pathways will results in increasing the selectivity of TOP1 inhibitors in tumours that have pre-existing deficiencies in relevant redundant pathways. Therefore, defects in cell-cycle checkpoints might adversely affect DNA repair and increase the antiproliferative activity of Camptothecin.

The abrogation of cell-cycle checkpoints can be achieved using small-molecule inhibitors of the protein kinases, such as CHK1, CHK2, ATM, ATR, DNA-PK and CDKs.

We chose to investigate the effects of the specific combination of Camptothecin and Roscovitine, a CDKs inhibitor. Hence we incubated A549 and H1299 cell lines for 48 hours either with Camptothecin alone or in combination with Roscovitine. The dose of Camptothecin used (0.5 μMol) was assessed to not induce a remarkable apoptotic process because the absence of a sub-G1 population (Fig.8). The dose of Roscovitine (10mM), instead was already demonstrated to not induce cell death but only inhibit cell proliferation (Crescenzi 2005). After the treatment cells were released and recultured in drug free medium for 8 to 10 days to investigate the effect of Roscovitine on clonogenic survival. These experiments surprisingly showed a significant increase in colony formation in the presence of Roscovitine if compared to the single treatment with Camptothecin alone (Fig. 7). This effect was verified as in A549 cells as in H1299 cells. This result was in some way similar to that obtained by Crescenzi et al. in 2005. They demonstrated that Roscovitine was able to protect A549 cells exposed to a sublethal dose of DNA-damaging agent resulting in a chemoprotective effect against doxorubicin. For H1299 cells, instead the effect verified by the authors was different. They underlined the ability of Roscovitine to potentiate the effect of doxorubicin inducing an effect of chemosensibilization. In our system we found an opposite effect.

To verify if Roscovitine was able to induce chemoprotection against Camptothecin independently from p53 status of cells we used other cell lines: HCT116 (colon cancer) that are p53 wt and a subclone p53 null derived from the same line for homologous recombination. In both strains Roscovitine was able to induce a significant increase in the number of colonies in comparison to those obtained after the treatment with Camptothecin alone. (Fig.7) This result suggested that the effect of Roscovitine was p53 independent because it was similar in all the cell lines analyzed.

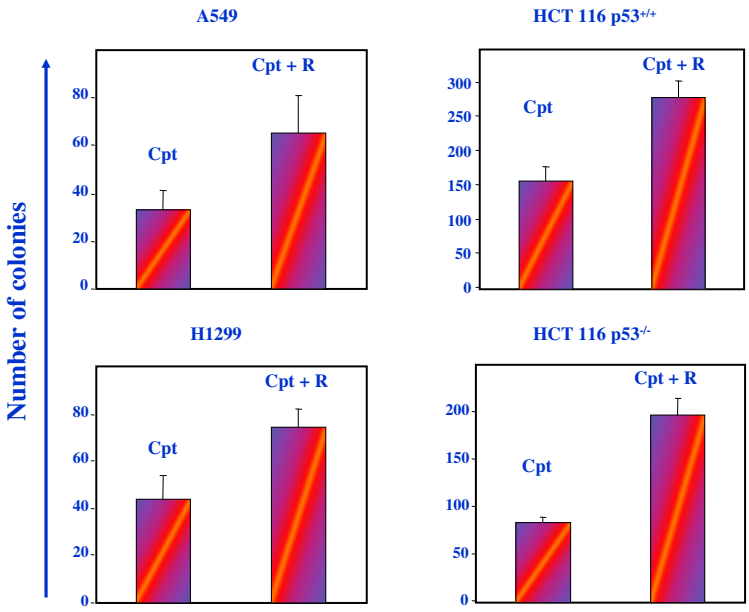


Fig.7 Clonogenic survivor. Triplicate samples of A549, H1299, HCT116 cells were incubated either with Camptothecin (Cpt) or with Camptothecin + Roscovitine (R) for 48 hours. Cells were replated in drug-freemedium. After 8 days, colonies were stained with methylene blue. $P < 0.05$, unpaired Student's t test.

4.2 Roscovitine renforces G1 and intra S checkpoints in Camptothecin-treated cells.

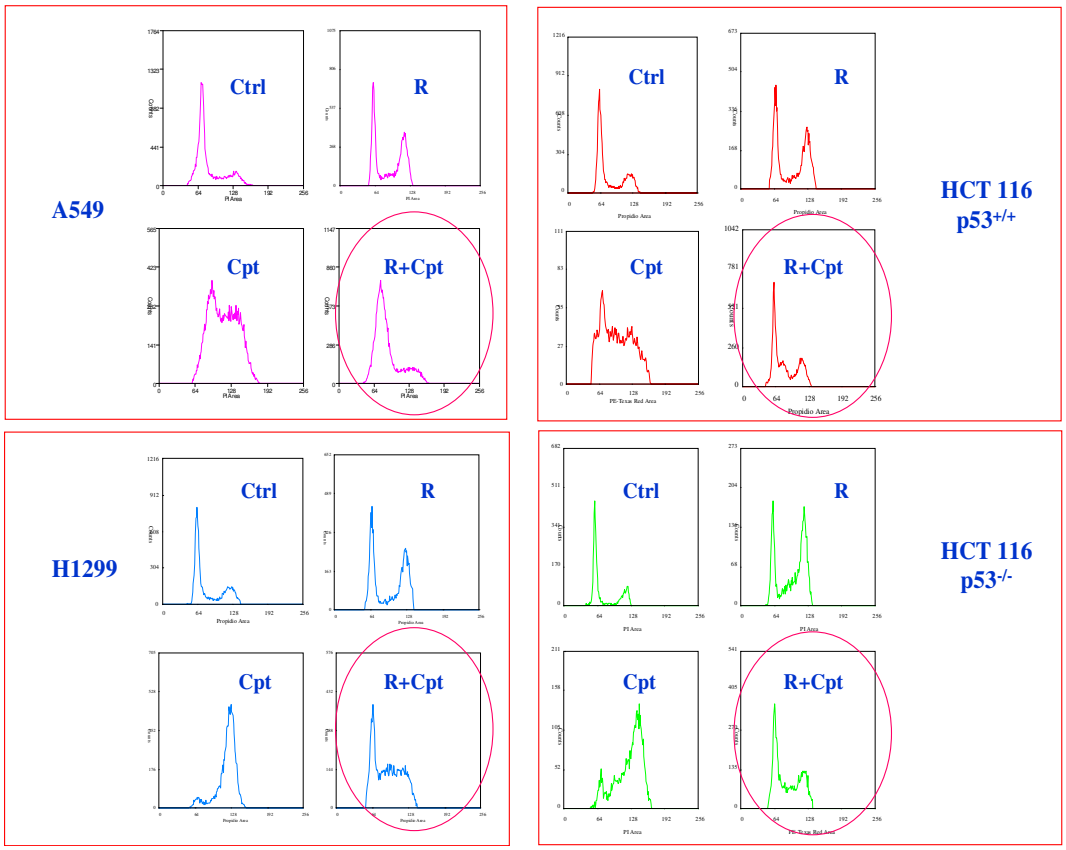
To determine the effect of Roscovitine and Camptothecin on cell distribution, cells were treated with individual drugs or with their combination and analyzed by flow cytometry. (Fig.8)

In all cell lines Roscovitine alone only slightly affected cell cycle inducing a modest but significant increase in G2 fraction as already described before (Crescenzi 2005) in all the cell lines analyzed. This increment is perhaps due to an apparent slowdown of the cell cycle without a significant effect on cell viability.

Incubation with Camptothecin 0.5 µM resulted in accumulation of cells in different phases of cell cycle that depends on the to p53 status. In fact, p53 positive cells (A549 and HCT116 wt) showed a block in S phase, instead p53 negative cells (H1299 and HCT116 $p53^{-/-}$) accumulated in G2/M phase. This was likely due to activation of intra S phase checkpoint in the presence of a functional p53 protein or in a more delayed activation of G2/M checkpoint where p53 is absent (Chen et al. 2003). In turn the accumulation of cells in G2-M or S phase, instead, was reduced in cells treated with Camptothecin plus Roscovitine and was accompanied by a concomitant increase of cells in G1 phase. Increased number of G1 cells in combined treatment indicates a reinforcement of G1 checkpoint as already described in our laboratory (Crescenzi et al 2005).

The protective effect of Roscovitine is related to a cell cycle inhibitory function, which results in an increased cell cycle block at G1 phase. G1 arrest has

been associated previously with decreased susceptibility to chemotherapeutic drugs (Sugiyama 1999; Lu 2000).



	G1	S	G2	G1	S	G2
CTRL	66	20	14	73	18	9
R	39	38	25	64	15	21
CPT	11	18	71	12	59	29
CPT+R	62	13	25	40	28	33
CTRL	62	25	13	72	17	11
R	42	32	26	66	20	14
CPT	9	17	74	10	51	39
CPT+R	54	16	30	43	15	24

A 549
H1299
HCT116p53$^{+/+}$
HCT116p53$^{-/-}$

Fig. 8 Cell cycle distribution after single and combined treatments. Cells were incubated with Roscovitine, Camptothecin, or both for 48 hours. Percentage of cells in G1, S, or G2-M phase are indicated in table.

4.3 Cell cycle proteins expression in Camptothecin – Roscovitine treated cells

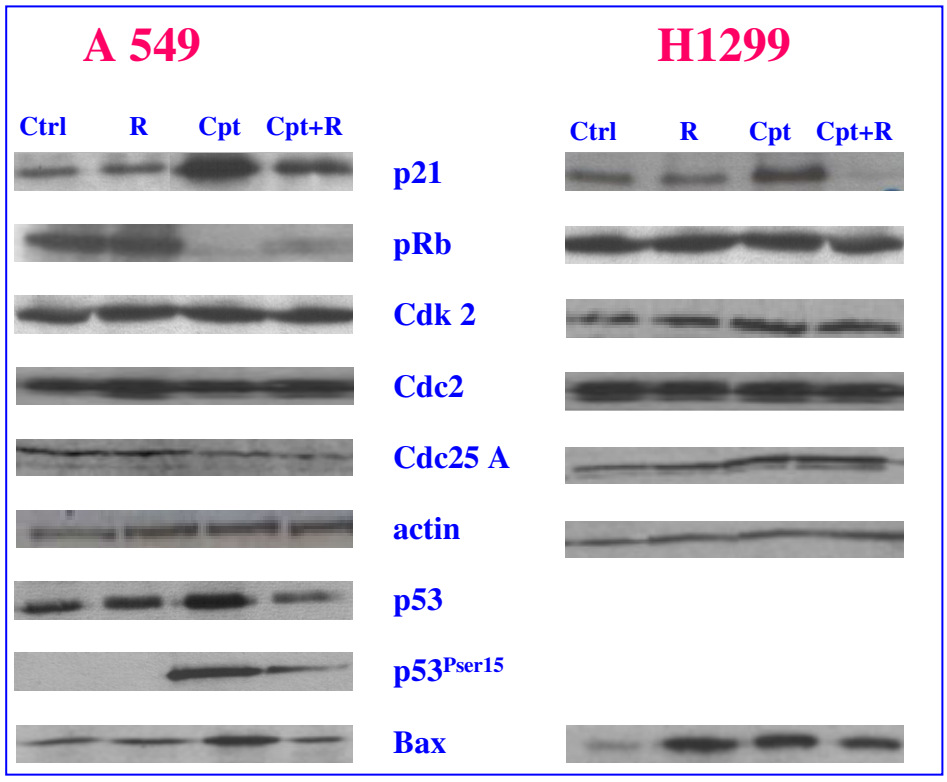
The ability of Roscovitine to modulate G1 and intra S cell cycle checkpoint might either depend on a direct inhibition of CDK activity or on the modulation of cell cycle inhibitory proteins.

To discriminate between these two possibilities, We examined the levels of cell cycle regulatory proteins by Western blot in all the cell lines. Incubation of p53 positive cells with the two drugs alone or in combination affects the level of expression of the protein and the phosphorylation in Ser 15 as determined with a specific antibody. In particular the total level of expression of the protein in A549 cells results downregulated in the combined treatment (Fig. 9A) if compared to Camptothecin alone. This effect is not demonstrable in HCT116 cells. However the effect of different treatments on the phosphorylation of Ser 15 in both cell lines is quite interesting. In fact it is induced upon treatment with Camptothecin alone and is significantly reduced upon combined treatment The tumor suppressor p53 is known to play a key role in cell cycle arrest as well as apoptosis in response to various stresses such DNA-damaging agents and anticancer drugs. Phosphorylation of p53 on Ser15 by the phosphatidylinositol-3-like kinases ATM and ATR triggers post-translational modifications that contribute to p53 stabilization (Banin et al. 1998; Canman et al., 1998) and subsequent activation. One of the major phosphatidylinositol-3 kinases that target p53 N-terminal residue Ser15 for phosphorylation is ATM (Yu et al. 2002). This observation may indicate that the presence of Roscovitine affects, in some way, the signalling ATM/ATR mediated. To verify this hypothesis we also investigated the levels of proteins that are downstream in p53 pathway. In particular we operated Western blot analyses of p21 protein and Bax protein. The levels of p21 protein are reduced in the presence of Roscovitine if compared with those observed with Camptothecin alone. This is detectable in all the cell lines used but is more significant in p53 negative cells (i.e. H1299 and HCT116 p53 $^{-/-}$). As already described by Dai and al. (2006) the downregulation of p21 is a consequence of CDK inhibitors and may change cellular response to chemotherapy.

According to our observation that cells treated with the combination of the two drugs were able to form more colonies than those treated with Camptothecin alone, the expression of Bax, a well known proapoptotic protein decrease when cells are treated with Camptothecin in the presence of Roscovitine. The downregulation of bax is observable in all the cell lines.

Because pRb plays a key role in G1 checkpoint (Bartek et al. 2001) and is also required for intra-S response to DNA damage (Knudsen 2000; Bosco 2004), we analyzed pRb phosphorylation status in A549, H1299, and HCT116 cells. An accumulation of hypophosphorylated, active pRb was detected in A549 and HCT116 p53$^{+/+}$ cells incubated with Camptothecin or with Camptothecin plus Roscovitine (Fig.9A e B). In contrast, no variation in pRb phosphorylation pattern was observed in both H1299 and HCT116 p53$^{-/-}$ after treatment with Camptothecin, Roscovitine or their combination (Fig 9A e B).

To check other proteins whose levels could have been regulated by Roscovitine or Camptothecin (such as Cdc2, Cdc25A and CDK2) we investigated their expression. Indeed none of these proteins appeared to have a role in this experimental system and their levels remained constant in all experimental conditions. Only Cdc25A resulted slightly downregulated in p53 positive lines in the presence of Roscovitine. These data suggest that the ability of Roscovitine to reinforce G1 and intra S checkpoint depends on direct inhibition of CDKs.



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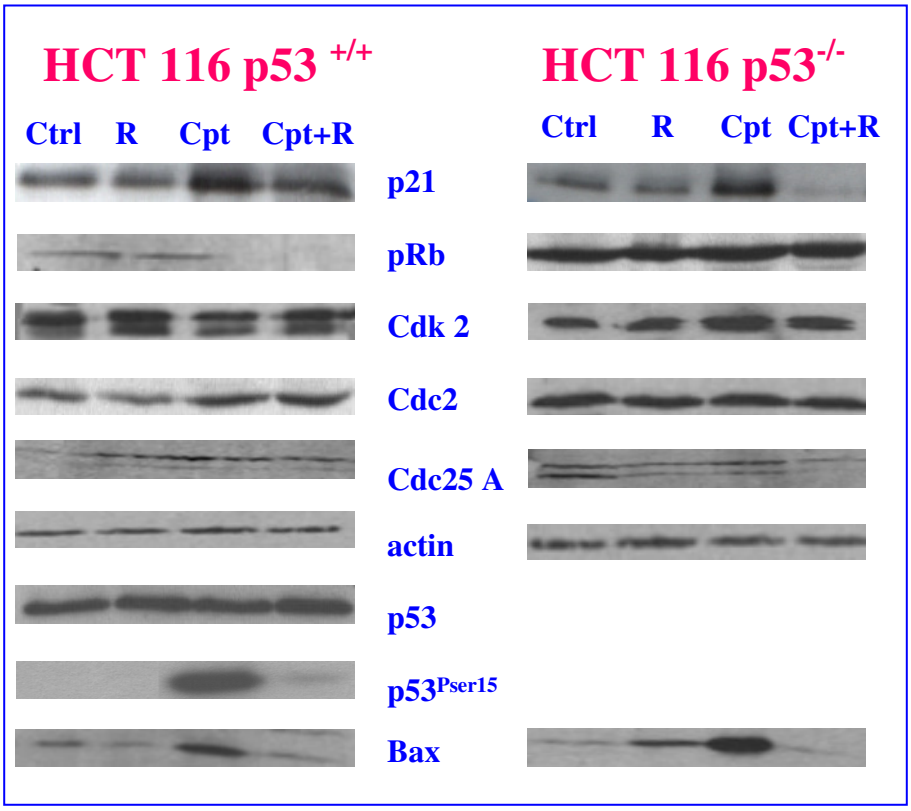


B

Fig. 9A e B Cell cycle proteins expression. A549, H1299 (panel A) and HCT116 cells (panel B) were incubated with roscovitine (R), Camptothecin (Cpt), or both (Cpt+R) for 48 hours and protein expressionwas detected by Western Blot. Filters were probed with anti-α-actin antibodies as loading control.

4.4 Roscovitine decreases Camptothecin-induced premature senescence

To evaluate the ability of Roscovitine to modulate cellular responses to Camptothecin, A549, H1299 and HCT116 cells were incubated for 48 hours either with Camptothecin alone or in combination with Roscovitine. Cells were then released and recultured in drug-free medium for 8 days. Treatment of cells with Roscovitine plus Camptothecin decreased the fraction of senescent cells (βgal-positivity or flatten morphology) by 40% compared with Camptothecin alone (Fig.10). The lower percentage of senescent cells correlates with the decreased level of p21 protein in the combined treatment in comparison to that of Camptothecin alone. Camptothecin is able to induce cellular senescence either in p53 positive and in p53 negative cell lines. It was extensively described that chemotherapeutic compounds used at sublethal doses can induce replicative senescence in tumour cell lines. In the presence of functional p53 the inhibition of Topoisomerase I leads to the activation of the G1/S checkpoint p53 dependent, block of cell cycle progression and induction of a replicative senescence program. Chang-Rung Chen (2005) have demonstrated that the activation of Chk2 by phosphorylation of Thr68 (induced in our conditions by Camptothecin treatment

in p53 negative cell lines) causes G2/M phase arrest and cellular senescence in a p53 independent manner.

In the case of combined treatment, the presence of Roscovitine prevents the accumulation of p21 and the activation of p53 or Chk2. This results in a lesser activation of replicative senescence program in favour of a DNA repair response to damage.

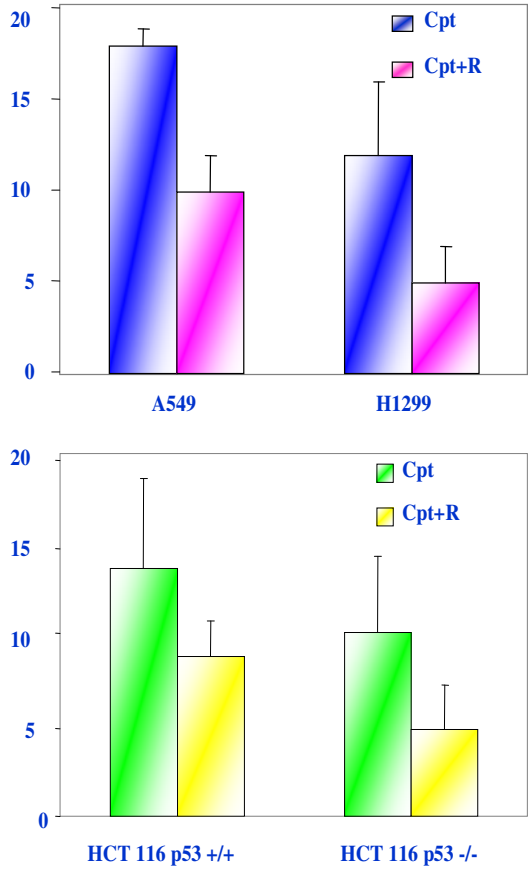


Fig. 10 Effect of combined treatment on senescence. Triplicate samples of A549, H1299 and HCT116 cells were incubated either with Camptothecin (Cpt) or with Camptothecin + Roscovitine (Cpt+R) for 48 hours. Cells were replated in drug-free medium. After10 days, cells were stained to detect SA-β-gal activity. The amount of senescent cells was determined by counting of three random fields. Columns, mean of three independent experiments

4.5 Chemoprotective effects of Roscovitine correlate with the extent of DNA damage.

The ability of Roscovitine to reinforce the G1 checkpoint in A549, HCT116 and H1299 cells is likely to be responsible for the increased resistance of the cells to Camptopthecin. To further investigate the mechanism of action of Roscovitine, it was examined the incidence of phosphorylation of histone H2AX (□-H2AX) in Camptothecin / Roscovitine treated cells. γ-H2AX is a sensitive signal for the detection of DNA DSBs (Rogakou et al 1998; Sedelnikova et al. 2002) since the amount of phosphorylated H2AX increases linearly with the severity of the damage.

Cells were incubated either with Camptothecin alone or with Camptothecin plus Roscovitine and subsequently analyzed by flow cytometry. Increased intensity of fluorescence in Camptothecin-treated cells compared with controls was readily detected by flow cytometry (Fig.11) Analyses of γ-H2AX in Camptothecin versus Camptothecin plus Roscovitine–treated cells confirmed the accumulation of cells with low fluorescence in the presence of Roscovitine. These data strongly suggest a chemoprotective effect of Roscovitine in A549, H1299 and HCT116 (both p53 wt or p53 $^{-/-}$) cells.

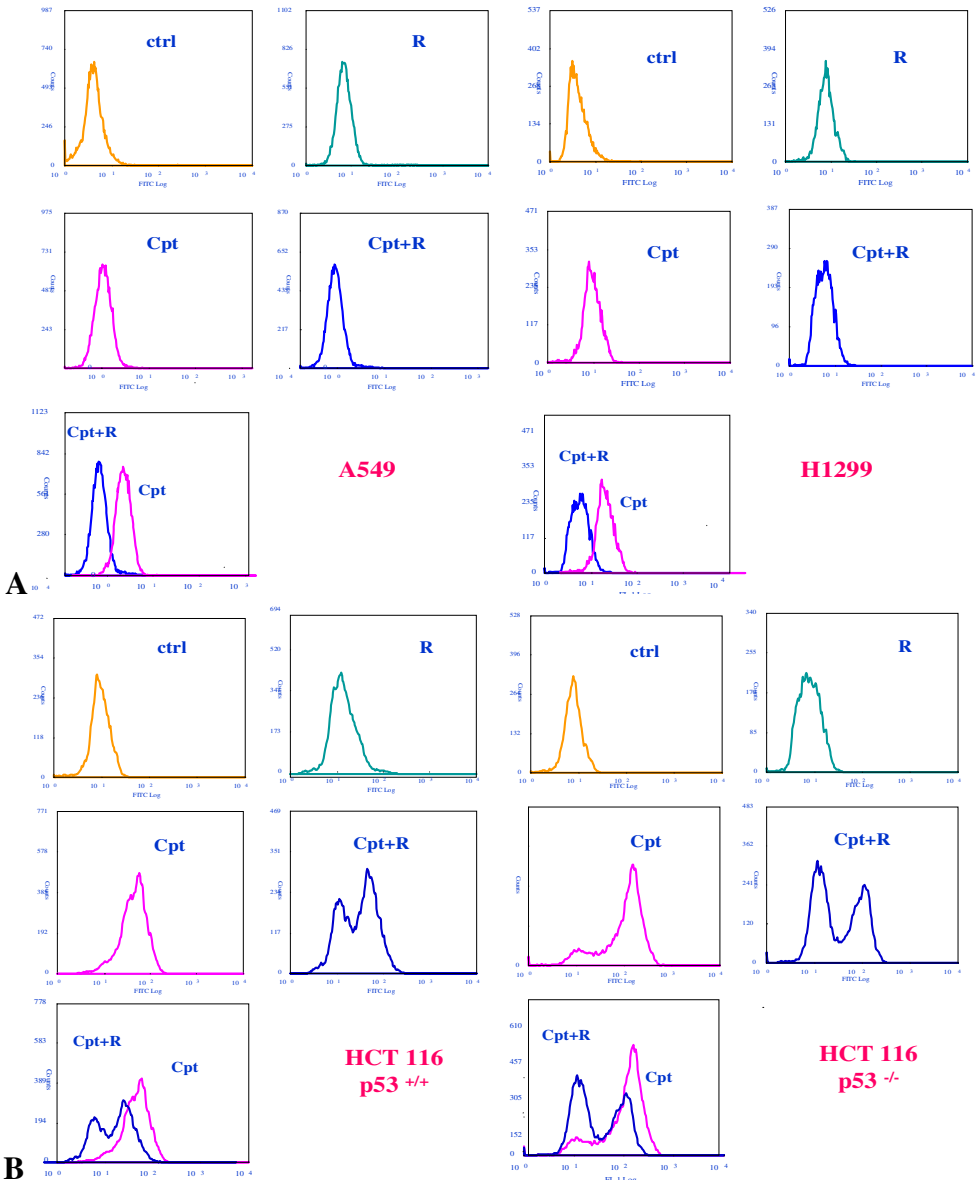
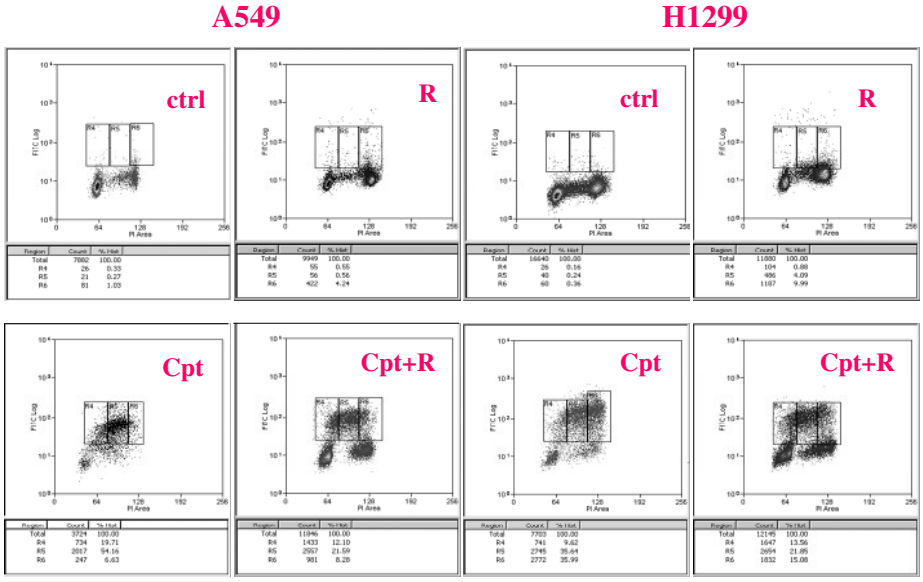


Fig. 11 A e B Effects of Roscovitine and Camptothecin on H2AX phosphorylation. A549, H1299 (panel A) and HCT116 (panel B) cells were incubated with Roscovitine or with Camptothecin in the presence or absence of Roscovitine for 48 hours. Cells were immunostained

with an anti-γ-H2AX monoclonal antibody followed by secondary fluorescein conjugate antibodies and analyzed by flow cytometry. In single panel it is showed the overlay of fluorescence peck of Camptothecin versus Camptothecin/ Roscovitine -treated cells.

It was also performed a double cytofluometric analysis of cell cycle by PI assay and H2AX phosphorylation evaluation to correlate DNA damage with cell cycle phase and induction of apoptosis. In both p53 positive cell lines, the highest degree of H2AX phosphorylation induced by Camptothecin alone was seen in S-phase cells, particularly during early stage of S. In cells not replicating DNA (G1, G2 and M) the level of H2AX phosphorylation was markedly lower than that in S-phase cells. Furthermore, the level of Camptothecin - induced γH2AX in G1 phase was much higher if cells were simultaneously contemporary treated with Roscovitine. The data are consistent with the notion that H2AX phosphorylation observed throughout S phase reflects formation of DSBs due to the collision of replication machinery with the complex Camptothecin-topoisomerase stalled on DNA. It has been suggested that the stalled replication forks attract cycle sensor proteins which trigger the ATR/Chk1- dependent checkpoint signalling cascade that involves activation of a variety of proteins including p53. Activated p53 (phosphorylated by ATR/Chk1 kinases) become stable and is able to arrest cell cycle progression, as well as to increase cells apoptosis in response to DNA damage (cell cycle 2005) . As already indicated, treatment of A549 and HCT cells with Camptothecin results in p53 phosphorylation. Since this phosphorylation remarkable decreases in the presence of Roscovitine it appears that the DNA damage is repaired more efficiently in this condition while p53 mediated are not induced.



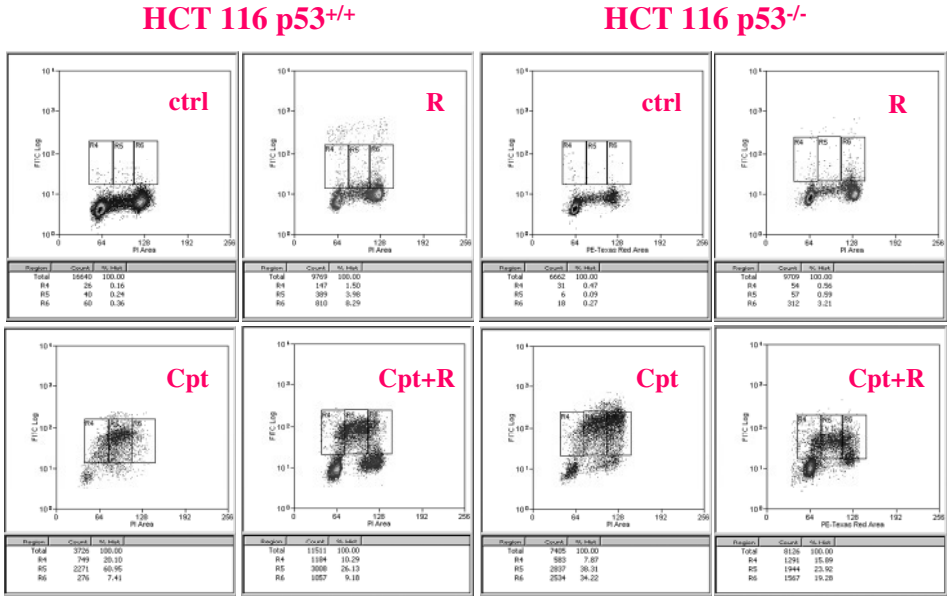


Fig. 12 Correlation of cell cycle distribution of cells with DNA damage (H2AX phosphorylation). It was performed a double cytofluometric analysis of cell cycle by PI assay and H2AX phosphorylation evaluation to correlate DNA damage with cell cycle phase and induction of apoptosis. Cells were immunostained with an anti-γ-H2AX monoclonal antibody followed by secondary fluorescein conjugate antibodies in the presence of Propidium Iodide and analyzed by flow cytometry. Dot plots report PI signal versus fluorescein fluorescence (FITC).

4.6 ATM/ATR substrates are phosphorylated in Camptothecin/ Roscovitine treated cells.

In order to establish if the chemo-protective effect of Roscovitine against Camptothecin was due to a more efficient activation of DNA damage sensors i.e. ATM and ATR proteins, the activity of these two proteins in the presence of Camptothecin, Roscovitine or both has been evaluated. ATM and ATR kinases preferentially phosphorylates the S/TQ motif as a consensus sequence in many of substrate proteins such as p53 NBS1, BRCA1, Chk1/Chk2, and SMC1 (Kim, S. 1999). Consequently it was used an antibody that detects endogenous levels of proteins containing the phosphorylated ATM/ATR substrate motif. The assay was conducted cytofluorimetrically assay using the indicated antibody and a secondary fluoresceinated antibody. Roscovitine alone wasn't able to efficiently activate ATM/ATR in our cells (Fig.13). The treatment with Camptothecin, instead, produced an increase in ATM/ATR activity that is underlined by the increase of phosphorylation of the consensus motif of the substrates proteins (Fig.13). This increment was more evident in cells treated simultaneously with the two drugs. This result obtained as in both cancer cell lines may indicate that Roscovitine is able to induce a more effective response to damage that Camptothecin caused to DNA while activating ATM/ATR dependent DNA repair pathways.

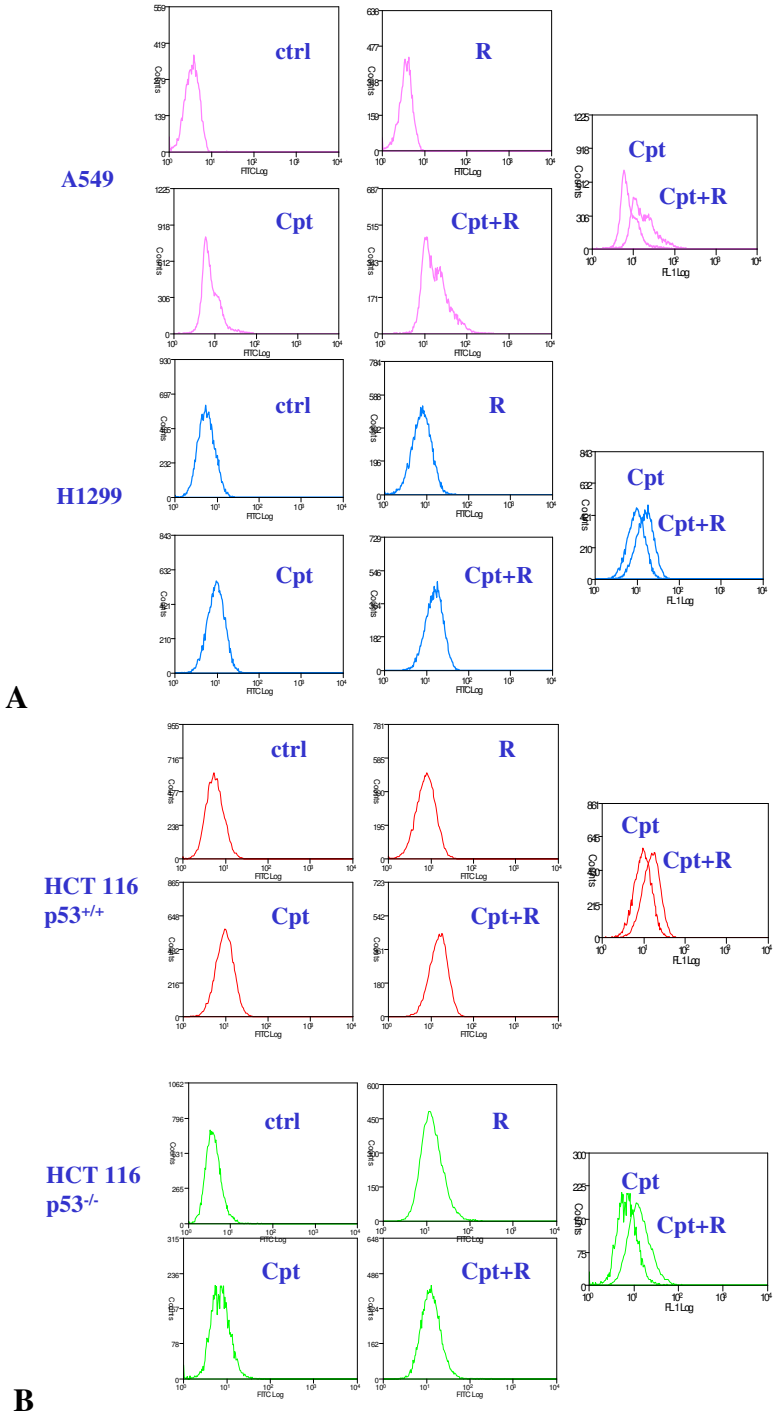


Fig. 13 A e B Effects of Roscovitine and Camptothecin on ATM/ATR substrates phosphorylation. A549, H1299 (panel A) and HCT116 (panel B) cells were incubated with Roscovitine or with Camptothecin in the presence or absence of Roscovitine for 48 hours. Cells were immunostained with an anti-S/TQ phosphorylated motif monoclonal antibody followed by secondary fluorescein conjugate antibodies and analyzed by flow cytometry. In single panel it is

showed the overlay of fluorescence peck of Camptothecin versus Camptothecin/ Roscovitine - treated cells.

4.7 Checkpoint kinases are involved in the response to Camptothecin

To further investigate the molecular response to single and combined treatments, checkpoint kinases were analyzed by Western Blot. These kinases act downstream in ATM/ATR pathway. ATM and ATR phosphorylate and activate Chk2 (Chehab et al., 1999; Caspari, 2000; Hirao et al., 2000) and Chk1 (Shieh et al., 2000), that, in turn directly phosphorylate serine 20 of p53. The stabilized p53 activates its transcriptional activity, leading to increased expression of p21 (Taylor and Stark, 2001; Kohn and Pommier,2005). The exposure to various DNA-damaging agents such as topoisomerase inhibitors rapid activates chk2, indicating that this kinase plays a role in cell cycle checkpoints. This has been proved directly though studies with dominant-negative Chk2, siRNA-mediated Chk2 ablation, or intrinsic cellular Chk2 deficiency which indicated the activation of the S and G2 checkpoints in response to double-strand breaks in various immortalized human cell types (Kwak 2006). The treatment with Camptothecin and Roscovitine induces the activation of ATM/ATR pathway and, in consequence, the phosphorylation of checkpoint kinases 1 and 2. In p53 positive cell lines (A549 and HCT116 wt) whichever of Camptothecin or Roscovitine are able to induce an increment in the basal level of total chk1 protein (Fig.14) but the two drugs display no evident effects on Chk2. In addition Chk1 is phosphorylated on Ser 345 following Camptothecin or Camptothecin /Roscovitine combined treatment. Indeed it is known that a significant fraction of Chk1 is phosphorylated following DNA damage induced by a variety of agents including UV light, ionizing radiation (IR), reduced DNA ligase activity, and Camptothecin too (Wan et al., 1999; Walworth and Bernards, 1996. Vertebrate Chk1 is phosphorylated by ATR, the ATM- and Rad3-related protein kinase (Zhao and Piwnica-Worms, 2001; Liu et al., 2000). This phosphorylation is essential for activation of the protein and consequently of S phase checkpoint. Activated chk1 induces prolonged cell cycle block to facilitate DNA repair. Apparently this effect is mediated by Cdc25A degradation (Lam and Rosen cell cycle 2004). Since its level after Camptothecin and Camptothecin/Roscovitine treated p53 positive cells result effectively downregulated (fig). In p53 negative cells (H1299 and HCT116 $p53^{-/-}$) only the combination of the two drugs is proficient to induce the upregulation and the phosphorylation of Chk1 kinase. In contrast Camptothecin alone induces the phosphorylation of Chk2 on Thr68. This site is recognized by ATM and the phosphorylation of the protein stimulates its activity. The activation of Chk2 kinase may cause activate a block of cell cycle in G2/M phase and promote cell death after DNA damage. This effect was clearly evident in p53 null cell lines, after Camptothecin. The G2/M block is reverted by concomitant use of Roscovitine that normalizes cell cycle distribution of cells and favours the recovery of G1 and S phases. In this case, probably there is an activation of intra S checkpoint that is Chk2 independent and Chk1 dependent.

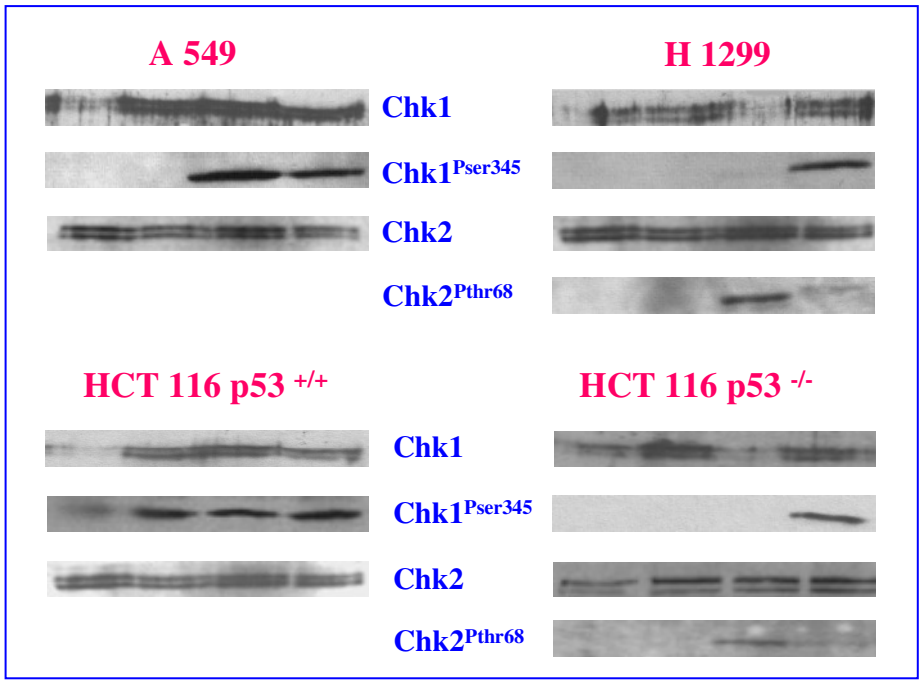
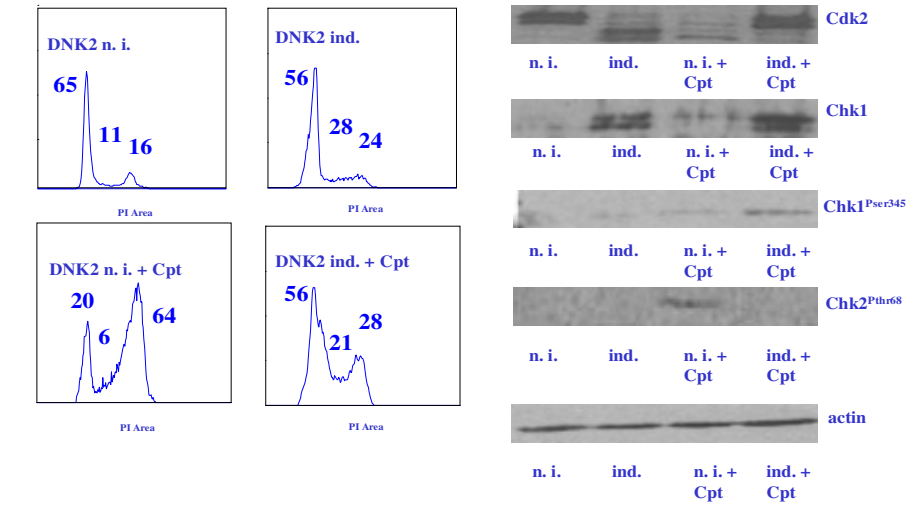


Fig. 14 Western blot analyses of Chk expression. Cells were incubated with Roscovitine (R), Camptothecin (Cpt), or both (Cpt+R) for 48 hours and protein expression was detected by Western blot.

4.8 Expression of dominant-negative cyclin-dependent kinase 2 increases Camptothecin-dependent G2 arrest and inhibits cell recovery after drug release.

Pleiotropic effects of Roscovitine have been reported in human tumor cell lines. Roscovitine has been shown to induce nucleolar fragmentation (David-Pfeuty 1999), p53 nuclear translocation, activation of mitogen-activated protein kinase pathway (Whittaker 2004), and inhibition of transcription (Hajduch 1999). Because Roscovitine protects H1299 cells against Camptothecin, we decided to further investigate the role of CDKs in chemoresistance. In fact the drug effect might be due or to the direct inhibition of CDK2 or to another secondary effect. We used two tetracycline-inducible clones of H1299 cells expressing a mutant dominant negative form of CDK2 (DNK2) (Hu 2001). These clones have already been isolated in our laboratory and selected for the expression of low level of DNK2 to mimic the effects of low concentrations of Roscovitine. Accordingly, induction of DNK2 only slightly affected the cell cycle distribution of asynchronously growing H1299 cells (Fig.15 A e B). Expression of DNK2 reverses the G2 arrest imposed by Camptothecin in both clones (Fig.15 A e B). This effect appears very similar to that obtained upon Roscovitine/Camptothecin treatment that results in a normalization of cell cycle distribution profile if compared to Camptothecin treatment. More importantly, overexpression of DNK2 results in the induction of expression of Chk1 protein and in its phosphorylation by ATR at the consensus site (ser 345) as already described in Roscovitine /

Camptothecin treated H1299 cells. The effect of Camptothecin alone on checkpoint kinases in not induced clones is identical to that observed in H1299. In fact, Chk1 expression is not affected and Chk2 results phosphorylated at ATM consensus site (Thr 68) as already described. These data indicate that the chemosensitizing effect of Roscovitine, observed in H1299 cells, is mediated via CDK inhibition.



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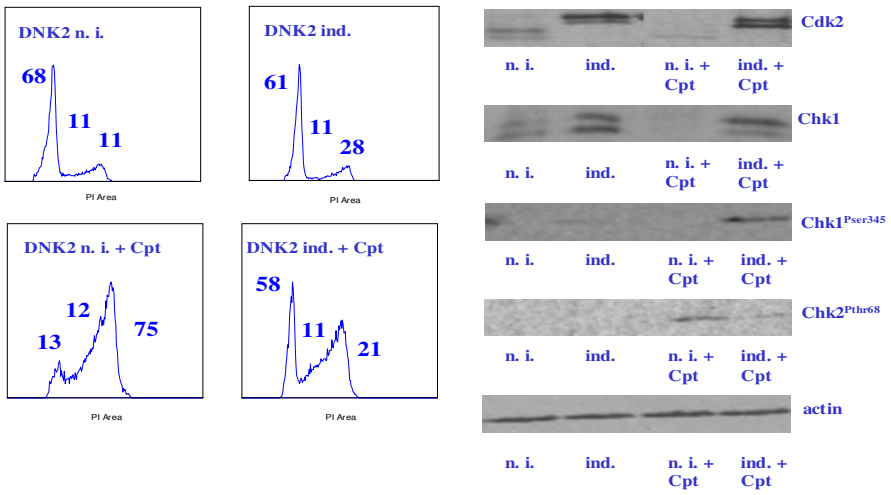

B

Fig. 15 Effect of dn-K2 on cell cycle and proteins expression. Two different clones of H1299 cells expressing a dominant negative form of CDK2 were pretreated (inducted) (i) or not (not inducted) (n.i.) with doxycycline for 24 hours and subsequently incubated with Camptothecin for 48 hours. Cytometric and Western Blot analyses were performed. Filters were probed with anti- α-actin antibody as loading control.

4.9 Roscovitine reduces tritiated thymidine [^{3}H]TTP incorporation of Camptothecin treated cells

We investigated the effect of Camptothecin and Roscovitine on tritiated thymidine ([^{3}H]TTP) incorporation. This is a sensitive marker of S phase entrance and proliferation rate. After 4 hours of incubation with the two drugs, alone or in combination, the incorporation of labelled nucleotide was significantly reduced in all cell lines if compared with treated control (Fig 15). This is in accord with a presumable effect of block of cell cycle in response to DNA damage. It's notable, however, that in Camptothecin treated cells the percentage of incorporation of thymidine was higher than in those treated with the combination Camptothecin/Roscovitine. This result is evident as in p53 positive cell lines as in p53 negative cell lines. The difference in percentage of incorporation between single and combined treatment appears statistically relevant after 4 hours of incubation with drugs and it's sustained up to 48hours although becoming progressively smaller. The entrance of Campothecin treated cells in S phase presumably requires the replication of a partially damaged DNA. The original single strand damages induced by Camptothecin cause the appearance of double strand breaks so that cells proceed to G2 phase carrying some damaged DNA. This presence induces the activation of G2/M checkpoint. The cell cycle arrest promotes the activation of DNA repair or, if not possible, the apoptotic response. The simultaneously presence of Roscovitine causes, on the contrary, the activation of G1 checkpoint and a reduction rate of S phase entrance (as demonstrated by lower tritiated thymidine incorporation). In addition it allows more efficient repair of DNA damage before replication. This favours the survival of cells to Camptothecin treatment and, as long term effect, enhances the capacity to form colonies as compared to cells treated with Camptothecin alone.

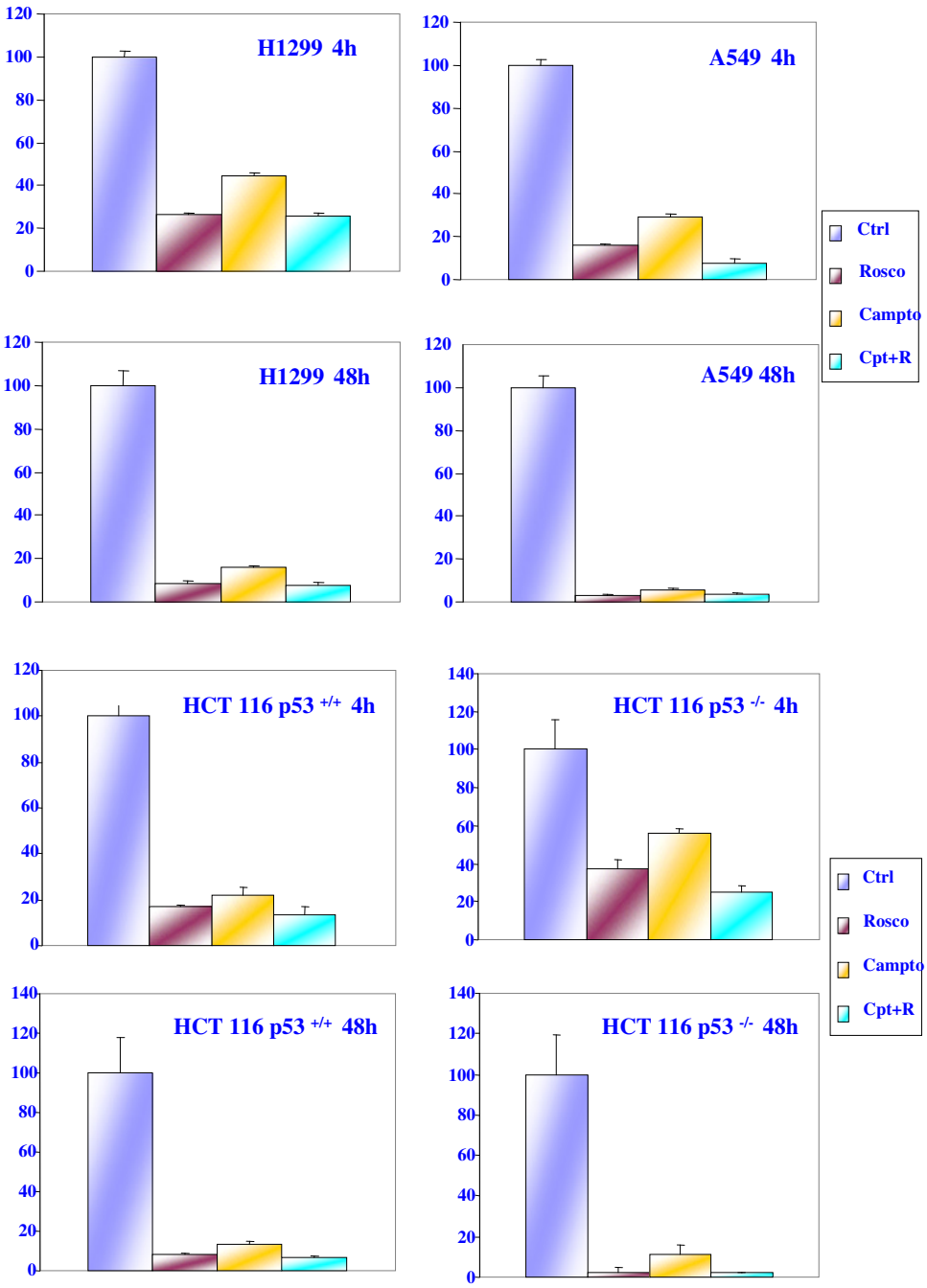

Fig. 16 Effects of single and combined treatment on Thymidine Incorporation. Cells were incubated complete medium (ctrl) or medium supplement with Camptothecin (0.5 µM), Roscovitine (10µM), or both. All samples were run in triplicate. After 4 h or 48h incubation, each the medium was replaced with fresh culture medium containing labelled [^{3}H]thymidine and the incorporation was measured after 1hours. Data are expressed as percentages of controls.

4.10 Camptothecin increases the amount of proliferating cell nuclear antigen (PCNA) loaded onto DNA in a p53 dependent way.

The observation of the ability of Roscovitine to reduce the entrance in S phase prompted us to analyze the effects induced on DNA replication proteins and, in particular, to measure the level of chromatin bound PCNA after Roscovitine, Camptothecin or combined treatment. Such analysis has been performed only in HCT116 cells because these are a good system to investigate the role of p53 protein in the molecular response to Camptothecin and Roscovitine. Cell extracts were separated in two fractions i.e. detergent soluble and chromatin-bound fractions, and analysed by Western blot for the intracellular distribution of PCNA after 48 hours of incubation with the two drugs. It appeared that the treatment with Camptothecin induces a remarkable increase in the amount of PCNA chromatin bound in p53 positive HCT116 cells. This effect in turn was undetectable in p53 negative subclone. The simultaneous treatment with Roscovitine reported the level of chromatin bound PCNA to that of control. No appreciable variations of the levels of PCNA in the different intracellular fractions in HCT116 $p53^{-/-}$ cells were detectable independently of the way they were treated. The major association of PCNA with DNA was then confirmed by immufluorescence assays. HCT116 p53 positive cells incubated for 48h with Camptothecin showed a more intense fluorescent signal of PCNA antibody in correspondence of chromatin clusters (Fig.16).

From the results described above, it seems that, in our system, p53 has a role in regulating PCNA bound to chromatin and this is mediated, probably, by p21. In fact recent works indicate that p21 is recruited with PCNA in the sites of DNA damage (Perucca 2006).The recruitment of PCNA on chromatin is counteracted by Roscovitine. It was suggested by Savio et al (2006) who demonstrated that the treatment with Roscovitine induces a disassembly of PCNA/chromatin complexes. Indeed, PCNA in the cell is present in multiple pools and is located replication sites alone or in complex with other protein such as CDK2 (Prosperi 2006), p21 and others. Thus, it is possible that overall levels of chromatin- bound PCNA were reduced in comparison with Camptothecin because Roscovitine inhibited new origin firing and induced disassembly of PCNA present at damaged sites (e.g., histone γ-H2AX foci). In addition the number of these sites is reduced as indicated by histone γ-H2AX cytofluorimetric assay (section 4.5).

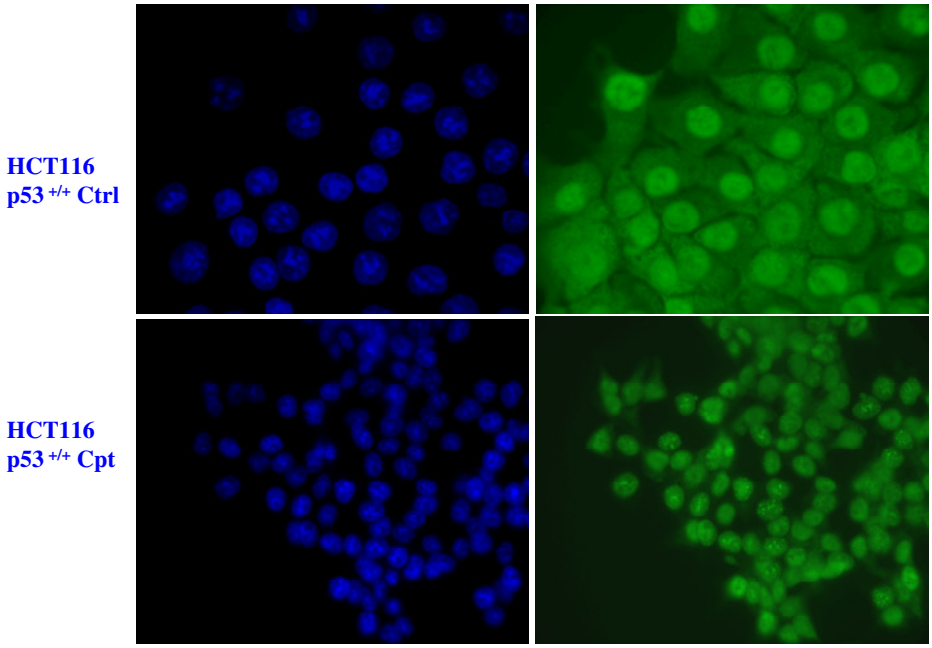

Fig. 17 Intracellular distribution of PCNA in HCT116 p53+/+ Camptothecin treated. Cells were immunostained with an anti-g-H2AX monoclonal antibody followed by secondary fluorescein conjugate antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). HCT116 cells were incubated with Camptothecin or vehicle (DMSO) for 48 hours and analyzed.

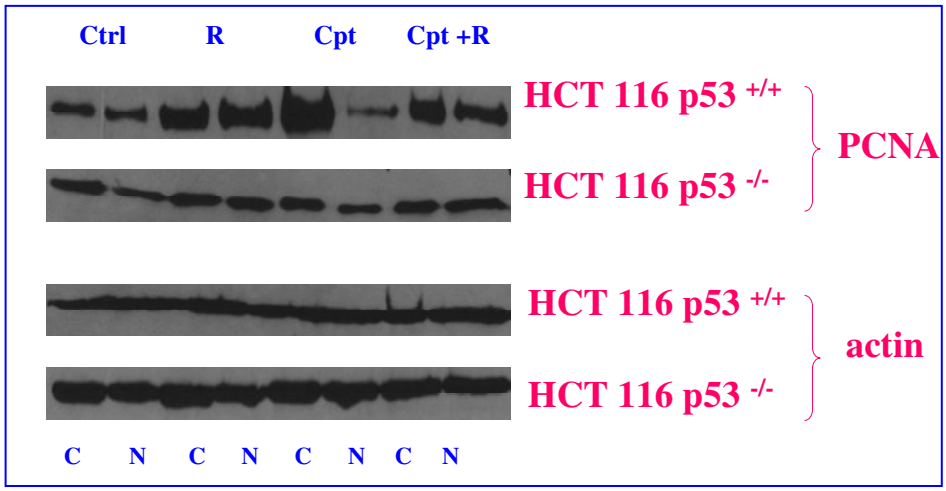

Fig. 18 Cytosolic and nuclear localization of PCNA after Camptothecin or Camptotheci/Roscovitine combined treatment. HCT116 cells were incubated for 48 hours in the presence of Camptothecin, Roscovitine or both. Different subcellular fractions: Cytosolic not chromatin bound (C) or Nuclear chromatin bound (N) were collected and analyzed. Levels of actin were used to normalized the quantity of proteins present in fractions.

4.11 Camptothecin induces Cdc6 p53 regulated degradation.

To further clarify the molecular mechanisms involved in cellular response to Camptothecin and Roscovitine, we decided to investigate the effect of drugs on Cdc6 levels (nuclear as in the cytosolic) form. To this purpose we evaluated the presence of the protein in the different intracellular fractions in p53 $^{+/+}$ and p53$^{-/-}$ HCT116 cells following single and combined treatments

Cdc6 is recruited by the origin recognition complex during G1, where it serves as a loading factor for the minichromosome maintenance (MCM) complex. This pre-replication complex (pre-RC) renders the genome competent for replication, that may occur after activation of the complex by cyclin-dependent kinases (CDKs) at the onset of S phase. It was already demonstrated that, in response to genotoxic stress, the ATM mediated activation of p53 results in the upregulation of p21. Duursma et al. demonstrated that the accumulation of this CDK inhibitor is necessary for Cdc6 degradation which is strictly p53 dependent. We found that Camptothecin treatment leads to a downregulation of Cdc6 in nuclear and cytosolic fractions analyzed but only in p53 positive line while no differences were detectable in HCT p53$^{-/-}$ (Fig. 19).

The decrease in Cdc6 levels has been strictly related to the induction of apoptotic processes. In fact Feng et al in 2003 demonstrated that the inhibition of the expression of DNA replication-initiation proteins such as Cdc6, induces apoptosis in human cancer cells. It has also been demonstrated that Cdc6 is also directly cleaved by caspase3 in ATM/ATR mediated apoptosis (Yim 2006).

The combined treatment with Roscovitine abrogates this degradation preventing apoptosis. It must be noted that in the presence of Roscovitine there is an increase in cytoplasmic, albeit not functional form of Cdc6. The traslocation of Cdc6 generally takes place at the onset of S phase (Saha 1998) but, in our conditions it is mediated by Roscovitine to probably prevent damaged DNA replication. These data are in accord to our hypothesis which consider Roscovitine as a factor favouring DNA repair and recovery of cells that, otherwise, are committed to apoptosis or senescence.

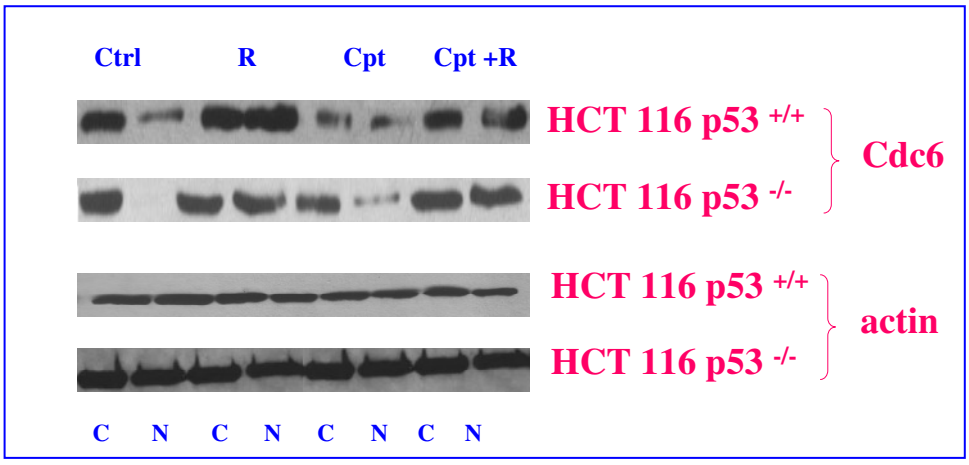

Fig.19 Cytosolic and nuclear localization of Cdcc6 after Camptothecin or Camptothecin/Roscovitine combined treatment. HCT116 cells were incubated for 48 hours in the presence of Camptothecin, Roscovitine or both. Different subcellular fractions: Cytosolic not chromatin bound (C) or Nuclear chromatin bound (N) were collected and analyzed. Levels of actin were used to normalized the quantity of proteins present in fractions.

5. CONCLUSIONS

Resistance to chemotherapeutic drugs is the most frequent reason for the failure of cancer treatments. In this reason new strategies have been developed in clinical practice including the use of "combined" regimens of therapy. The response of cancer cell lines to the treatment with new compounds or their combination may give useful indications for the effective advantage for their use *in vivo*. In this work we tried to better understand molecular and cellular mechanisms activated by tumour cells when treated with the combination of two chemotherapeutic drugs i.e. Camptothecin (Topoisomerase I inhibitor) and Roscovitine (CDK inhibitor). We showed that this particular combination favours cellular surviving compared with the single treatment with Camptothecin alone. This effect was p53 independent in fact, although we have used four different tumour cell lines having different p53 status they behaved similarly upon the various treatments. This was shown by mean of several approaches including cell cycle progression analysis, protein expression evaluation, DNA damage response, replicative senescence pathway and cellular proliferation rates. All experiments suggested that Roscovitine, by inhibiting CDK and, in particular Cdk2, protects cells from Camptothecin induced damage. When in combination Roscovitine, in fact, favours a response of DNA repair rather than apoptosis or senescence pathways activation. This effect is due to its capacity to induce 1) an earlier activation of cell cycle checkpoints with an increased activity of the damage sensor kinases ATM and ATR, 2) a reduction of chemical induced replicative senescence 3) a decrease in the amount of DSBs. Roscovitine, in addition**,** reduced thymidine incorporation in Camptothecin treated cells and the amount of chromatin bound PCNA. These facts prevent the entrance and progression of cells in S phase in the presence of Camptothecin-damaged DNA.

It's also clear that p53 plays an important role because its activity is regulated indirectly by Camptothecin and Roscovitine. In the presence of the first compound p53 is phoshorylated in Ser15 resulting immediately activated. The activation causes several effects including p21 is upregulation, cdc6 degradation and, at longer times, triggering of apoptosis. In the presence of Roscovitine p53 does not result active so there are no further consequences; cells do not undergo apoptotic processes, the DNA repair is favoured and there is a rapid recovery from damage .

Our results in whole raise an important question because suggest possible dangerous effects deriving from this particular combination of drugs in human therapy.

6. ACKNOWLEDGEMENTS

At the end of these four year I want to thank all persons who helped me during the period of my PhD program.

First of all I thank Prof. *Giuseppe Palumbo* for giving me the opportunity to work in his laboratory welcoming in his group from the first moments.

I want to thank *Prof Hugh J.M. Brady* for the scientific and economical support to my PhD project and for giving me the possibility to spend a short period in his laboratory in London.

I'm thankful to *Prof. Giancarlo Vecchio*, co-ordinator of the International Doctorate in Molecular Oncology and Endocrinology for to let me attend this Doctorate program under his supervision.

I would like to thank *Dr. Elvira Crescenzi* who daily followed my scientific work and taught me how to accept critics using them for becoming better.

I'm very thankful to my colleague and dear friend *Dr. Angela Chiaviello* for being near me in every moments listening and understanding me without judging.

A special thank to *Dr Rosa Russo* for making me understand that even when you teach something to someone you will never end to learn.

A thank to all my colleagues of the Department of Biologia e Patologia Cellulare e Molecolare in Naples and in particular *Dr Ilaria Postiglione* for her "noisy" presence in laboratory very important for a good state of humour and *Dr Annalisa Morano* for the "scientific" (more or less…) conversations under the flow boxes.

Finally I want to spend more than a word for my family who helped me in any possible way.

A big thank to *my sister Giorgia* who represents my family here in Naples and is always capable to understand and help her "bigger" (only in age, I think) sister. Thanks to *my sister Sara* for the long encouraging phone calls, to *my mother and my father* for their constant and precious presence in my life.

Last but not least a special thought to all of my friends in Naples, too many to thank one by one but too important to not thank.

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8. ORIGINAL PAPERS

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 5, pp. 2631–2638, February 3, 2006

Central Role of the Scaffold Protein Tumor Necrosis Factor Receptor-associated Factor 2 in Regulating Endoplasmic Reticulum Stress-induced Apoptosis*[S]

Received for publication, February 25, 2005, and in revised form, October 17, 2005 Published, JBC Papers in Press, November 18, 2005, DOI 10.1074/jbc.M502181200

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The endoplasmic reticulum represents the quality control site of the cell for folding and assembly of cargo proteins. A variety of conditions can alter the ability of the endoplasmic reticulum (ER) to properly fold proteins, thus resulting in ER stress. Cells respond to ER stress by activating different signal transduction pathways leading to increased transcription of chaperone genes, decreased protein synthesis, and eventually to apoptosis. In the present paper we analyzed the role that the adaptor protein tumor necrosis factor-receptor associated factor 2 (TRAF2) plays in regulating cellular responses to apoptotic stimuli from the endoplasmic reticulum. Mouse embryonic fibroblasts derived from TRAF2$^{-/-}$ mice were more susceptible to apoptosis induced by ER stress than the wild type counterpart. This increased susceptibility to ER stress-induced apoptosis was because of an increased accumulation of reactive oxygen species following ER stress, and was abolished by the use of antioxidant. In addition, we demonstrated that the NF-κB pathway protects cells from ER stress-induced apoptosis, controlling ROS accumulation. Our results underscore the involvement of TRAF2 in regulating ER stress responses and the role of NF-κB in protecting cells from ER stress-induced apoptosis.

In eukaryotic cells, proteins must be correctly folded and assembled before to transit to intracellular organelles and the cell surface (1, 2). A number of cellular stress conditions can interfere with protein folding, leading to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER)[3] lumen. The ER has evolved specific signaling pathway to deal with the potential danger represented by the misfolded proteins. This adaptive response is named unfolded protein response (3). Activation of unfolded protein response results in attenuation of protein synthesis, and up-regulation of genes encoding chaperones that facilitate the protein folding process in the ER. Thus, unfolded protein response reduces accumulation and aggregation of malfolded proteins, giving the cell the possibility of correcting the environment inside the ER (3, 4). However, if the damage is too strong and homeostasis cannot be restored, the mammalian unfolded protein response initiates apoptosis. In mammalian cells, three transmembrane proteins Ire1α (5), Ire1β (6), and PERK (7) act as ER stress sensor proteins and play important roles in transducing the stress signals initiated by the accumulation of malfolded proteins from the ER to the cytoplasm and nucleus. Ire1s and PERK are kept in an inactive state through association of their N-terminal lumen domain with the chaperone BiP. Following accumulation of malfolded proteins in the lumen of the ER, BiP dissociates to bind the malfolded proteins and Ire1s and PERK undergo oligomerization and transphosphorylation within their cytoplasmic kinase domains (8, 9).

Other stress response pathways are activated following ER stress, such as the JNK/SAPK and NF-κB pathways (10, 11). Activation of these pathways following ER stress is mediated by the physical and functional interaction of Ire1α and TRAF2 (10). The central role played by TRAF2 in mediating cellular response to ER stress has been proposed based upon the observation that ectopic expression of a dominant negative mutant of TRAF2 lacking the N terminus Ring finger domain, blocks ER stress-induced NF-κB and JNK/SAPK activation, and that mouse embryonic fibroblast derived from TRAF2 knock-out mice failed to activate NF-κB following ER stress (10, 11). TRAF2 was initially identified as a TNF receptor 2 interacting protein (13). Interestingly, TRAF2-deficient MEFs are very sensitive to cell death induced by TNF and other members of the TNF receptor family (14, 15). At least part of the anti-apoptotic effect of TRAF2 can be explained by its function as a mediator of NF-κB activation, thus leading to NF-κB-dependent expression of anti-apoptotic genes. The anti-apoptotic activity of NF-κB also involves inhibition of the JNK cascade via at least two distinct mechanisms: through GADD45-β-mediated blockade of MKK7 and interference with ROS production (16, 17). It is well known that ROS or oxidative stress plays an important role in various physiological and pathological processes such as aging, inflammation, and neurodegenerative diseases (18–20). Recently, it has been demonstrated that accumulation of misfolded protein within the lumen of the ER causes accumulation of ROS and cell death (21). However, it is currently unknown whether some of the key molecules involved in ER stress response, such as TRAF2, are involved in modulation of ROS and induction of apoptosis. Here we use MEFs derived from TRAF2 knock-out mice to study the role of TRAF2

* This work was supported by grants from the Associazione Italiana Ricerca sul Cancro, MIUR-FIRB RBNE0155LB, and Centro di Competenza GEAR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. A and B.

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[3] The abbreviations used are: ER, endoplasmic reticulum; NF-κB, nuclear factor-κB; MEF, mouse embryonic fibroblast; TRAF, tumor necrosis factor receptor-associated factor; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; WT, wild type; H_2DCFDA, dichlorodihydrofluorescein diacetate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; FL, full-length; NAC, *N*-acetyl-L-cysteine.

in the regulation of pro-survival or pro-apoptotic pathways following ER stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—Wild type (WT) and TRAF2$^{-/-}$ murine embryonic fibroblasts (MEFs) were provided by Drs. T. W. Mak and W. C. Yeh (14). WT and JNK1/2$^{-/-}$ and WT and p65$^{-/-}$ MEFs were provided by Dr. R. Davis and Dr. G. Franzoso, respectively (22, 23). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Thapsigargin was from Calbiochem and used at 5–50 nM; tunicamycin was purchased from Roche and used at 50–150 ng/ml. Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Calbiochem) was dissolved in Me_2SO and used at 5 μM; L-NAC was dissolved in sterile water and used at 5 mM. Anti-TRAF2, anti-IκBα, and anti-JNK antibodies were purchased from Santa Cruz Biotechnology. The TRAF2 full-length expression vector was previously described (24).

Western Blot Analysis—Subconfluent monolayer of murine embryonic fibroblasts were washed with phosphate-buffered saline and then lysed in a lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, supplemented with a mixture of protease inhibitors (Roche). Equal amounts of total proteins (50 μg) were resolved by SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 4 °C for Western blot analysis. Filters were blocked for 1 h at room temperature with 10% nonfat dry milk in TBS-T buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20). Then, filters were probed with specific antibodies in the same buffer for 14–16 h at 4 °C. After TBS-T washing to remove excess primary antibodies, the blots were incubated in horseradish peroxidase-coupled secondary antibody for 1 h followed by enhanced chemiluminescence detection of the proteins with Hyper-film ECL detection (Amersham Biosciences).

Luciferase Assay—For luciferase assay, WT, TRAF2$^{-/-}$, and TRAF2FL MEFs (4×10^5 cells per well) were seeded in 6-well (35 mm) plates. After 12 h cells were transfected with 0.5 μg of Ig-κB-LUC reporter gene plasmid using Lipofectamine. Cells were stimulated with thapsigargin or tunicamycin for 8 h, and reporter gene activity was determined by the luciferase assay system (Promega). A pRSV-β-galactosidase vector (0.2 μg) was used to normalize for transfection efficiencies.

Retroviral Infection—Full-length hemagglutinin-tagged TRAF2 was subcloned into the retroviral expression vector pBMN by standard cloning techniques. pBMN vector was then transfected in a packaging cell line using Lipofectamine. 48 h after transfection, the viral supernatants were supplemented with Polybrene (9 mg/ml) and filtered through a 0.45-mm filter. TRAF2$^{-/-}$ fibroblasts (1×10^6) were incubated with viral supernatants for 48 h. The expression of exogenous protein was assayed by Western blot analysis on total cell extracts using anti-TRAF2 antibodies.

ER Stress Induction and Measurements of Apoptosis—5×10^3 cells/well were seeded in 96-well culture plates and incubated for 24 or 48 h at 37 °C with different concentrations of thapsigargin or tunicamycin. Cell survival was examined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and an electron coupling reagent (phenazine methosulfate), according to the manufacturer's instructions (Promega). Cell death was assessed by staining the exposed phosphatidylserine on cell membranes with fluorescein isothiocyanate-conjugated annexin V (BD Pharmingen), or propidium iodide staining according to Nicoletti *et al.* (25). Samples were analyzed by flow cytometry using a FACScalibur (Beckman Coulter, Fullerton, CA), equipped with ModFit Software. Results were mean ± S.D. of at least three separate experiments.

Measurement of ROS Production—Reactive oxygen species were detected with H_2DCFDA (Calbiochem). H_2DCFDA diffuses into the cells where it is converted into a non-fluorescent derivative (H_2DCF) by endogenous esterases. H_2DCF is oxidized to green fluorescent DCF in the presence of intracellular ROS. Cells were routinely treated with either tunicamycin or thapsigargin for 24 or 48 h, washed, and incubated at 37 °C for 30 min in the presence of H_2DCFDA in serum-free medium. Me_2SO-treated cells were used as controls. After incubation, cells were washed twice with phosphate-buffered saline, resuspended in phosphate-buffered saline, and analyzed by flow cytometry using a FACScan Cell Scanner (BD Biosciences).

Kinase Assay—JNK immunoprecipitates were used for the immune complex kinase assay that was performed at 30 °C for 10 min with 2 μg of substrate, 10 μCi of [γ-^{32}P]ATP in a total of 20 μl of kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 μM Na_3VO_4, and 50 μM dithiothreitol). The substrate was glutathione *S*-transferase-c-Jun (amino acids 1–79). The reaction was terminated by boiling in SDS sample buffer, and the products were resolved by 12% SDS-PAGE. Phosphorylated proteins were detected by autoradiography.

RESULTS

Increased Susceptibility of TRAF2$^{-/-}$ MEFs to ER Stress-induced Apoptosis—TRAF2 is a scaffold protein that transduces signals from membrane receptors and the ER membrane (10–12). To assess the role of TRAF2 in apoptosis induced by ER stress, we treated MEFs derived from TRAF2$^{-/-}$ mice and WT MEFs with increasing concentrations of thapsigargin and tunicamycin. Both drugs induce ER stress by inhibiting ER-resident Ca^{2+}-ATPase, and *N*-glycosylation, respectively. After a 48-h treatment, some morphological changes were observed. In particular, WT MEFs showed an extended shape, typical of cellular stress response, whereas TRAF2$^{-/-}$ MEFs appeared detached and shrunken (Fig. 1*A*). Because these morphological changes were reminiscent of apoptosis, we performed annexin V staining on WT and TRAF2$^{-/-}$ MEFs. As shown in Fig. 1*B*, treatment with thapsigargin or tunicamycin caused a dramatic increase in apoptosis in TRAF2$^{-/-}$ MEFs but not in WT MEFs. The higher sensitivity to apoptosis observed in TRAF2$^{-/-}$ MEFs was not because of an intrinsic defect of these cells, given that reintroduction of TRAF2 (TRAF2FL) completely rescued cell viability (Fig. 1, *C–E*). TRAF2$^{-/-}$ MEFs showed the same susceptibility as WT MEFs to serum starvation- and doxorubicin-induced cell death (Fig. 1*F*). These results suggest a specific role for TRAF2 in modulating survival signals from the ER.

ROS Mediate Increased Apoptosis in TRAF2$^{-/-}$ MEFs—ER stress has recently been shown to promote oxidative stress and apoptosis (21). Hence, to have some insight on the molecular mechanism determining the increased susceptibility to ER stress-induced apoptosis, we compared ROS production in WT and TRAF2$^{-/-}$ MEFs. As shown in Fig. 2, treatment with thapsigargin or tunicamycin caused an increase in ROS production in TRAF2$^{-/-}$ MEFs but not in WT. Reconstitution of these cells with TRAF2 (TRAF2FL) blocked ROS accumulation following treatment with thapsigargin and tunicamycin (Fig. 2, *A* and *B*). To investigate whether the increased production of ROS was responsible for the susceptibility of TRAF2$^{-/-}$ MEFs to ER stress-induced apoptosis, TRAF2$^{-/-}$ MEFs were treated with thapsigargin or tunicamycin in the presence of different antioxidants and 48 h later cell viability was measured by MTS assay and the ROS level by flow cytometry. As shown in Fig. 3, NAC abolished ROS accumulation and protected these cells from

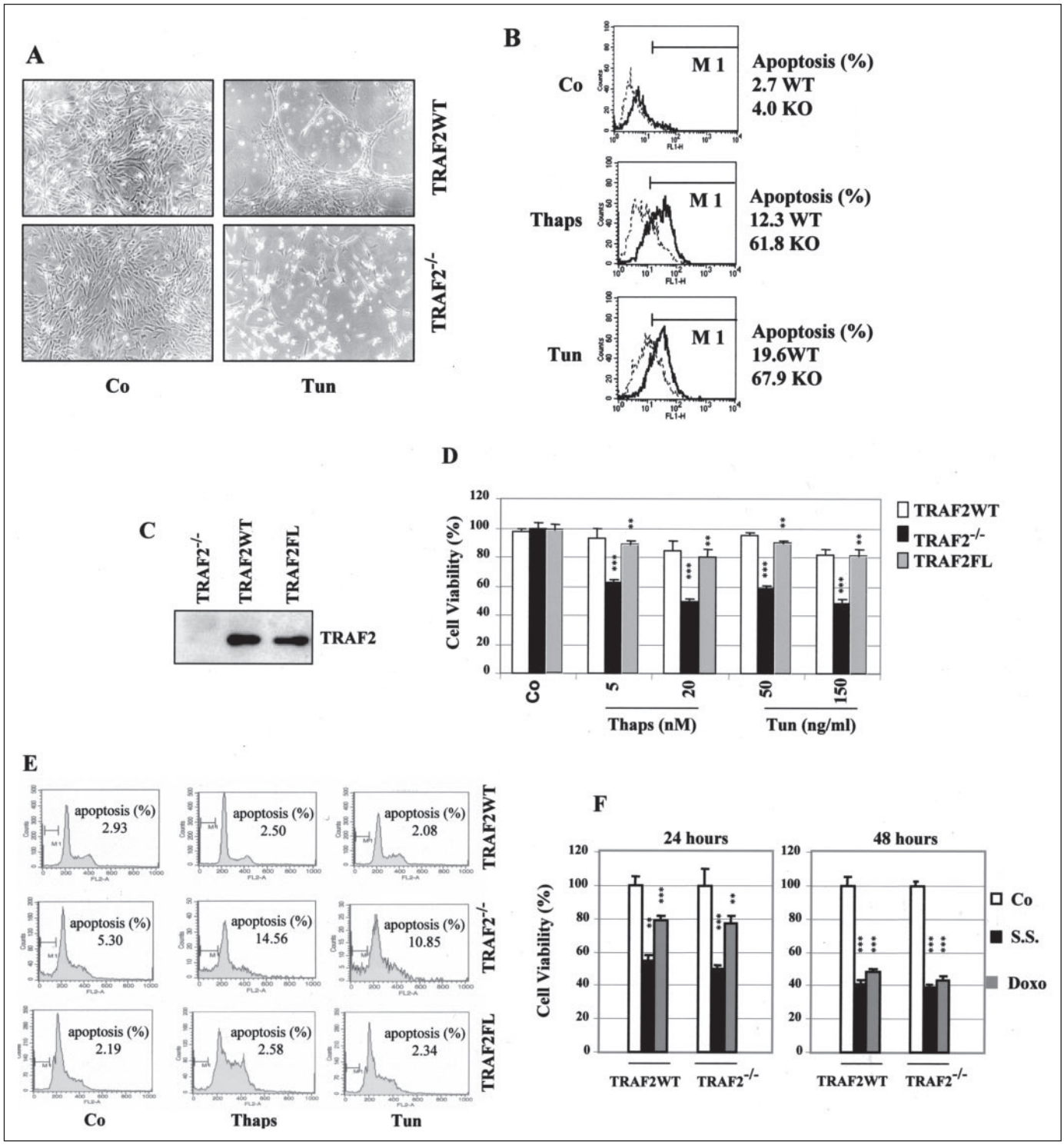
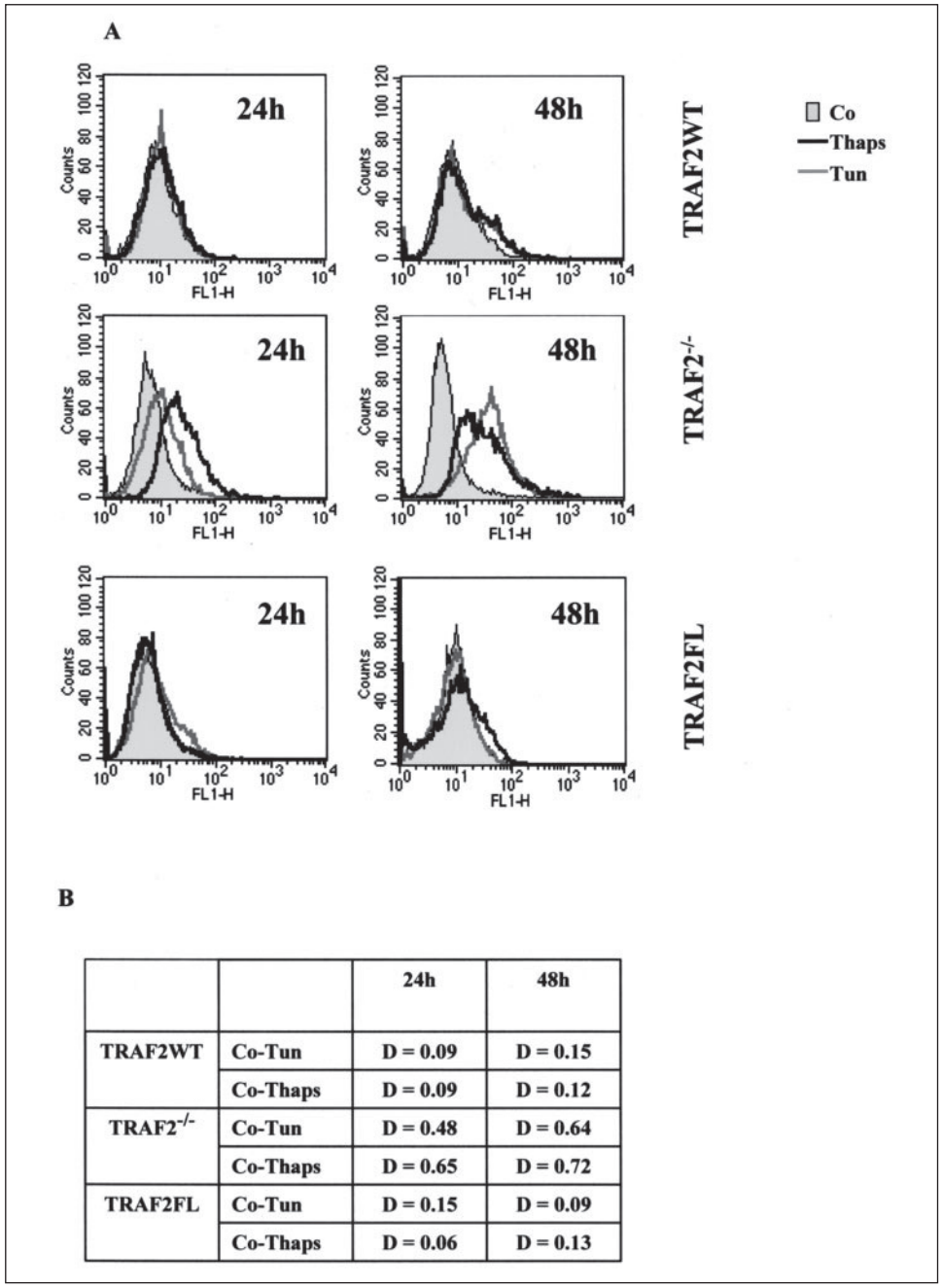


FIGURE 1. **ER stress causes apoptosis in TRAF2$^{-/-}$ MEFs.** *A*, WT and TRAF2$^{-/-}$ MEFs were treated with 100 ng/ml tunicamycin or vehicle for 48 h. Cell death was examined by morphological changes under a phase-contrast microscope. *B*, WT and TRAF2$^{-/-}$ MEFs were treated with 5 nM thapsigargin or 50 ng/ml tunicamycin for 48 h. Apoptosis was assessed by flow cytometry after staining with fluorescein isothiocyanate-conjugated annexin V. Percentage of the apoptotic cell is indicated. *C*, restoration of TRAF2 protein expression. TRAF2$^{-/-}$ MEFs were infected with an expression vector encoding full-length TRAF2. Expression of the TRAF2 protein was assessed by Western blot in WT, TRAF2$^{-/-}$, and TRAF2-reconstituted cells (TRAF2FL). *D*, restoration of TRAF2 protein expression rescues TRAF2$^{-/-}$ cells from ER stress-induced apoptosis. WT, TRAF2$^{-/-}$, and TRAF2FL MEFs were treated with Me_2SO (*Co*), tunicamycin (*Tun*), or thapsigargin (*Thaps*) for 48 h. Cell viability was assessed by MTS assay. Data are mean ± S.D. from five independent experiments. Statistical analysis was by unpaired Student's *t* test: **, $p < 0.002$; ***, $p < 0.0001$. *E*, restoration of TRAF2 protein expression rescues TRAF2$^{-/-}$ cells from endoplasmic reticulum stress-induced apoptosis. TRAF2$^{-/-}$, WT, and TRAF2FL MEFs were treated with Me_2SO (*Co*), 20 nM thapsigargin, or 150 ng/ml tunicamycin for 48 h and analyzed by flow cytometry. Percentage of sub-G_o cells is indicated. *F*, TRAF2$^{-/-}$ and WT MEFs were serum starved for 24 and 48 h, or treated with 0.2 μM doxorubicin for 24 and 48 h, and cell viability was assessed by MTS assay. Data are mean ± S.D. from three independent experiments. Statistical analysis was by the unpaired Student's *t* test: **, $p < 0.002$; ***, $p < 0.0001$. *KO*, knock-out.



B

		24h	48h
TRAF2WT	Co-Tun	D = 0.09	D = 0.15
	Co-Thaps	D = 0.09	D = 0.12
TRAF2$^{-/-}$	Co-Tun	D = 0.48	D = 0.64
	Co-Thaps	D = 0.65	D = 0.72
TRAF2FL	Co-Tun	D = 0.15	D = 0.09
	Co-Thaps	D = 0.06	D = 0.13

FIGURE 2. **Susceptibility of TRAF2$^{-/-}$ MEFs to endoplasmic reticulum-dependent oxidative stress.** *A*, WT, TRAF2$^{-/-}$, and TRAF2FL MEFs were treated with Me_2SO (*Co*), 20 nM thapsigargin (*Thaps*), or 150 ng/ml tunicamycin (*Tun*) for 24 or 48 h. Cells were labeled with 5 μM H_2DCFDA and analyzed by flow cytometry. *B*, Kolmogorov-Smirnov statistical analysis of flow cytometric data were used according to Cell Quest Software (BD Biosciences Immunocytometry Systems). *D* values by Kolmogorov-Smirnov analysis ($p \leq 0.001$) are shown.

apoptosis. Similar results were obtained by using dithiothreitol as antioxidant (data not shown). Interestingly, also the small percentage of WT MEFs and reconstituted TRAF2$^{-/-}$ MEFs undergoing apoptosis following treatment with tunicamycin and thapsigargin were almost completely protected by both antioxidants (Fig. 3 and data not shown). These results demonstrated that susceptibility of TRAF2$^{-/-}$ MEFs to ER stress-induced apoptosis was because of increased accumulation of ROS. It is worth noting that in TRAF2$^{-/-}$ cells, higher levels of ROS and apoptosis were detected, even in the absence of ER stressing agents (Fig. 1, *B* and *E*, and data not shown).

TRAF2-mediated NF-κB Activation Protects Cells from ER Stress-induced Apoptosis—Given the central role played by TRAF2 to correctly signal activation of NF-κB and JNK from ER, we investigated which of these pathways control ROS accumulation and protect cells from ER stress-induced apoptosis. MEFs derived from p65 knock-out and JNK1/2 double knock-out mice were treated with thapsigargin or tunicamycin in the presence or absence of NAC. As shown in Fig. 4*A*, p65$^{-/-}$ MEFs showed very high levels of ROS following treatment with thapsigargin and tunicamycin. As expected, treatment with NAC decreased ROS accumulation by about 40%. In contrast, JNK1/2$^{-/-}$ MEFs showed an accumulation of ROS similar to WT MEFs (Fig. 4*A*). Statistical analysis is reported in Fig. 4*B*. We next investigated the susceptibility of p65$^{-/-}$ and JNK1/2$^{-/-}$ MEFs to apoptosis induced by thapsigargin or tunicamycin in the presence or absence of NAC. As shown in Fig. 4*C*, p65$^{-/-}$ MEFs were highly susceptible to apoptosis compared with WT MEF and treatment with NAC significantly increased cell viability. JNK1/2$^{-/-}$ MEFs did not show susceptibility to ER stress-induced cell death, as compared with WT MEFs. These results suggest that NF-κB protects cells from ER stress-induced apoptosis by controlling ROS accumulation.

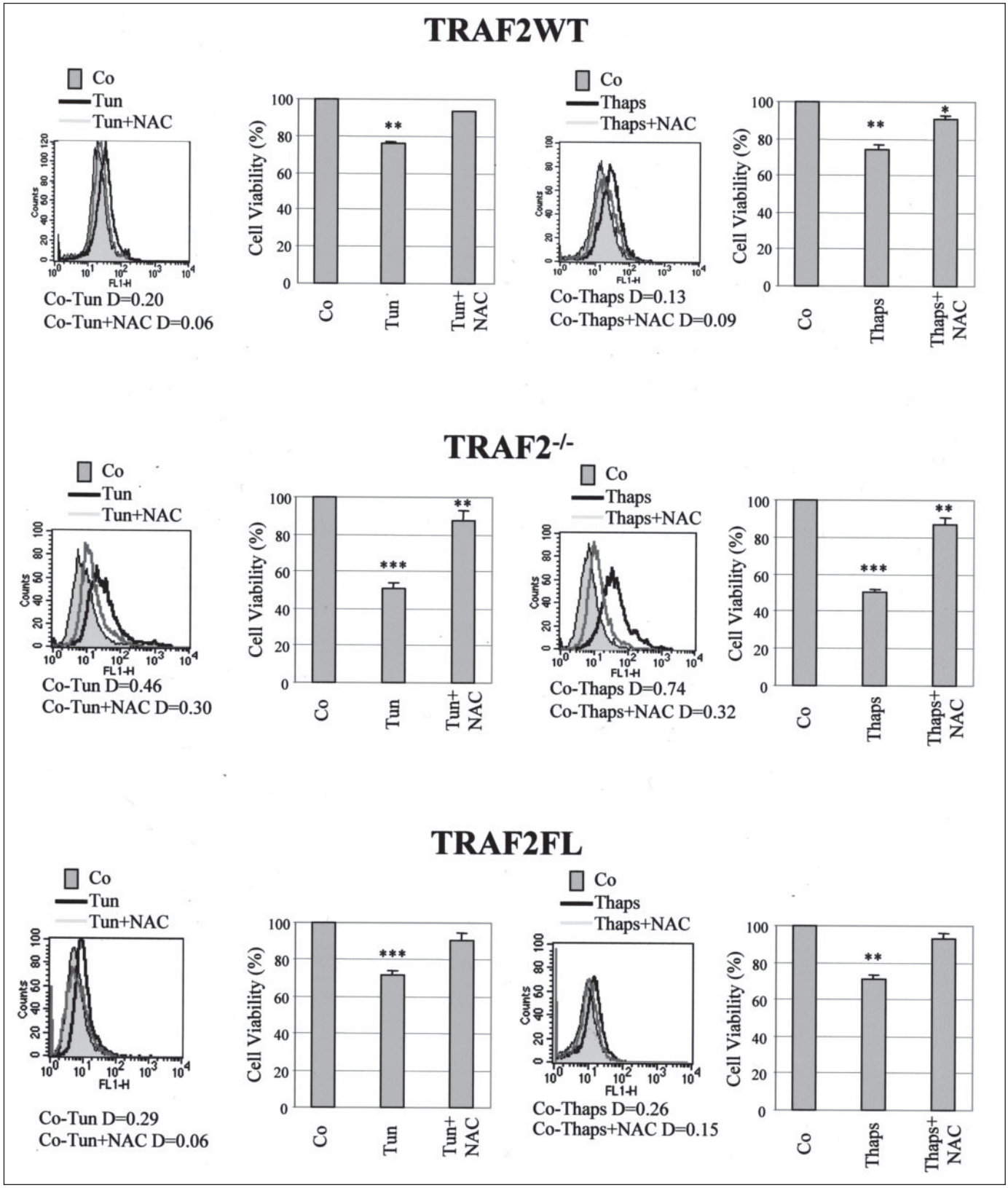
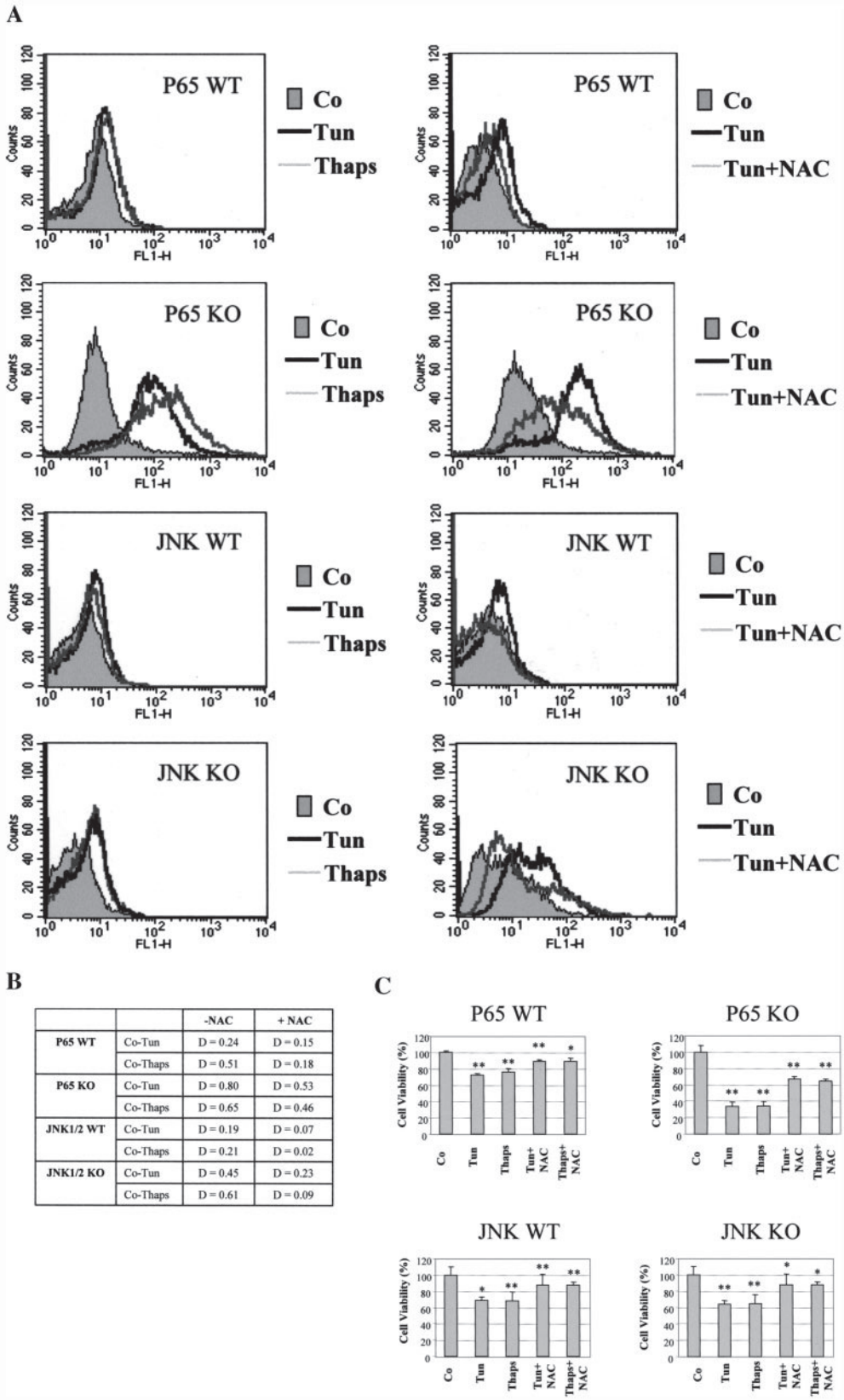


FIGURE 3. **ROS production correlates with endoplasmic reticulum stress-induced apoptosis.** WT, TRAF2$^{-/-}$, and TRAF2FL MEFs were treated with Me_2SO (*Co*), 150 ng/ml tunicamycin (*Tun*), or 20 nM thapsigargin (*Thaps*) for 48 h, in the presence or absence of antioxidants (NAC or dithiothreitol). ROS production was assessed by flow cytometry after labeling with H_2DCFDA. Cell viability was evaluated by MTS assay. Data are mean ± S.D. from three independent experiments. Statistical analysis was by the unpaired Student's *t* test: *, $p < 0.02$; **, $p < 0.002$; ***, $p < 0.0001$.



		-NAC	+ NAC
P65 WT	Co-Tun	D = 0.24	D = 0.15
	Co-Thaps	D = 0.51	D = 0.18
P65 KO	Co-Tun	D = 0.80	D = 0.53
	Co-Thaps	D = 0.65	D = 0.46
JNK1/2 WT	Co-Tun	D = 0.19	D = 0.07
	Co-Thaps	D = 0.21	D = 0.02
JNK1/2 KO	Co-Tun	D = 0.45	D = 0.23
	Co-Thaps	D = 0.61	D = 0.09

FIGURE 4. **Differential susceptibility of p65$^{-/-}$ and JNK1/2$^{-/-}$ MEFs to endoplasmic reticulum-dependent oxidative stress.** *A*, JNK1/2$^{-/-}$ and p65$^{-/-}$ MEFs were treated with Me_2SO (*Co*), 20 nM thapsigargin (*Thaps*), or 150 ng/ml tunicamycin (*Tun*) for 24 h, in the presence or absence of 5 mM NAC. ROS production was assessed by flow cytometry after labeling with H_2DCFDA. *B*, Kolmogorov-Smirnov statistical analysis of flow cytometric data were used according to Cell Quest Software (BD Biosciences). *D* values by Kolmogorov-Smirnov analysis ($p \leq 0.001$) are shown. *C*, p65$^{-/-}$ and JNK1/2$^{-/-}$ MEFs were treated with Me_2SO (*Co*), thapsigargin (*Thaps*), or tunicamycin (*Tun*) for 24 h, in the presence or absence of 5 mM dithiothreitol. Cell viability was evaluated by MTS assay. Data are mean ± S.D. from three independent experiments. Statistical analysis was by the unpaired Student's *t* test: *, $p < 0.02$; **, $p < 0.002$. *KO*, knock-out.

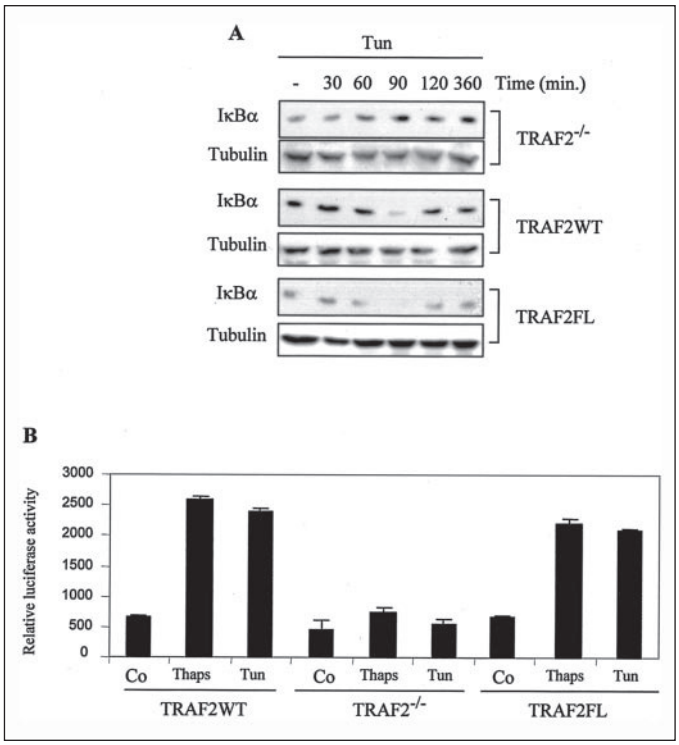


FIGURE 5. **Defective activation of the NF-κB pathway in TRAF2$^{-/-}$ MEFs after endoplasmic reticulum stress.** *A*, WT, TRAF2$^{-/-}$, and reconstituted TRAF2FL MEFs were treated with tunicamycin (*Tun*) (150 ng/ml), and expression of the IκBα protein was analyzed by Western blot. Filters were stripped and reprobed with anti-α-tubulin antibodies, as loading control. *B*, relative luciferase activity observed in WT, TRAF2$^{-/-}$, and TRAF2FL MEFs transfected in triplicate with 0.5 μg of the Ig-κB-luciferase reporter plasmid, stimulated with thapsigargin or tunicamycin, as indicated. Values shown (in arbitrary units) represent the mean (±S.D.) of two independent experiments, normalized for β-galactosidase activity of a cotransfected Rous sarcoma virus-β-galactosidase plasmid.

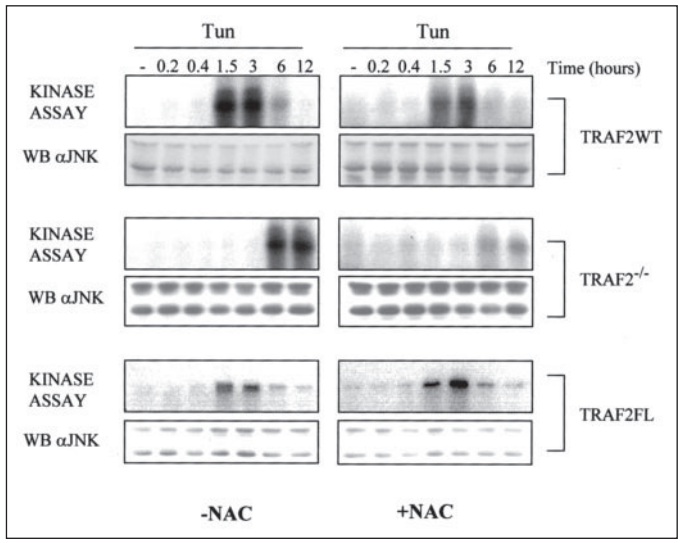


FIGURE 6. **Sustained activation of the JNK pathway in TRAF2$^{-/-}$ MEFs after endoplasmic reticulum stress.** WT, TRAF2$^{-/-}$, and TRAF2FL MEFs were treated with tunicamycin (150 ng/ml) for the indicated periods of time in the presence or absence of the antioxidant NAC, and activity of endogenous JNK was assessed by kinase assay using glutathione *S*-transferase-c-Jun as substrate. *Lower panel* shows a Western blot for total JNK. *WB*, Western blot.

To have further insight on the cross-talk between the NF-κB and the JNK pathways after ER stress, we evaluated activation of both pathways in WT, TRAF2$^{-/-}$, and reconstituted TRAF2FL MEFs. Treatment with tunicamycin caused activation of NF-κB in WT and TRAF2FL MEFs, as demonstrated by the disappearance of the inhibitory subunit IκBα (Fig. 5*A*) and by the increased activity of a κB-driven luciferase reporter gene (Fig. 5*B*). In the absence of TRAF2 it was not possible to detect activation of NF-κB. The observed activation of NF-κB was functional as demonstrated by the reappearance of the inhibitory subunit IκBα, a known early target gene of NF-κB (Fig. 5*A*).

Activation of JNK in WT and TRAF2FL MEFs stimulated with tunicamycin was detected 90 min after stimulation and decreased thereafter. Treatment with antioxidant did not affect JNK activation (Fig. 6). In contrast, in TRAF2$^{-/-}$ MEFs, activation of JNK was detectable only 6 h after stimulation and remained sustained for up to 12 h. This sustained activation of JNK was almost completely suppressed by NAC (Fig. 6). This result confirms that TRAF2 was necessary to activate JNK after ER stress, and suggests that the increased level of ROS detected in the absence of TRAF2 may mediate the sustained activation of JNK. This is in agreement with previous reports showing that after TNF stimulation the early activation of JNK depends on TRAF2 and that the sustained activation of JNK depends on ROS (26). Altogether these results suggested that following ER stress, the TRAF2-mediated activation of NF-κB was responsible for protection from apoptosis by decreasing ROS levels and controlling sustained JNK activation.

DISCUSSION

The endoplasmic reticulum is the principal site for protein synthesis and folding, and also serves as a cellular storage site for calcium. Agents that interfere with protein folding or export lead to ER stress and eventually cell death. Although initiation of apoptosis induced by death receptors and mitochondria is well studied, the mechanism by which ER stress triggers apoptosis is still not clear. In the present paper, we present evidence supporting a central role played by TRAF2 in regulation of pro-apoptotic and anti-apoptotic pathways initiated at the ER. We demonstrate that TRAF2$^{-/-}$ MEFs have increased susceptibility to ER stress-induced apoptosis. This increased susceptibility to ER stress-induced apoptosis was because of accumulation of ROS following ER stress, and was abolished by the use of antioxidants, such as NAC. In addition, we demonstrated that NF-κB was protecting cells from ER stress-induced apoptosis by controlling ROS accumulation.

TRAF2 has been demonstrated to be involved in signaling from endoplasmic reticulum being able to interact with Ire1 (10), one of the ER transmembrane proteins involved in initiating signals from the ER. TRAF2 mediates activation of both the JNK/SAPK and the NF-κB pathways following ER stress (10, 11). This scenario is reminiscent of TNF signaling, in which TRAF2 mediates simultaneous activation of the NF-κB survival pathway and pro-apoptotic JNK pathway, and the fate of the cell would be determined by interplay between these opposing signals. NF-κB exerts its anti-apoptotic activity by inhibiting caspase function (28–30), preserving function of mitochondria (31), and down-regulating JNK activity (23, 32). The latter function is mediated by at least two different mechanisms: by blocking activation of MKK7 via GADD45β (16) and decreasing ROS accumulation via the ferritin heavy chain (17). The importance of ROS in regulating sustained activation of JNK following TNF receptor triggering has been recently investigated in a NF-κB null cell model (26). Based on this study, TRAF-mediated NF-κB activation suppresses the TNF-induced ROS accumulation that, in turn, induces prolonged JNK activation and cell death. Our result supports this model and suggests that a similar mechanism may also operate for the ER. In fact, induction of ER stress causes activation of both NF-κB and JNK. In the absence of TRAF2 or p65, the NF-κB

pathway is not activated, and the late, ROS-dependent JNK activity is not counteracted, leading to cell death.

How does ROS affect JNK activation? ROS may affect JNK activation by at least two different mechanisms: by oxidizing and inhibiting mitogen-activated protein kinase phosphatase (33) and activating the protein ASK1 (34). This kinase may be activated via ROS and TRAF2 and has been demonstrated to be essential for inducing cell death after ER stress, at least in neuronal cells (34, 35). It may be possible that after ER stress and in the absence of a functional NF-κB activation, ASK1 is activated by the increased level of ROS and mediates sustained JNK activation and cell death.

Whereas it is clear from our results that the presence of a functional NF-κB is necessary for survival, counteracting increased induction of ROS following ER stress, the mechanism by which NF-κB exerts this function is not fully understood. It has been recently demonstrated that NF-κB up-regulates expression of ferritin heavy chain, an enzyme involved in iron metabolism and suppression of ROS accumulation (17). However, it is possible that in addition to up-regulation of genes involved in disposal of the ROS, NF-κB may also control transcription of genes that suppress production of ROS.

Our results confirm the central role played by TRAF2 in regulating activation of NF-κB following ER stress, and also sheds light on the functional significance of this activation. Recently, it has been demonstrated that in addition to the TRAF2-mediated NF-κB activation, another mechanism leading to activation of NF-κB following ER stress might exist. Based on this model, following ER stress, phosphorylation of eukaryotic initiation factor 2 represses synthesis of the inhibitory subunit IκBα, leading to activation of NF-κB (36). The two models of activation of NF-κB following ER stress, the TRAF2-mediated and the eukaryotic initiation factor 2-mediated, are not mutually exclusive. It is possible that both mechanisms contribute to activate NF-κB upon ER stress. However, whereas the biological significance of the link between eukaryotic initiation factor 2 phosphorylation and NF-κB activation is not fully understood, the functional significance of TRAF2-mediated NF-κB activation seems to be clear, at least in our experimental system. In fact, cells lacking TRAF2 or functional NF-κB undergo massive cell death after ER stress.

In conclusion, in the present study we provide evidence, for the first time, that the adaptor protein TRAF2 plays a central role in regulating signaling from the ER and that the activation of NF-κB, mediated by TRAF2, protects cells from ER stress-induced apoptosis. Therefore TRAF2 and NF-κB may be potential targets to control ER stress-induced apoptosis.

Acknowledgment—We thank Dr. Kostas Pantopoulos (McGill University, Canada) for helpful advice on ROS detection.

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