Modulation of Apoptosis and Signalling in Cancer Cells after Treatment with Epigenetic Modulators

Dissertation

for the obtainment of the academic degree

doctor rerum naturalium (Dr. rer. nat)

presented to the Council of the Faculty of Biology and Pharmacy of the Friedrich-Schiller-Univeristät Jena

seit 1558

by

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I dedicate this work to My Eternal Father,
My Husband and My Family

Declaration

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

The thesis has not been previously submitted either to the Friedrich Schiller University, Jena or to any other University.

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Abbreviations

Ac-DEVD-AFC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoro methylcoumarin
Ac	Acetylation
Acetyl-p53	Acetylated p53
Ac-H4	Acetylated histone-4
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride
AP24	24 kDa apoptotic protease
ATM	Ataxia telangiectasia mutated kinase
ATR	ATM- and rad3-related kinase
Bad	Bcl-associated death promoter
Bak	Bcl-2-homologous antagonist/killer
Bax	Bcl-2 assoiciated X protein
Bcl-2	B-cell lymphoma gene
Bcl-xL	Long splicing variant of a Bcl-2 gene
BSA	Bovine serum albumin
CD95	Apoptosis receptor (Fas)
CDKs	Cyclin D-dependent kinases
CTL	Cytotoxic T-lymphocytes
cyt C	Cytochrome C
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
DIABLO	Direct IAP binding protein of low PI
$DiOC_6(3)$	3,3'-dihexyloxacarbocyanine-iodide
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNMT	DNA methyltransferases
DR	Death receptor
DR	Death receptor
DSBs	DNA double strand breaks
DTT	[-]-1, 4-L-Dithio-L-threitol

EDTA	Ethylenediamine tetra-acetic acid
ES	Ewing's sarcoma
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
FDA	Food and drug administration
FCS	Foetal calf serum
GAPDH Gy	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour/s
HAT	Histone acetyltransferase
HDACi	Histone deacetylase inhibitor/s
HDACs	Histone deacetylases
HSP90	Heat shock protien 90
IAP	Inhibitors of apoptosis protein
MDM2	Mouse double murine 2
MDM4/X	Mouse double murine 4/X
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MOMP	Mitochondrial outer membrane permeabilisation
mt-p53	Mutated p53
NaB	Sodium butyrate
NF-κB	Nuclear factor-κB
ng	Nanogram
Noxa	Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family
NSAIDs	Nonsteroidal anti-inflammatory drugs
p53	Tumor suppressor protein
pATM	Phosphorylation of ATM
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIKK	(PI3K)-related protein kinase
PUMA	p53-upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species

rpm	Rotation per minute
SAHA	Suberoyl anilide hydroxamic acid
SC-560	5-(4-chloropheynyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondrial activator of cell death
TNF-α	Tumour necrosis factor-α
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl) aminomethane
TSA	Trichostatin A
Ub	Ubiquitin
Ubl	Ubiquitylation
UW	Ultraviolet radiation
VPA	Valproic acid
w/v	Weight/volume
wt-p53	Wild-type p53
z-VAD-fmk	z-Val-Ala-Asp(OMe)-CH_2F-fluoromethylketone
γH2AX	Gamma H2AX / phosphorylation of H2AX
$\Delta\psi_m$	Mitochondrial membrane potential
µg	Microgram
µl	Microliter
µM	Micromolar

Summary

Cancer has long been considered to be a genetic disease. However, it has recently been realised that epigenetic alterations also have a significant role in carcinogenesis. The most important epigenetic modifications are DNA methylation and histone acetylation. Dysregulation of these modifications can favour cancer growth. Therefore, targeting of aberrant epigenetic modifications may be a promising strategy for cancer treatment.

In particular, histone deacetylase inhibitors (HDACi) are an emerging new class of anticancer agents. HDACi can exert antineoplastic activities through inducing growth arrest, apoptosis, cellular senescence, mitotic cell death, autophagic cell death, anticancer immune response and antiangiogenesis. The present study focuses on elucidating the mechanisms by which HDACi induce cell cycle arrest and cell death either as single agent or in combination with other anticancer agents. The work addresses four objectives:

1) HDACi-induced G_2/M arrest,
2) Synergistic effects of HDACi in combination with the p53 activator nutlin-3,
3) Anticancer effects of nutlin-3 in Ewing's sarcoma cells,
4) Role of serine proteases in HDACi-induced apoptosis.

To achieve these objectives, cancer cells were treated with HDACi at various concentrations either alone or in combination with other anticancer agents and were analysed for their effects by flow cytometric analysis of cell cycle and different cell death parameters, by Alamar Blue assay, by determination of caspase-3 activity, by Western blot analysis and by real-time RT-PCR.

Earlier, our group has demonstrated that HDACi were capable of inducing a significant G_2/M arrest when their apoptosis-inducing activity was blocked by the pan-caspase inhibitor, z-VAD-fmk. Hence, we hypothesised that caspase-3-deficient MCF-7 cells would respond to HDACi treatment by undergoing G_2/M cell cycle arrest rather than apoptosis. Indeed, we found the HDACi vorinostat to cause a strong G_2/M arrest in MCF-7 cells. On investigating the mechanism of vorinostat-induced G_2/M arrest, we found that vorinostat induced phosphorylation of H2AX (γH2AX) at Ser139, a marker for DNA damage, suggesting that vorinostat treatment produces DNA damage. In addition, we found vorinostat to mediate phosphorylation of the protein kinase ATM (ataxia telangiectasia mutated), a master regulator of cellular responses to DNA damage. However, inhibition of ATM had no significant effect on vorinostat-induced cell

cycle arrest. Thus, the role of other factors involved in cell cycle regulation, i.e. the proteasome, NF-κB and p53, were studied. We noted that the proteasome inhibitor bortezomib, different NF-κB inhibitors and the p53 activator nutlin-3 all had a significant impact on vorinostat-induced G_2/M arrest. As an accessory finding of the experiments employing nutlin-3, we made the interesting observation that vorinostat and nutlin-3 cooperated in exerting anticancer activity.

Nutlin-3 is a murine double minute-2 (MDM2) inhibitor, which restores p53 function and, thus, is considered an interesting candidate for the treatment of wild-type p53 (wt-p53) cancers. However, nutlin-3 alone may be insufficient for an effective cancer therapy. Thus, in the second part of this study, we investigated the anticancer activity of nutlin-3 in combination with HDACi in more depth. We observed that the combination of nutlin-3 with various HDACi [vorinostat, sodium butyrate (NaB), MS-275 and apicidin] synergistically induced cell death in wt-p53 cell lines (A549 and A2780), but not in a p53 null cell line (PC-3). In contrast, nutlin-3 antagonised the cytotoxicity of paclitaxel. On exploring the mechanism of the synergistic activity of nutlin-3 and HDACi, we observed the induction of p53 acetylation and downregulation of MDM2 and MDM4 gene expression. These findings suggest that HDACi may enhance the antitumour action of nutlin-3 by inducing p53 hyperacetylation and/or downregulation of MDM2 and/or MDM4 gene expression in wt-p53 cancer cells. Hence, this combination of drugs could be efficiently used for treating wt-p53 tumours.

Ewing's sarcoma (ES) is the second most common malignant bone tumour in childhood. p53 mutations have been detected in only about 10 % of patients with ES. Thus, about 90 % of ES patients are potentially responsive to p53-based targeted therapies. Considering this, we hypothesised that administration of nutlin-3 could be a novel method for treating ES. Therefore, in the third part of this study, we explored the antitumour activity of nutlin-3 in wt-p53 and mutated p53 (mt-p53) ES cell lines. We observed that nutlin-3 treatment elevated p53 level and activated p53 target gene expression, such as MDM2, p21 and PUMA, in wt-p53 ES cells, but not in mt-p53 ES cells. In accordance, nutlin-3-induced apoptosis was found only in wt-p53 ES cells. Furthermore, we have shown that nutlin-3 stimulated cellular senescence, implicating that nutlin-3 produced pleiotropic antitumour effects in ES. In addition, we found nutlin-3 to synergise with a NF-κB inhibitor to induce antineoplastic activity in ES cells. These findings imply that the direct activation of p53 by nutlin-3 treatment may be an effective strategy for treating ES patients.

Our earlier findings had pointed to an important role of serine proteases in HDACi-induced cytotoxicity. Here, in the fourth part of this study, we revisited the involvement of serine

proteases in HDACi-induced apoptosis. We observed that the serine protease inhibitor AEBSF prevented trichostatin A (TSA)- or vorinostat-induced apoptosis, whereas it had no effect on NaB-induced apoptosis. Further, TSA-mediated cytotoxic effect was strongly reduced by AEBSF as analysed by clonogenic assay. Therefore, the effect of AEBSF appeared too strong to be true and, thus, suggested that AEBSF may directly inactivate vorinostat and TSA. Hence, we measured HDAC activity and observed strong prevention of TSA- or vorinostat-mediated HDAC inhibition by AEBSF, whereas AEBSF left NaB action untouched. As well, we also found that AEBSF prevented TSA- or vorinostat-induced, but not NaB-induced, histone 4 hyperacetylation. Taken together, our findings propose that AEBSF directly inactivates TSA and vorinostat, that way restraining these HDACi from inhibiting HDAC activity and from inducing apoptosis.

Zusammenfassung

Krebs wurde lange Zeit als eine genetische Erkrankung angesehen. Inzwischen ist bekannt, dass auch epigenetische Veränderungen für die Krebsentstehung wesentlich sind. Die derzeit am besten verstandenen epigenetischen Modifikationen sind DNA-Methylierung und Histon-Acetylierung, deren Fehlregulierung Krebswachstum stimulieren kann. Aberrante epigenetische Modifikationen und deren auslösende Mechanismen erscheinen daher als Angriffsziel in der Krebstherapie viel versprechend.

Unter den epigenetisch modulierenden Substanzen besitzen besonders Histondeacetylase-Inhibitoren (HDACi) als neue Klasse von Krebsmedikamenten ein hohes Potenzial für zukünftige Therapieverbesserungen. HDACi wirken gegen Tumorzellen, indem sie Wachstumsarrest, Apoptose, zelluläre Seneszenz, mitotischen und autophagischen Zelltod induzieren, immunstimulatorisch wirken und die Angiogenese hemmen. In der vorliegenden Arbeit wurden Mechanismen untersucht, mit denen HDACi als Monosubstanzen oder in Kombination mit anderen Wirkstoffen Zellzyklusarrest und Zelltod auslösen. Im Einzelnen ist die Arbeit in vier Abschnitte eingeteilt:

a) HDAC-induzierter G_2/M-Arrest,

b) synergistische Effekte von HDACi in Kombination mit dem p53-Aktivator Nutlin-3,

c) antineoplastische Wirkungen von Nutlin-3 auf Ewing-Sarkom-Zellen,

d) Beteiligung von Serin-Proteasen an der HDACi-induzierten Aoptose.

In den Untersuchungen wurden Krebszellen entweder mit HDACi allein, oder mit HDACi in Kombination mit anderen Wirkstoffen behandelt. Der Zelltod wurde durchflusszytometrisch durch Propidiumiodid-Aufnahme bestimmt, die Wirkung auf Mitochondrien durch Messung des mitochondrialen Membranpotenzials und die Arretierung im Zellzyklus durch Zellzyklusanalyse. Des Weiteren wurden Alamar Blue-Assays vorgenommen, Caspase-Aktivitäten gemessen und Western-Blot- und "Real Time"-RT-PCR-Analysen durchgeführt.

In früheren Untersuchungen hatte unsere Arbeitsgruppe festgestellt, dass HDACi einen signifikanten G_2/M-Arrest induzierten, wenn ihre apoptotische Wirkung durch den pan-Caspase-Inhibitor z-VAD-fmk gehemmt war. Wir stellten daher die Hypothese auf, dass Caspase 3-defiziente MCF-7-Zellen auf die HDACi-Behandlung nicht mit Apoptose, sondern bevorzugt mit Ausbildung eines Zellzyklusarrests reagieren würden. In der Tat beobachteten wir, dass der

HDACi Vorinostat einen deutlichen G_2/M-Arrest in MCF-7-Zellen auslöste. Die Analyse des zugrunde liegenden Mechanismus zeigte, dass Vorinostat die Phosphorylierung von H2AX (γH2AX) herbeiführt, einem Marker für DNA-Schädigung, was darauf hinweist, dass die Vorinostat-Behandlung zu DNA-Schäden führt. Zudem konnten wir zeigen, dass Vorinostat die Phosphorylierung der Proteinkinase ATM (ataxia telangiectasia mutated) bewirkt, einem Schlüsselenzym in der zellulären Reaktion auf DNA-Schäden. Die Hemmung von ATM hatte jedoch keine Wirkung auf den Vorinostat-ausgelösten G_2/M-Arrest. Wir untersuchten daher weitere Faktoren, die typischerweise an der Regulierung des Zellzyklus beteiligt sind. Hierzu gehörten das Proteasom, NF-κB und p53. Wir konnten im Rahmen dieser Analysen beobachten, dass der Proteasom-Hemmstoff Bortezomib, verschiedene NF-κB-Inhibitoren wie auch der p53-Aktivator Nutlin-3 den Vorinostat-induzierten G_2/M-Arrest deutlich modulierten. Als zusätzliches Ergebnis dieser Analysen konnte gezeigt werden, dass Vorinostat und Nutlin-3 eine kooperative antineoplastische Wirkung ausübten.

Nutlin-3, ein Inhibitor von mouse double minute-2 (MDM2), aktiviert p53 und wird daher als ein viel versprechender Wirkstoff zur Behandlung von Krebsformen mit Wildtyp-p53 (wt-p53) angesehen. Dabei erscheint Nutlin-3 jedoch als Monotherapeutikum möglicherweise nicht ausreichend effektiv. Im zweiten Abschnitt dieser Arbeit haben wir darum die Kombination von Nutlin-3 und HDACi untersucht und beobachtet, dass Nutlin-3 in Kombination mit verschiedenen HDACi (Vorinostat, Natriumbutyrat (NaB), MS-275 und Apicidin) in wt-p53-Zelllinien (A549 und A2780), aber nicht in p53-Null-Zellen (PC-3) synergistisch Zelltod auslöste. Im Gegensatz dazu antagonisierte Nutlin-3 den Paclitaxel-induzierten Zelltod. Die Untersuchungen zum Mechanismus des Nutlin-3/HDACi-Synergismus zeigten, dass HDACi die p53-Acetylierung induzierten und die Genexpression von MDM2 und MDM4 reduzierten. Diese Befunde legen nahe, dass HDACi die antineoplastische Wirkung von Nutlin-3 verstärken, indem sie die p53-Hyperacetylierung und/oder die Genexpression von MDM2 und/oder MDM4 herbeiführen, und dass die Nutlin-3/HDACi-Kombinationsbehandlung eine wirkungsvolle Strategie zur Therapie von wt-p53-Tumoren sein könnte.

Das Ewing-Sarkom (ES) ist die zweithäufigste Knochenkrebsform bei Kindern. Bei Patienten mit ES wurde in nur zehn Prozent der Fälle p53-Mutationen gefunden, so dass potenziell 90 % auf p53-gerichtete Behandlungen ansprechen sollten. Wir stellten daher die Hypothese auf, dass Nutlin-3 ein sehr geeigneter Wirkstoff für die Therapie des Ewing-Sarkoms sein könnte. Im dritten Teil dieser Studie überprüften wir darum die Wirksamkeit von Nutlin-3 in Ewing-Sarkom-Zelllinien mit wt-p53 und mutiertem p53 (mt-53). Wir beobachteten, dass Nutlin-3 in

wt-p53-, nicht aber in mt-p53-Zellen p53 stabilisierte und die Expression von p53-regulierten Genen (MDM2, p21, PUMA) induzierte. Im Einklang mit diesen Befunden löste Nutlin-3 nur in wt-p53-Zellen die Apoptose aus. Darüber hinaus fanden wir, dass Nutlin-3 die zelluläre Seneszenz induzierte, was darauf hinweist, dass Nutlin-3 pleiotrope antitumorale Effekte auf Ewing-Sarkom-Zellen ausübt. Zudem stellten wir eine synergistische Wirkung von Nutlin-3 in Kombination mit einem NF-κB-Inhibitor fest. Unsere Befunde legen nahe, dass die Aktivierung von p53 mittels Nutlin-3 ein effektives Behandlungsverfahren für Ewing-Sarkom-Patienten sein könnte.

Frühere Untersuchungen unseres Labors hatten auf eine wichtige Rolle von Serin-Proteasen beim HDACi-vermittelten Zelltod hingewiesen. Im letzten Abschnitt dieser Arbeit wurde diese Beobachtung nochmals überprüft. Dabei stellten wir fest, dass der Serin-Protease-Hemmstoff AEBSF den Vorinostat- und Trichostatin A- (TSA), jedoch nicht den NaB-induzierten Zelltod inhibierte. Zudem unterband AEBSF vollständig den von TSA ausgelösten zytotoxischen Effekt in Langzeit-Messungen. Diese eklatant starke Wirkung von AEBSF erschien untypisch für rein biologische Effekte und legte den Verdacht nahe, dass es Vorinostat und TSA durch chemische Interaktion direkt inaktivieren könnte. Wir bestimmten daher die HDAC-Aktivität und stellten fest, dass AEBSF tatsächlich die Vorinostat- und TSA-vermittelte Hemmung der HDAC-Aktivität deutlich verminderte, während es keine Wirkung auf die NaB-vermittelte Hemmung hatte. Damit übereinstimmend fanden wir, dass AEBSF die Vorinostat- und TSA-, nicht aber die NaB-induzierte Hyperacetylierung von Histon H4 verhinderte. Diese Ergebnisse deuten stark darauf hin, dass AEBSF Vorinostat und TSA durch direkte Interaktion inaktiviert und so daran hindert, die HDAC-Aktivität zu hemmen und den Zelltod auszulösen.

1 INTRODUCTION

1.1 Cancer

The unusual and uncontrolled proliferation of tissues is considered as 'cancer'. This is due to the disturbance in the tissue homeostasis, which mainly because of the progression of self-derived or external derived factors over a highly regulated normal cell proliferation. The basis for cancer development is the instability of host genome by sequential mutations (Weinberg, 1996;Kinzler and Vogelstein, 1996a) of four classes of genes. They are growth-promoting oncogenes (Hanahan and Weinberg, 2000), growth-inhibiting tumour suppressor gene, genes that are responsible for regulating apoptosis and genes that are regulating DNA repair (Greenblatt et al., 1994). Many external factors including exposure to virus, xenobiotics (foreign chemicals) and radiation can contribute to the mutation and induces the malignant transformation of cells (Yuspa, 2000).

Generally, the malignant tumour has two kinds of characteristic growth 1. Benign tumour is an encapsulated abnormal tissue mass that never disseminates 2. Malignant tumour invades adjacent tissues by infiltrative destructive growth. Such invasive growth may progress to metastasis after spreading of cancer cells through blood circulatory system and establish a new growth at distance parts of the body (Tannock et al., 2005).

1.1.1 Cell cycle

The hallmark of eukaryotic cellular proliferation is the cell cycle through which a cell undergoes division to give two daughter cells. A cell division cycle comprises four precisely controlled sequential phases 1. G_1 Phase: a gap follows on from M phase to S phase, and is a period when the cell is responsive to both positive and negative growth signals 2. S phase, during this period the cell undergoes replication and synthesises DNA 3. G_2 phase: a gap session after S phase and prepares the cell to enter M phase and 4. M phase in which the cell divides into two complete cells. Most cells in normal tissue are in quiescent G_0 state and eventually removed from G_1 phase. On exposure with mitogenic agents, cells in G_0 or G_1 are stimulated to progress to restriction point R beyond which cells are committed to enter S phase. The cells beyond R point do not require further stimulation and progress into S phase and M phase to complete the cycle (Garrett, 2001).

The fidelity of cell cycle is maintained by regulatory mechanisms also known as cell cycle checkpoint at three major positions. First cell cycle checkpoint arises at the G_1/S phase transition to monitor DNA damage and also restrains S phase due to incomplete DNA replication. Next checkpoint activated at G_2/M to prevent entry of incompletely replicated DNA into mitosis. Third check point evolves as checkpoint due to improper spindle formation during mitosis (Pietenpol and Stewart, 2002). Apart from these restraining mechanisms, cells also respond to DNA damage through activating apoptosis, a programmed cell death. In transformed cells, dysregulation of these controlling mechanisms allows the cells to behave independent of signals from growth activators or inhibitors for cell division and leading the cells to pass R point and progress into subsequent cell cycle phases.

Numbers of studies demonstrated that the association between cell cycle controls and regulation of apoptosis is intrinsically established by players common to both processes. On double strand DNA damage, the halted replication forks activate evolutionarily conserved signalling pathways such as phosphoinositide 3-kinase (PI3K) that consist of damage-sensing complexes and the large protein-serine/threonine (Ser/Thr) kinases ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) (Bartek et al., 2004;Harper and Elledge, 2007). ATM and ATR in turn activate the CHK2 and CHK1 protein kinases, respectively. These proteins phosphorylate and inhibit CDC25 phosphatases, which have a main role in activating CDKs, essential players in the cell cycle (Bartek et al., 2004;Harper et al., 2007). They also stabilise p53 through phosphorylation. p53 can induce the transcription of both CDK inhibitors and proapoptotic proteins such as PUMA and Noxa (Vousden and Lu, 2002), and NF-κB, which at the same time represses the antiapoptotic protein Bcl-xL (Perkins, 2004;Perkins, 2007). Moreover, direct functional relationship between checkpoint components and regulators of the mitochondrial apoptotic pathway was put forward in a number of investigations (Pietenpol et al., 2002). Moreover, DNA damage-induced cell cycle arrest may also lead the cells into senescence in which the cell proliferation is irreversibly blocked (Jian-Hua Chen et al., 2007). Phenotypically, senescence is characterised by an increase in cytoplasmic volume and an accumulation of lysosomes, leading to increased granularity and elevated level of β-galactosidase expression (Dimri et al., 1995). In contrast to apoptosis, in which cytotoxic signals converge to a programmed death, senescence is typically a delayed stress response involving multiple effector mechanisms. These effector mechanisms include epigenetic regulation, the DNA damage response, and the senescence-associated secretion phenotype.

1.1.2 ATM

Mammalian cells have evolved with cellular pathways as DNA damage response to protect DNA from DNA damages by ionising radiation like gamma rays and X-rays and genotoxic agents. ATM is a core sensor protein activated in response to DNA double strand breaks (DSBs), which acts in all phases of cell cycle (Liang et al., 2009). Activated ATM regulates cell cycle check points through phosphorylating downstream signalling molecules to control cell cycle and facilitate DNA repair or apoptosis. Biochemically, ATM is a Ser/Thr protein kinase, and is a member of phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family. ATM comprises a Fat domain, a protein kinase domain, FACT domain at C terminus and a substrate binding domain (SBS) for p53, NBS1, BRCA1 at N-terminus (Banin et al., 1998;Canman et al., 1998;Khanna et al., 1998).

The activated ATM-mediated DNA damage response is a complex process that initiates from the relaxation of chromatin as a consequence of a DBSs. In the DNA flanking regions, ATM is partially activated and phosphorylates p53 and possibly other substrates. ATM is then recruited to the site of the break by the MRE11-RAD50-NBS1 (MRN) complex and phosphorylates members of the complex and other downstream substrates. As result of activation, inactive ATM dimer is monomerised, and parallel transphosphorylation (autophosphorylation) occurs on at least three sites: Ser367, Ser1893 and Ser1981. However, the activation of ATM is controlled by phosphatases such as protein phosphatase-2A (PP2A), PP5 and WIP1. Acetylation (Ac) of ATM at Lys3016 within the C-terminal FATC domain by acetyltransferase TIP60 also brings the process of activation.

It is noteworthy that in the G_1/S checkpoint, ATM phosphorylates p53 on Ser15 and other sites; although this modification does not stabilise and activate p53, it is considered as a marker for that process. Indeed, stabilisation of p53 is also mediated by ATM phosphorylation of the checkpoint kinase CHK2, MDM2 and MDM4. Phosphorylation of MDM2 suppresses its affinity for p53, and prevents ubiquitylation (Ubl) and proteasomal degradation of p53 and contributes to its stabilisation. Furthermore, ATM phosphorylates MDM4 on Ser403 and mediates CHK2 phosphorylation of MDM4 on Ser342 and Ser367. These phosphorylation events, together with ubiquitylation by MDM2 (stimulated by CHK2), lead to MDM4 degradation and to p53 stabilisation. By this way, ATM control over G_1/S check point as well as in intra-S phase and G_2/M phase check point, DNA repair control (Figure 1).

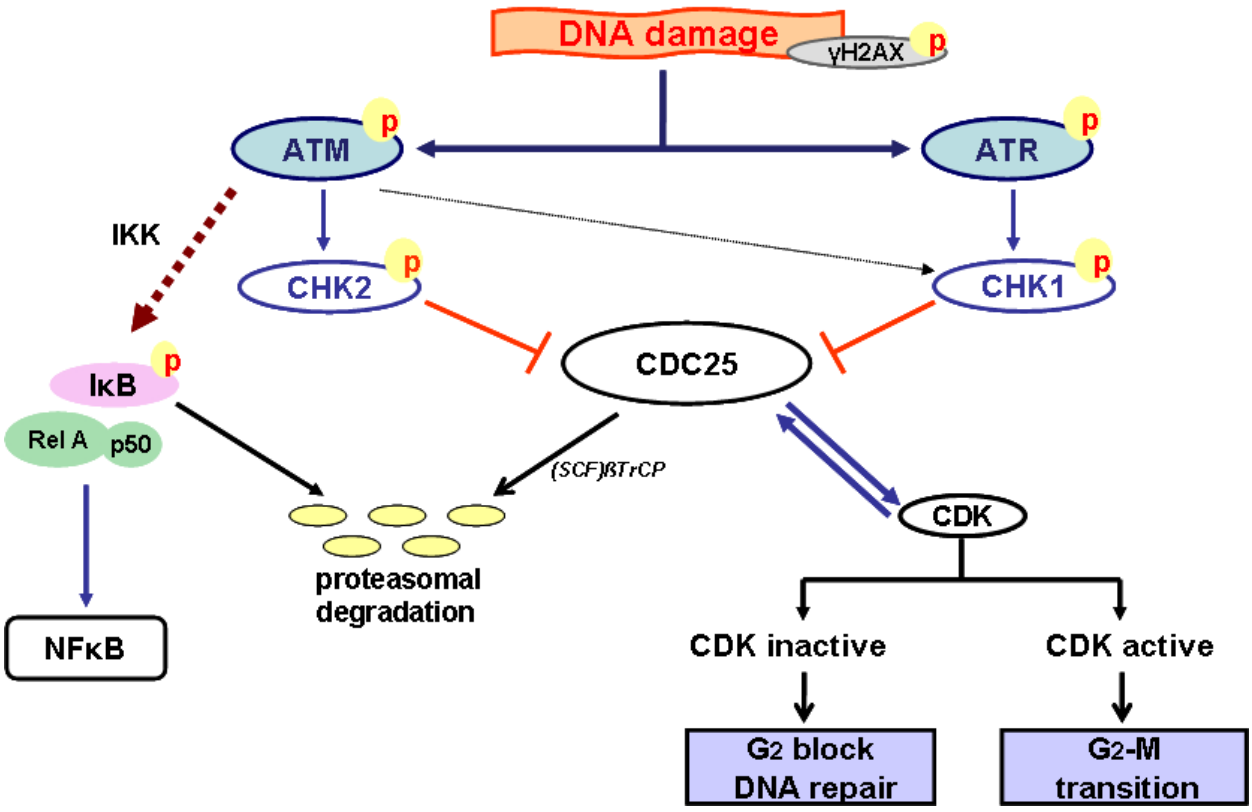

Figure 1 Role of ATM in cell cycle arrest through DNA damage.

Cell cycle checkpoints pathway induced in response to DNA damage. The two kinases ATM and ATR are activated in response to different types of DNA damage. ATM is mainly activated by the DSBs whereas ATR is activated by single stranded DNA and stalled forks. ATM and ATR phosphorylate different targets, including checkpoint kinase-1 (CHK1) and CHK2. The active form of CDK is a main player in governing cell cycle progression. This CDK activation depends on the level of the cyclins, which are controlled by the ubiquitin proteasome system. Phosphorylation of CDC25 by CHK1 and CHK2 activates SKP1-CUL1-F-box (SCF)$^{\beta TrCP}$ and promotes its degradation. The activation of DNA damage-induced checkpoint leads to CDC25 degradation, low CDK activity and G_2 arrest. However, completion of DNA repair restores CDK activity that allows G_2-M transition. Arrows indicate activation and T-shaped lines indicate the inhibitory mechanism (modified from Branzei and Foiani, 2008).

1.1.3 p53

p53, a transcription factor, acts as a gatekeeper in normal cells by preventing abnormal proliferation of cells via complex cell cycle, DNA replication and repair and apoptosis regulatory mechanisms. By this way, p53 maintains tissue homeostasis of multicellular organisms (Kinzler and Vogelstein, 1996b).

Under cellular stress, DNA damage and oncogene activation, p53 level increases and in turn transactivates p53 target genes, such as p21, GADD 45, Bax, PUMA and Noxa, leading to cell cycle arrest and/or apoptosis (Vousden and Prives, 2009). Translocation of p53 to mitochondria induces apoptosis through transcriptional independent mechanism.

Furthermore, oncogene activation may also stimulate the expression of p14Arf protein which inhibit MDM2, leading to p53 accumulation. As a whole, both the cellular level and status of p53 play an important role in inducing cell cycle arrest or apoptosis in malignant cells. Therefore, p53 could be considered as an important clinical target, perhaps the reactivation of p53 by downregulating p53-MDM2 or MDM4 binding would be a novel therapeutic strategy for treating p53 wild-type cancers.

1.1.4 MDM2

The murine double minute 2 (mdm2) gene was originally identified by virtue of its amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM) (Cahilly-Snyder et al., 1987). Later the MDM2 protein was identified as a binding partner of p53 inhibiting p53-mediated transcription activation (Momand et al., 1992), indicating its potential role in neoplastic transformation.

Human mdm2 gene (hdm2 gene) amplification was observed in one third of wt-p53 human sarcomas (Oliner et al., 1992), suggesting that induction of MDM2 protein expression is an molecular mechanism by which the cell can inhibit p53 and stimulate tumour formation. MDM2 consists of several functional domains. The N-terminal region contains the p53-binding region (residues 26 ± 108) (Chen et al., 1993;Picksley et al., 1994). The central region contains a short highly acidic region, that regulates transcriptional activity (residues 211 ± 299) (Leveillard and Wasylyk, 1997;Thut et al., 1997) and the C-terminus contains a RING finger domain (residues 442 ± 481) essential for E3 ligase activity and ubiquitin-mediated degradation of p53 (Fang et al., 2000;Honda and Yasuda, 2000;Brooks and Gu, 2006). It has also been shown to be modified by SUMO in the C-terminus of Mdm2 (Buschmann et al., 2000). MDM2 contains several cellular localisation sequences including a nuclear localisation sequence (NLS, residues 178 ± 182) (Olson et al., 1993), a nuclear export sequence (NES, residues 183 ± 195) (Roth et al., 1998) and a nucleolar localisation sequence (NoLS, residues) (Lohrum et al., 2000;Weber et al., 2000). MDM2 negatively regulates both stability and activity of p53 by directly binding to p53 within its N-terminal transactivation domain (residues 19 ± 26). Further targets it for ubiquitin-mediated degradation by the proteasome. Under normal conditions, the mdm2 gene itself is transcriptionally activated by p53 and forms a regulatory feedback loop of two proteins (Freedman et al., 1999). However, under cellular stress conditions this feedback loop is disrupted, leading to rapid stabilisation and activation of p53 by a variety of mechanisms that are dependent on the particular type of stress. Many of these mechanisms converge on MDM2 itself

and involve regulation of mRNA expression, nucleolar sequestration, and posttranslational modification (Woods and Vousden, 2001).

1.1.5 MDM4

MDM4 (also known as MDMX) is a prominent regulator of MDM2 activity. MDM4 is also a critical negative regulator of p53 like MDM2 (Marine and Jochemsen, 2005). MDM2 and MDM4 interact with each other via their C-terminal RING domains. Interestingly, MDM4 stabilises both MDM2 and p53 and also promotes the E3-ligase activity of MDM2 (Linares et al., 2003;Poyurovsky et al., 2007). Therefore, the MDM4 has the ability to promote MDM2 function as well as its own direct impact on p53. This suggests that MDM4 may be a potential therapeutic target for the regulation of p53 activity (Shangary and Wang, 2009).

1.1.6 Apoptosis

Apoptosis or programmed cell death is a physiological mechanism through which most multicellular organisms attain their own cell death. Thereby they maintain the balance between senescence and cell proliferation and regulate overall tissue homeostasis. Dysregulation of apoptosis in a cell can manifest to cancer, autoimmune disease and degenerative disorders. The apoptotic cells have been identified with distinct morphological changes such as membrane blebbing, cytoplasmic shrinkage, alteration of asymmetrical distribution of membrane components and condensation of the nucleus (Reed et al., 2000) and later they become into apoptotic bodies that are eventually eliminated by phagocytic cells without triggering an inflammatory response.

In general, apoptosis is triggered by endogenous stimuli like growth factor deprivation as well as exogenous stimuli like ultra violet (UV) or gamma radiation or other DNA damaging agents such as chemotherapeutic drugs. When the cells undergo apoptosis due to inadequate cellular matrix interaction it is known as anoikis (Frisch and Screaton, 2001). So far two major pathways have been demonstrated in relation to the governing the regulation of apoptosis. Those are 1. Extrinsic or death receptor pathway 2. Intrinsic or mitochondrial pathway.

1.1.6.1 Extrinsic or death receptor pathway

The mammalian death receptor (DR) apoptotic pathway is triggered by ligands which bind to the death receptor members of the TNF receptor family: Fas (also called DR2, CD95 or APO-1), TNF receptor type 1 (TNFR-1, also called DR1, p55, p60 or CD120a), DR3, TRAIL (TNF-

related apoptosis-inducing ligand) receptors DR4, DR5, and DR6 (Nagata, 1999); (Suliman et al., 2001). For example, stimulation of Fas by FasL or TNFR-1 by TNF has been implicated in the elimination of unwanted lymphocytes and transformed cells

Death receptors share the presence of a death domain in their cytoplasmic tails (Ashkenazi, 2002). Death receptor signalling is amplified by the events that occur following engagement of Fas by its ligand FasL on the cell surface (Figure 2). Following Fas/FasL interaction, the Fas receptor proteins aggregate to form a trimer and recruit the adaptor protein FADD (Fas-associated death domain protein) that contains two protein interaction domains, a death domain and a death effector domain (DED). Fas/FADD interaction allows the recruitment of DED-containing initiator caspase such as caspase-8 or caspase-10 to the complex, resulting in their activation. Activated caspase-8 and caspase-10 in turn process downstream caspases such as caspase-3, thereby finalising the commitment of apoptosis.

1.1.6.2 Intrinsic or mitochondrial pathway

The hallmark of intrinsic pathway is in the activation of mitochondria which are structurally composed of outer membrane, inner membrane and inter membrane space (IMS). The IMS have membrane proteins such as cytochrome C (cyt C), apoptosis-inducing factors (AIF), Omi/HtrA2, EndoG and Smac/DIABLO. Apoptotic stimuli, such as deprivation of growth factors, DNA damage, oxidative stress, hypoxia, or chemotherapeutic drugs, induce the opening of membrane pores, loss of membrane potential and trigger the release of sequestrated proapoptotic proteins into cytosol, including cyt C. Indeed, release of cyt C is positively regulated by proapoptotic proteins BID and Bax and negatively regulated by antiapoptotic proteins Bcl-2 and Bcl-xL. The released cyt C stimulates formation of apoptosome by forming a complex with Apaf-1 (Apoptosis protease activating factor-1) and caspase-9. The activated caspase-9 in turn activates other effector caspases like caspase-3, -6 and -7, which catalyse cleavage of various cellular substrates, resulting in cell death. The proapoptotic protein Smac/DIABLO and Omi/HtrA2, released from mitochondria along with cyt C, down regulate inhibitors of apoptosis proteins (IAP) such as XIAP, which directly inhibit the proteolytic function of caspase-3, -7 and -9 and suppress apoptosis.

The DR and mitochondrial pathways are linked by caspase-8 cleavage of BID (a proapoptotic member of the Bcl-2 family) that generates a proteolytic fragment that cooperates with Bax and forms supramolecular openings in the outer mitochondrial membrane leading to release of

mitochondrial cyt C. Hence, BID may serve to amplify death signals initiated by engagement of the DR, as BID deficiency does not affect caspase-8 activation, but drastically diminishes

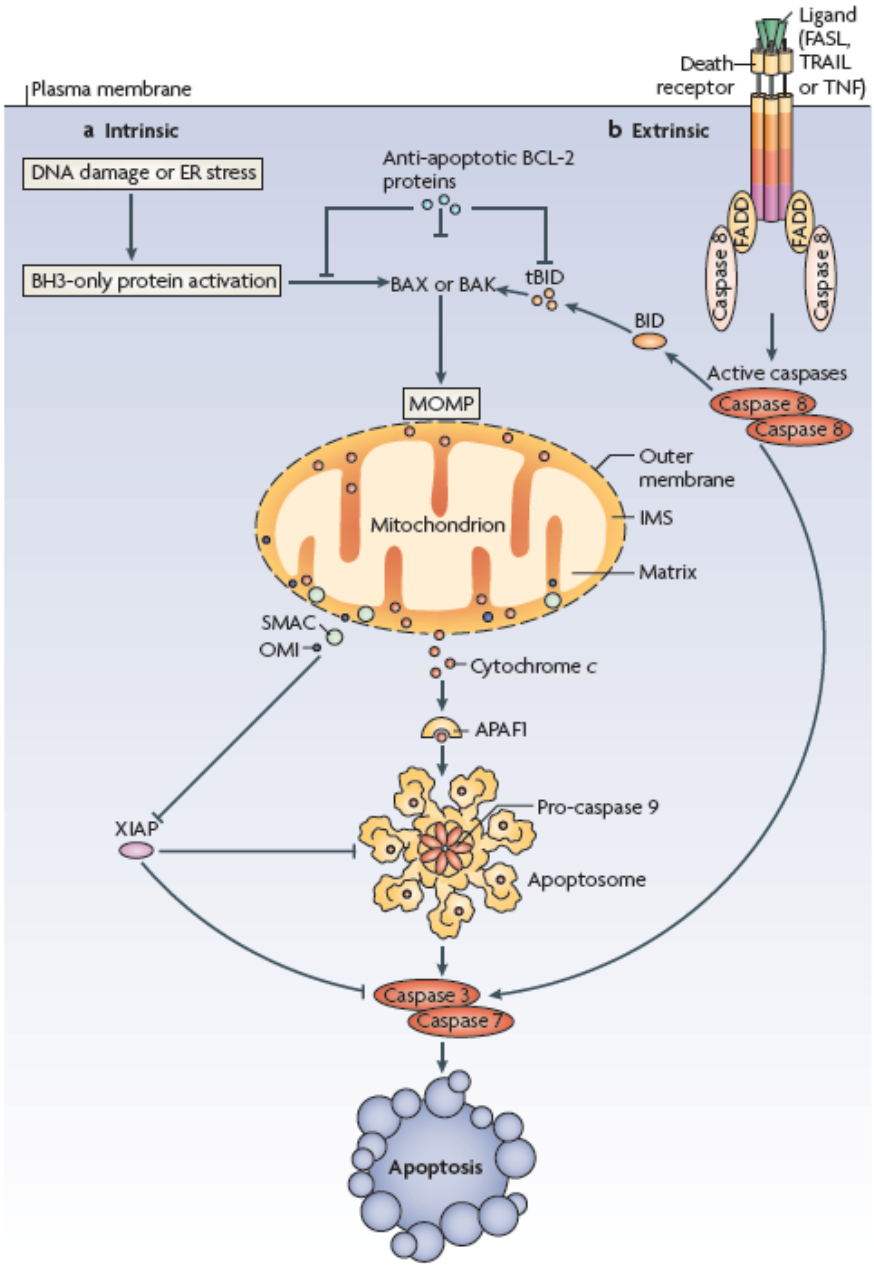

Figure 2 Apoptotic pathways.

A) *Intrinsic apoptotic pathway is induced by DNA damage or endoplasmic reticulum (ER) stress, leading to mitochondrial outer membrane permeabilisation (MOMP) and cyt C release mediated by activated proapoptotic proteins Bax and Bak. cyt C complex with APAF-1 to form apoptosome to activate procaspase-9 into caspase-9 which in turn activates caspase-3, to execute apoptosis.* ***B)*** *Extrinsic apoptotic pathway is induced by binding of death receptors with their ligands, leading to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD) and then caspase-8. This causes dimerization and activation of caspase-8, which directly activates caspase-3 and caspase-7, leading to apoptosis. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase-8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), the product of which (truncated BID; tBID) is required in some cell types for death receptor-induced apoptosis. FASL, FAS ligand; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing Ligand (Tait and Green, 2010).*

caspase-3 processing. Defective apoptosis has been observed in mice and cells deficient for Fas, FasL, caspase-8 or FADD (Ranger et al., 2001).

1.1.7 Serine proteases

Among the proteases, one group of proteolytic enzymes are serine proteases. These enzymes contain a serine residue at their active centre, which participates in the formation of an intermediate ester to transiently form an acyl-enzyme complex. The role of serine proteases in apoptosis has been mostly studied by observing whether particular apoptotic events can be prevented by inhibitors of these enzymes. Gorczyca et al. have shown that fragmentation of DNA in HL-60 cells treated with DNA topoisomerase inhibitors to induce apoptosis was prevented by irreversible inhibitors of serine proteases. But little is known about apoptosis-specific serine proteases. Granzymes A and B are serine proteases, which are abundant in granules of cytotoxic T-lymphocytes (CTL) (Masson and Tschopp, 1987;Pardo et al., 2004;Pardo et al., 2008) and natural killer (NK) (Ida et al., 2005) are the best characterised. Granzymes B can cleave procaspase-3, -6, -7, -8, -9 and -10 and most likely it activates endogenous caspases of the lymphocyte target cells, thereby inducing their apoptosis (Van de et al., 1997;Vandenabeele et al., 2005). But Granzyme A appears not to be associated with activation of caspases and it cleaves proteins independently of the latter (Martinvalet et al., 2008).

Compared to other class of non-caspase proteases, with the exception of Granzymes B, relatively little is known regarding the role of serine proteases in apoptotic signalling events (MacDonald et al., 1999;Zhao et al., 2007;Guo et al., 2008).

The limited number of apoptosis serine proteases characterised to date includes HtrA2/Omi and AP24 (Suzuki et al., 2001;Hegde et al., 2002;Seong et al., 2004). Omi can mediate apoptosis by interfering with inhibitor of apoptosis protein's function or via an independent trypsin-like serine protease activity (Martins et al., 2002;Yang et al., 2003). AP24 represents an alastase-like serine protease activity capable of inducing endonuclease L-DNase II that translocates to the nucleus and activates DNA fragmentation (Altairac et al., 2003).

1.1.8 NF-κB

Nuclear factor-κB (NF-κB) are transcription factors, critically regulate the outcome of cellular effect in normal and neoplastic cells. It has multitude functions in various pathways, such as synthesis and release of cytokine, intracellular signal transduction, and the inflammatory

response in both normal and abnormal cells (Zhou et al., 2010). They consist of five homologous subunits, RelA/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2 which dimerise and form inactive ternary complex with IκBs, the inhibitors of NF-κBs including IκBα in the cytoplasm. On activation, IκBα is phosphorylated and undergoes ubiquitin-proteasome mediated degradation and the released dimeric p65/p50 complex becomes active. The free NF-κB translocates to and accumulates within the nucleus where it is free to associate with cognate κB elements in target gene promoters (Karin and Ben Neriah, 2000;Naugler and Karin, 2008).

It has long been known that NF-κB signalling promotes the development of cancer by inhibiting apoptosis. (Van Antwerp et al., 1996). Several target genes of NF-κB include Bcl-2 family members such as Bcl-xL, IAPs (inhibitors of apoptosis), and c-FLIP (Karin, 2006) prevent apoptosis. NF-κB also indirectly prevents mitochondria-mediated apoptosis through neutralisation of ROS (through induction of manganese superoxide dismutase or ferritin heavy chain). Many investigators have observed that resistance to apoptosis in human cancer cell lines may be dependent on activation of NF-κB because when NF-κB is inhibited, apoptosis can be triggered more readily (Bernal-Mizrachi et al., 2006;Chen et al., 2006;Mi et al., 2007;Singh et al., 2007). NF-κB-induced apoptosis resistance has been implicated in chemotherapeutic failures in cancer treatment, and thus inhibiting NF-κB activation could be a potential way of therapy to treat tumours.

1.2 Epigenetics in cancer

Formerly, cancer was considered to be a genetic disease, caused by mutations of genes in somatic cells by mutagens, chemicals that damage DNA, or viruses. As a matter of fact, manifestation of cancer has been thought to be a result of alteration in the genes of cell cycle check points, tumour suppressor genes and proto-oncogenes (Boveri, 2008), Later, epigenetic events, such as histone modification and DNA methylation of CpG islands located in the promoter regions of a number of tumour suppressor genes have also been correlated with aberrant gene expression. It has now been realised that dysregulation of epigenetics has also a significant contribution to the process of carcinogenesis (Jones and Baylin, 2007).

Epigenetics is heritable changes in gene expression without changing the DNA sequence (Yoo and Jones, 2006;Goldberg et al., 2007). Epigenetic events play an important role in the initiation and progression of cancer. The most important epigenetic events are DNA methylation and histone tail modifications such as acetylation, methylation, phosphorylation and ubiquitination (Yoo et al., 2006). Alteration or dysregulation of these events can favour manifestation of cancer

growth. Therefore it has been now realised that the reactivation of epigenetically inactivated genes could be a novel strategy for cancer treatment.

1.2.1 DNA methylation

DNA methylation is a covalent chemical modification in which the addition of a methyl group to the carbon-5 position of cytosine residues occurs in DNA. Most cytosine methylation occurs in the sequence context 5'CG3' (CpG dinucleotide). This reaction is catalysed by at least three DNA methyltransferases (DNMT): DNMT1, DNMT3a and 3b in mammals. (Ramsahoye et al., 2000;Prokhortchouk and Defossez, 2008). Association of DNMT and establishment of newly methylated promoters were shown with proteins including Rb, E2F1, HDACs, histone methyltransferase and transcription repressor (Robertson et al., 2000;Fuks et al., 2003).

Methylation of DNA may affect the transcription of genes in two ways: First, it involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters which contain methyl-CpG residues (Singal and Ginder, 1999), and the second mode of repression involves a direct binding of specific transcriptional repressors to methylated DNA (Singal et al., 1997;Singal et al., 2001;Prokhortchouk and Hendrich, 2002). It can also affect histone modifications and chromatin structure (Das and Singal, 2004). DNA methylation is one of the most studied epigenetic mechanisms, it plays a crucial role in the development of nearly all kind of cancer (Jaenisch and Bird, 2003). Methylation changes have been implicated in various types of cancers such as colon cancer (Veigl et al., 1998), renal cell cancer (Morrissey et al., 2001), breast cancer and lung cancer (Virmani et al., 2001), GIT cancer (Kawakami et al., 2000) and lymphoma (Garcia et al., 2002).

1.2.2 Histones

Histones are among the most evolutionarily conserved and major proteins bound with DNA in chromatin in the nucleosome of eukaryotic cells (Kornberg and Lorch, 1999). Biochemically, they are small basic proteins with molecular weight of between 11 kDa and 20 kDa. They contain a high proportion of the positively charged amino acids lysine and arginine. They are classified into five classes, such as H1, H2A, H2B, H3, and H4, and further these proteins are categorised into two groups: core histones (H2A, H2B, H3 and H4) and linker histone (H1). Structurally, each core histone comprises a central fold domain, an N terminal and C terminal tails. The fold domain is involved in the formation of assembly of the histone octamer by association of an H3-H4 tetramer and two H2A-H2B dimers. The extending tails are responsible

for normal functioning of cellular process such as replication and transcription (Hadnagy et al., 2008). H3 and H4 tails are the more susceptible region for various posttransitional modifications. Furthermore, each histone octamer with C terminal at core and N terminal domain extending out is wrapped with 147 base pairs of DNA to become a nucleosome which again is organised into repetitive units of nucleosomes linked by H1 to form a higher order complex as chromatin (Luger and Hansen, 2005;Luger, 2006;Mendez-Acuna et al., 2010).

1.2.3 Histone posttranslational modifications

Histones are the core proteins of nucleosomes. Posttranslational modification of histones occurs through the formation of reversible covalent modifications in amino acids such as serine and threonine phosphorylation, lysine acetylation, lysine and arginine methylation, lysine ubiquitylation, biotinylation and sumoylation, as well as poly-ADP-ribosylation in arginine and glutamate, and regulate in part gene expression by altering chromatin organization (Mendez-Acuna et al., 2010).

1.2.3.1 Histone methylation

Methylation of histone H3 at lysine 79 (H3-K79-me) by HMT DOT1 is an important event for the localisation of 53BP1 at DSBs, implying an alternative strategy for recruitment of 53BP1 in DSBs. This lysine is constitutively methylated and changes in chromatin conformation after DSB induction to expose it, leading to 53BP1 recruitment (Huyen et al., 2004).

In addition, methylation of histone H3 at lysines 4, 36 and 79 (H3-K4-me, H3-K36-me and H3-K79-me) represents the transcription activation, whereas methylation of lysines 9, 27 from histone H3 and lysine 20 from histone H4 (H3-K9-me, H3-K27-me and H4-K20-me) is correlated with transcription repression (Kouzarides, 2007). H3-K79 and H4-K20 are also methylated in response to UV irradiation and are important for an efficient repair of UV-induced damage (Sanders et al., 2004;Bostelman et al., 2007).

1.2.3.2 Histone phosphorylation

Histone phosphorylation has a critical role in the regulation of mitosis, cell death, repair, replication and recombination (Ito, 2007). ATM kinase or ATR kinase or DNA-dependent protein kinase (DNA-PK) mediated serine 139 phosphorylation in H2AX, termed γH2AX, also occurs in response to DNA double-strand breaks for recruitment of DNA-damage-response proteins, including DNA damage checkpoint proteins (Fernandez-Capetillo et al., 2002;Unal et

al., 2004). In addition, histone H2B or H4 phosphorylation also have a key role in response to DNA double-strand breaks, apoptosis, meiosis and transcription activation events (Ito, 2007). However, the detailed mechanism is yet to be identified.

1.2.3.3 Histone ubiquitylation

Ubiquitin (Ub) is a 76 amino acid protein that is ubiquitously distributed and highly conserved throughout eukaryotic organisms. In histones, ubiquitylation is induced by ATR-dependent ubiquitylation on histone H2A and UV-damaged DNA-binding (DDB) complex and by the ubiquitin ligase (E3) CUL4A complex on temporary H3 and H4 due to UV irradiation (Bergink et al., 2006). Moreover, H3 and H4 are ubiquitylated early in the DDR than H2A ubiquitylation. It is important to note that ubiquitylated H3 and H4 can reduce nucleosomal stability, and the UV-DDB complex brings a chromatin environment that facilitates the assembly of the NER complex on damaged DNA (Wang et al., 2006).

Furthermore, ubiquitylated lysines facilitate an environment that favours interaction of proteins containing ubiquitin-interacting motifs, and leads to accumulation of downstream DDR factors at sites of DNA damage (Yan et al., 2007). The recently identified ubiquitin ligase enzyme RNF8 (RING finger-containing nuclear factor 8) has shown to catalyse regulatory ubiquitylation at sites of DSBs (Panier and Durocher, 2009), that is later maintained by RNF168 (van Attikum and Gasser, 2009). Moreover, the RNF8-mediated ubiquitylation induce recruitment of downstream signalling molecules such as 53BP1 and BRCA1 (Huen et al., 2007;Mailand et al., 2007;Wang et al., 2007).

1.2.3.4 Histone acetylation

Acetylation and deacetylation of ε-amino group of conserved lysine residue in histone tail is an important reversible biochemical reaction in which an acetyl moiety is enzymatically transferred by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Since this process has a key role in the alteration of chromatin or nucleosomal architecture that influences the accessibility of regulatory factors in the regulation of transcription and expression of genes, much attention has been paid, and it is now a well characterised posttranslational modification (Grant, 2001;Gregory et al., 2001;Ellis et al., 2009;Lane and Chabner, 2009a;Sawan and Herceg, 2010).

1.2.3.5 Histone acetyltransferases (HATs)

HATs are categorised into nuclear or A-type HAT proteins and cytoplasmic B-type HATs, and grouped into three major families: the MYST family containing MOZ, Ybf2/Sas3, Sas2, Tip60, the Gcn5 related N-acetyltransferase or GNAT/PCAF family, and the p300/CBF family (Sterner and Berger, 2000;Roth et al., 2001;Vetting et al., 2005;Hodawadekar and Marmorstein, 2007;Allis et al., 2007). In addition, there are a number of putative acetyltransferases (i.e. Spt10), which contain motifs similar to those found in HATs but their acetyltransferase activity remains to be identified (Neuwald and Landsman, 1997). Though the nuclear HATs utilise common acetyl-CoA and acetyl-lysine as substrate, they were observed with diverged protein sequence and provide explanation for difference in the substrate specificity and biological activity. The Gcn5 and PCAF consist of three conserved sequence motifs (A, B and D) that are shared with other acetyltransferases, including serotonin acetyltransferase and spermidine acetyltransferase (Neuwald et al., 1997). Some of the other GNAT members contain another conserved C motif whereas the MYST proteins have sequence homology only with motif A of GNAT proteins. However, the p300/CBP family has no homology sequence with either of the Gcn5/PCAF or MYST HATs. Furthermore, several HATs within the Gcn5/PCAF and MYST family have been identified as multisubunit complexes in vivo and believed to act on nucleosomes (Marmorstein and Roth, 2001). HATs, such as PCAF and p300/CBP, have also a role in the acetylation of non-histone proteins, e.g. transcription factors such as p53 and MyoD (Glozak et al., 2005;Zhang and Dent, 2005).

1.2.3.6 Molecular mechanism of HATs

Recent studies on the mechanism of HATs have supported the single step catalytic mechanism, in which lysine directly attacks the acetyl-CoA from the ternary complex enzyme acetyl-CoA histone-peptide (Berndsen et al., 2007) (Figure 3). In this mechanism, acetyl-CoA and the protein substrate bind the active site of HAT to form a ternary complex. Simultaneous deprotonation of the lysine by an active-site glutamate (base) allows nucleophilic attack on the carbonyl carbon of acetyl-CoA unstable tetrahedral intermediate forms, which then turn into CoA and the acetylated protein as the reaction products.

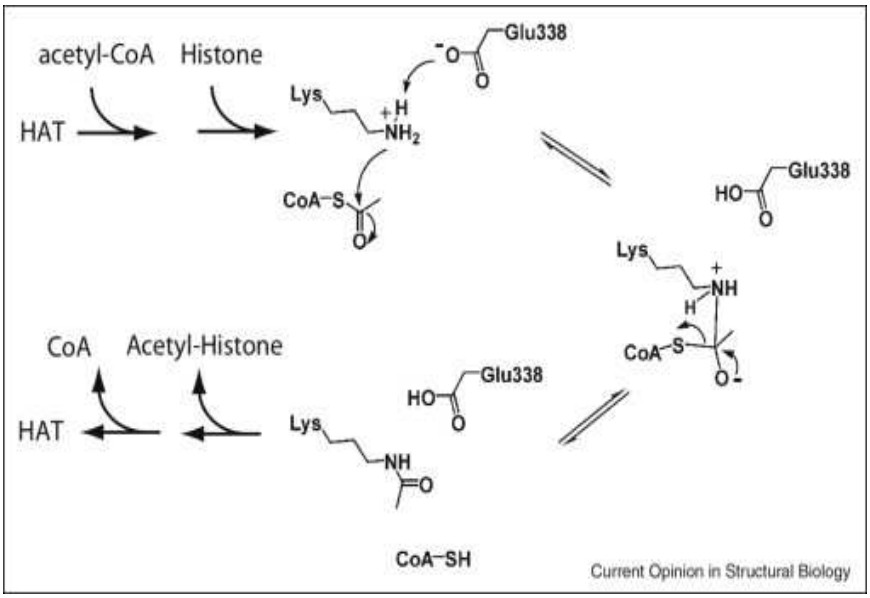

Figure 3 Enzymatic of HATs.

The acetyl-CoA and peptide substrate bind on active site of HAT to form a ternary complex as acetyl-CoA, peptide substrate and active site glutamate (e.g. Glu338 from Esa1). Deprotonation of the ε-amine of substrate lysine allows lysine attacks the carbonyl carbon of the acetyl moiety of acetyl-CoA forming a tetrahedral intermediate, which later forms CoA and acetylated product (Berndsen et al., 2007).

1.2.3.7 Histone deacetylases (HDACs)

HDACs are enzymes responsible for removal of the acetyl moiety from acetylated histones. Currently, eighteen HDACs have been identified in humans and grouped into four classes (Mariadason, 2008): Classes I, II and IV are zinc-dependent metalloproteins and class III are NAD^+-dependent enzymes.

i) Class I HDACs are HDAC 1, 2, 3 and 8, which are homologous to yeast Rpd3. They are ubiquitously expressed and mostly found in nucleus, smaller in size (350-500 amino acids). In the deacetylase domain, a small C-terminal region is often subject to posttranslational modifications, like phosphorylation, ubiquitination and sumoylation (de Ruijter et al., 2003;Yang and Seto, 2008).

ii) Class II has two subgroups. a) Class IIa is represented by HDAC4, 5, 7 and 9 and b) Class IIb consists of HDAC6 and 10. Class II enzymes are able to shuttle in and out of the nucleus in response to certain cellular signals, and they share domains similar with HDAC I (Verdin et al., 2003).

iii) Class III HDACs consist of the large family of sirtuins (SIRs) from group SRIT 1 to 7 that are evolutionarily distinct, with a unique enzymatic mechanism dependent on the

cofactor NAD^+, and are not targeted by the currently available HDAC inhibitors under development (Finnin et al., 2001;Witt et al., 2009).

iv) Class IV HDAC (HDAC 11) is found in the nucleus, and interacts with HDAC6 in vivo (Gao et al., 2002;Ledent and Vervoort, 2006).

Table 1 Properties of zinc-dependent HDACs (Bertrand, 2010)

Group	Size (aa)	Location chromosome	Cellular distribution	Complex	Role
Class I (type Rpd3)					
HDAC1	483	1p34	N	Sin3, NURD	TC
HDAC2	488	6q21	N	Sin3, NURD	TC
HDAC3	428	5q31	N	NCOR1/NCOR2-GPS2-TBL1X	
HDAC8	377	Xq13	N		TC
Class II (type Had1)					
IIa					
HDAC4	1084	q37.2	N, C	NCOR1/NCOR2	TC
HDAC5	1122	17q21	N, C		TC
HDAC7	855	12q13.1	N, C	Sin3, NCOR2	TC
HDAC9	1011	p21-p15	N, C		
IIb					
HDAC6	1215	Xp11.22–23	N, C		
HDAC10	669	22q13.31	N, C	NCOR2	TC
Class IV					
HDAC11	347	3p25.2	N		

Size is expressed in amino acid number, N: nuclear, C: cytoplasm, TC: transcription corepressor.

The differences between the zinc-dependent classes I, II and IV HDACs (Gregoretti et al., 2004;Ledent et al., 2006;Ocker and Schneider-Stock, 2007;Witt et al., 2009) are presented in Table 1 according to size (number of amino acids), cellular distribution and interactions with transcription factors.

1.2.3.8 Molecular mechanism of HDACs

The mechanism of action of the HDAC enzymes involves removing of the acetyl group from the histones. Hypoacetylation results in DNA condensation leading to transcriptional repression. The catalytic domain of HDAC is formed by a stretch of around 390 amino acids consisting of a set of conserved amino acids. The active site consists of a gently curved tubular pocket with a wider bottom. Removal of an acetyl group occurs via a charge-relay system (Finnin et al., 1999). An essential component of the charge-relay system is the presence of a Zn^{2+} ion. This ion is bound to the zinc binding site on the bottom of the pocket. However, other cofactors are required for HDAC activity (Figure 4).

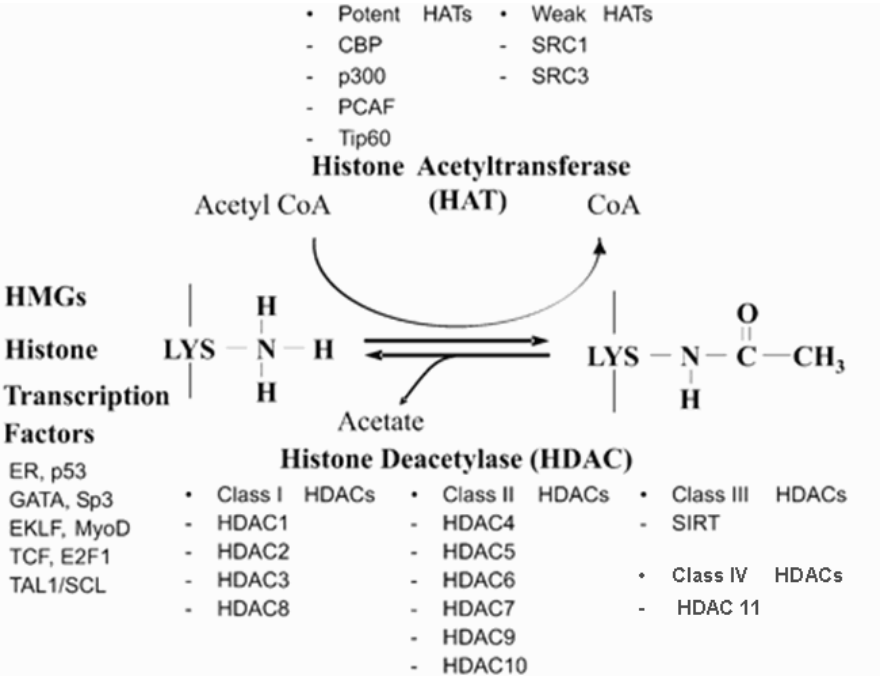

Figure 4 Dynamic histone acetylation is catalysed by HATs and HDACs (Davie, 2003).

HDAC enzymes deacetylase not only histone proteins, they also have the ability to deacetylase non-histone proteins both in cytoplasm and nucleus. These non-histone proteins include transcription factors, e.g. p53, E2F1-3 (Mujtaba et al., 2004), transcriptional regulators Rb, CtBP2, signal transduction mediators STAT3 and viral proteins E1A (Yang et al., 2008;Ray et al., 2008).

1.3 Histone deacetylase inhibitors (HDACi)

Histone deacetylase inhibitors (HDACi) are a novel class of anticancer agents, initially identified by their ability to reverse the malignant phenotype of transformed cells (Kouraklis and Theocharis, 2002;Yoshida et al., 2003;Papeleu et al., 2005). It is now widely accepted that HDACi have broad spectrum anticancer activity in various cancer cells and animal model studies (Witt et al., 2009). Vorinostat or suberoyl anilide hydroxamic acid (SAHA) was the first

compound approved by the FDA for the treatment of refractory cutaneous T cell lymphoma (CTCL) (Lane et al., 2009;Tan et al., 2010).

Studies have shown that HDACi like vorinostat can induce growth arrest in both normal and transformed cells in culture. However, HDACi proficiently induce cell death only in various cancer cells including neuroblastoma, melanoma, leukaemia, multiple myeloma and breast, prostate, lung, ovary, colon carcinoma and many others (Marks and Breslow, 2007). Indeed, normal cells are up to tenfold more resistant to vorinostat-induced cell death compared to transformed cells (Ungerstedt et al., 2005). Vorinostat can also cause growth arrest, caspase-dependent apoptotic cell death and/or caspase-independent autophagic cell death (Rosato et al., 2003;Shao et al., 2004;Guo et al., 2004;Marks and Jiang, 2005). Furthermore, vorinostat as well as other HDACi have been shown to augment the efficacy of other anticancer therapy modalities, such as radiation therapy and several anticancer agents, including anthracyclins, fludarabine (Fludara), flavopiridol, imatinib (Gleevec), proteasome inhibitor bortezomib (Velcade), antiangiogenic agents and nuclear receptor ligands, such as all-trans retinoic acid and APO2L/TRAIL (Fuino et al., 2003;Yoshida and Melo, 2004;Marks and Dokmanovic, 2005;Bolden et al., 2006;Sonnemann et al., 2006). Trichostatin A (TSA) and valproic acid (VPA) have been shown to induce sensitisation of multidrug-resistant cancer cells to etoposide (Hajji et al., 2010).

1.3.1 Mechanisms of HDACi

The molecular mechanism of HDACi was determined in studies such as HDAC-like protein-vorinostat interaction (Finnin et al., 1999), HDAC8-hydroxamate interaction (Somoza et al., 2004;Vannini et al., 2004). These studies found that vorinostat binds in the active site pocket and chelates the zinc ion at the base of the catalytic pocket of HDAC. Since class I, II, and IV HDAC have zinc ion and highly conserved enzymatic pocket, majority of these compounds do not selectively inhibit individual HDACs. Except class III (sirtuins), all other HDACs or several members of HDAC family are simultaneously susceptible for HDACi-mediated inhibition (Witt et al., 2009;Lane and Chabner, 2009b).

1.4 Biological effects of HDACi on cancer cells

HDACi can mediate a diverse range of effects on cell growth and survival. HDACi have a global effect on gene expression through the activation and or repression of genes following hyperacetylation of histones and chromatin remodelling in transformed cells specifically than

normal cells (Lane et al., 2009). HDACi also have a profound inhibitory effect on many non-histone protein substrates of HDAC such as DNA binding transcriptional factors, transcriptional regulators, signal transductional mediators, DNA repair enzymes, nuclear import regulators, hormone receptors, inflammation mediators, chaperone proteins and cytoskeleton proteins, which regulate cell proliferation, differentiation and cell death (Xu et al., 2007). Hence, HDACi induce transformed cell death through transcription-dependent and transcription-independent mechanisms such as induction of growth arrest, extrinsic and intrinsic apoptosis pathway, senescence, mitotic cell death, autophagic cell death and antiangiogenesis (Minucci and Pelicci, 2006;Xu et al., 2007).The above magnitude of biological effects varied depending on the nature of HDACi, concentration, exposure time and cell context.

1.4.1 Differentiation

HDACi have drawn much attention because of their ability to induce differentiation of malignant cells in cultures. TSA, hexamethylene bisacetamide and their analogs have been shown to induce differentiation in vitro. Indeed, vorinostat was identified through its differentiation-inducing property, and later its inhibitory effect on HDAC was recognised (Marks et al., 2007).

1.4.2 Cell cycle arrest

HDACi are capable of inducing cell cycle arrest effectively in various malignant cells. At low concentration, HDACi favourably induce G_1 arrest whereas at high concentration they induce both G_1 and G_2/M cell cycle arrest (Richon et al., 2000). The G_1 and G_2/M cell cycle arrest brought by inhibition or downregulation of HDAC which in turn favours the activation of cyclin-dependent kinase inhibitor 1A, (p21Waf1/cip1) (Richon et al., 1996;Rajgolikar et al., 1998;Richon et al., 2000) which has been shown to be upregulated by almost all HDACi (Johnstone, 2002). The activation of p21 occurs in a p53-independent manner and is necessary for the inhibition of CDK4/6 regulating G_1 progression, CDK2 regulating G_1/S transition, proliferating cell nuclear antigen that is required for DNA replication (Vidal and Koff, 2000) and cdc2/CDK1 regulating G_2/M transition (Xu et al., 2007). Moreover, TSA also promotes G_1 cell cycle arrest in cells without p21 through the activation of p15 (INK 4b), a cyclin D-dependent kinase inhibitor.

1.4.3 Apoptosis

HDACi have been accounted for inducing cell death through efficiently activating both extrinsic and intrinsic apoptotic pathways in many transformed cells in vitro as well as in vivo (Xu et al., 2007).

1.4.3.1 Activation of the extrinsic apoptotic pathway by HDACi

A number of studies have demonstrated the ability of HDACi to activate the genes encoding both death receptors and cognate ligands and suppressing the genes responsible for inhibiting components of death receptor pathway (Insinga et al., 2005b;Frew et al., 2009) in transformed cells but not in normal cells. The Fas and FasL were activated by HDACi in neuroblastoma cells (Glick et al., 1999), osteosarcoma xenografted mice and mouse model of APL (Insinga et al., 2005a). TRAIL, DR-5, and TNF-α have been shown to be upregulated in various cancer cells (Rosato et al., 2003).

1.4.3.2 Activation of the intrinsic apoptotic pathway by HDACi

Induction of intrinsic apoptotic pathways is crucial pathway through which HDACi mediate cell death in cancer cells. HDACi cause cancer cells to release cyt C from mitochondrial intermembrane space and activate caspase-9 (Bolden et al., 2006). Moreover, treatment with these compounds results in upregulation of a large number of Bcl-2 family proapoptotic genes such as Bim, Bmf, Bax, Bak, Noxa, PUMA and repression of antiapoptotic genes Bcl-2 and Bcl-xL (Zhao et al., 2005;Xu et al., 2006). It has been reported that HDACi enhance Bim gene expression by activating E2F1 (Zhao et al., 2005).

1.4.4 Antiangiogenesis

HDACi are capable of inducing antiangiogenesis through repressing proangiogenesis factors genes such as HIF-1α and VEGF in various malignant cells (Bolden et al., 2006;Liang et al., 2006). Moreover, these compounds can induce degradation of HIF-1α by VHL dependent and independent mechanism (Kong et al., 2006;Lane et al., 2009) and prevent endothelial cell from angiogenic stimulus generated by VEGF (Deroanne et al., 2002). Apart from the above biological effect, HDACi also mediate other antitumour effect through formation of reactive oxygen species (ROS), inducing autophagy, senescence, mitotic cell death, inactivation of chaperonin HSP90, and disruption of aggresome pathway. These diverse biological effects of HDACi contribute to their antineoplastic behaviour in cancer cells but not normal cells.

1.5 Structural classes of HDACi

Around 80 HDAC inhibitors have been purified as natural products or synthetically produced (Acharya et al., 2005) and these can be subdivided into six groups: Short-chain fatty acids: e.g.

Table 2 Classification of HDACi (Bolden et al., 2006;Zhou and Zhu, 2009)

Classes	HDACi	Tumours with clinical benefits (http://clinicaltrials.gov/)	Clinical trial	References
Class I Short chain fatty acids	Valproic acid (VPA)	Acute myeloid leukaemia	(I/II)	(Bolden et al., 2006)
	Phenylbutyrate (PB)	Acute myeloid leukaemia	(I/II)	
	Sodium butyrate (NaB) (only in vitro)			
	Phenylacetate (PA) AN-9			
Class II Hydroxamic acid derived compounds	Suberoyl anilide hydroxamic acid (SAHA/vorinostat)	Cutaneous T-cell lymphoma (approved), thyroid cancer, bladder cancer	(I/II/III/IV)	(Marks et al., 2007;Tan et al., 2010)
	Trichostatin A (TSA), (only in vitro studies)			
	LBH589, LAQ824,	Cutaneous T-cell lymphoma	LBH589 (I/II/III)	(Bhalla, 2005)
	PXD101 and tubacin	Chronic lymphocytic leukemia	PXD101 (I/II)	(Bhalla, 2005)
Class III Benzamides	MS-275	Acute myeloid leukemia, melanoma	(I/II)	(Bolden et al., 2006)
	CI-994	Colon cancer, renal cancer	(I)	
Class IV Epoxyketones	Trifluromethyl ketone			
	α-ketoamides			
Class V Cyclic peptide	Depsipeptide (romidepsin, FK228/FR901228)	Cutaneous T-cell lymphoma, (approved) pancreatic cancer, prostate cancer	FK228 (I/II/III)	(Grant et al., 2010)
	CHAPs, HC-toxin and trapoxin			
	Apicidin	Acute myeloid leukaemia		
Class VI Hybrid Molecules	Depudecin and MGCD0103	Acute myeloid leukaemia, colon cancer and chronic lymphocytic leukemia	MGCD0103 (I/II)	(Blum et al., 2009)

VPA, phenylbutyrate, phenylacetate. (Newmark et al., 1994;Carducci et al., 2001). Hydroxamic acid derived compounds: e.g. trichostatin A (TSA) and vorinostat (Melnick and Licht, 2002;Marks and Xu, 2009), Cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-

decanoyl (AOE) moiety: e.g. romidepsin, apicidin, CHAPs, trapoxin (Taunton et al., 1996), Ketones: trifluromethyl ketone, alpha-ketoamides and benzamides: CI-994, MS27-275 (Saito et al., 1999). Different structural classes of HDACi used are described with clinical benefits in the Table 2.

1.6 Small molecule inhibitors

1.6.1 ATM inhibitors

Caffeine is a known inhihitor of both ATM and the related kinase, ATM and Rad3-related (ATR) protein kinase, and both are central components of the DNA damage response. ATM is activated by DNA DSBs, whereas ATR is preferentially activated by ssDNA regions, which can occur at stalled DNA replication forks (O'Driscoll et al., 2003). Caffeine is a relatively non-specific inhibitor of ATM, but the small molecule inhibitor 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933) has been shown to specifically inhibit ATM in the low nanomolar range (IC_{50}:12.9 nM). KU-55933 is a novel, specific, and potent inhibitor of the ATM kinase. It has been shown to sensitise cancer cells to both IR and chemotherapeutic agents but the impact of senescence suppression on this sensitising effect remains unclear (Hickson et al., 2004); (Cowell et al., 2005). A more recent study has shown that caffeine and KU-55933 induce cell death in prematurely senescent breast cancer cells (Crescenzi et al., 2008).

1.6.2 Serine protease inhibitors

Serine protease inhibitors are chemical substances that act by binding with serine residues of the active site of serine proteases and inhibit their proteolytic function. AEBSF (4-(2-aminoethyl)-benzenesulfonylfluoride), also known as Pefabloc or ISP, is one of the potent irreversible inhibitor of serine proteases (Rideout et al., 2001;Garcia-Morales et al., 2005). It mainly acts by sulfonylating the serine residue of the active site of the enzyme (Powers et al., 2002), but it may also covalently modify other proteins (Conboy et al., 2008).

In some apoptotic systems, the use of AEBSF has suggested that serine proteases participate in the activation of executioner caspases (de Bruin et al., 2003;King et al., 2004). Moreover, Egger et al., 2003 have utilised AEBSF to show indirect inhibitory effect on caspase-3 processing in FDC-P1 cell extracts and described endogenous serine protease requirement upstream of caspase-3 processing.

1.6.3 Nutlin-3

Nutlin-3 is a small-molecule, chemically (±)-4-(4,5-Bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl)-piperazin-2-one (Figure 5). It is a MDM2 inhibitor which restores p53 function and is, thus, an appealing candidate for the treatment of cancers retaining wt-p53. Nutlin-3 exerts antineoplastic effects in tumours retaining wt-p53 (Shangary et al., 2009). The imidazoline compound nutlin-3 was the first reported to have anticancer activity in vivo (Vassilev et al., 2004). Subsequent analyses revealed that nutlin-3 was efficacious in several tumour models (Tovar et al., 2006), and it has recently entered phase I clinical trials with patients suffering from advanced solid cancers (Hoffmann-La Roche, 2007) or hematologic malignancies (Hoffmann-La Roche, 2008).

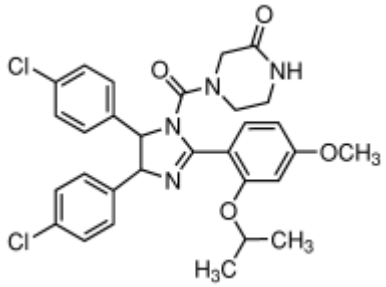

Figure 5 Structure of nutlin-3

2 OBJECTIVE

The objective of the current study was to elucidate the mechanisms by which HDACi induce cell cycle arrest and cell death either as single agent or in combination with other anticancer agents. The work deals with four parts:

1. In addition to eliciting cell death, HDACi have been shown to induce G_1 and/or G_2/M cell cycle arrest. HDACi-induced G_1 cell cycle arrest is accomplished by induction of $p21^{WAF1}$ expression, but little is known on the mechanism of HDACi-induced G_2/M arrest. Hence, the first part of this study was to investigate the mechanism of HDACi-induced G_2/M cell cycle arrest.

2. Of the fifty percent of human tumours with wt-p53, many are thought to have compromised p53 function due to increased MDM2 levels, which is a negative regulator of p53. Activation of the p53 pathway by using nutlin-3, a MDM2 antagonist, might offer a new therapeutic approach for tumours with wild-type p53. HDACi have been observed to activate p53 through acetylation. Thus, the second part of this study was to explore whether the anticancer activity of nutlin-3 could be enhanced by combination with HDACi.

3. Only about 10% of ES patients have been found with p53 alterations. Thus, the 90% of ES patients with wt-p53 are potentially amenable to nutlin-3 treatment, suggesting that targeting and activation of p53 may be an effective therapeutic strategy for ES. Hence, the third part of this study was to investigate the anticancer effects of nutlin-3 in ES cells.

4. The aim of the last part of this work was to verify the involvement of serine proteases in HDACi-induced apoptosis by using serine protease inhibitor.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell lines used

- WE-68, VH-64 are wt-p53 ES cell lines and SK-ES-1 cells are mt-p53 ES cell line, were provided by Dr. F. van Valen from Münster, Germany and CADO-ES-1 cells (wt-p53 ES cell line) were obtained from the DSMZ from Braunschweig, Germany, and were maintained in RPMI-1640. (PAA, Cölbe, Germany).
- A549 cells are lung cancer cell line with p53 wild-type (O'Connor et al., 1997) were obtained from ATCC (Manassas, VA, USA), and cells were maintained in Ham's F12K (PAA).
- PC-3 cells are prostate cancer cell line with p53 null type (O'Connor et al., 1997) were obtained from the DSMZ. Cells were maintained in RPMI-1640 (PAA).
- A2780 cells were maintained in RPMI-1640 and SKOV-3 cells were maintained in McCoy's 5A medium. (Dr. J. Braunger, Altana Pharma, Konstanz, Germany). Both cells are ovarian cancer cell lines.
- MCF-7 cells are breast cancer cell line without caspase-3, were maintained in RPMI-1640.

3.1.2 Reagents for cell culture

Reagent	**Source**
Dulbecco's PBS 1x (without Ca^{2+} and Mg^{2+})	PAA, Cölbe
Fetal calf serum (FCS)	PAA, Cölbe
Ham's F-12 (without L-Glutamin)	PAA, Cölbe
Collagen solution	Roche, Darmstadt
Penicillin	PAA, Cölbe
PCR mycoplasma test kit	Applichem, Darmstadt
RPMI-1640 (with stable Glutamine)	PAA, Cölbe
Stable glutamine	PAA, Cölbe
Streptomycin	PAA, Cölbe

Trypan blue	Biochrom AG, Berlin
Trypsin/ Ethylene diamine tetra acetate (EDTA) (1x)	PAA, Cölbe

3.1.3 Agents for functional test

Cytostatics agent	**Source**
Doxorubicin	Alexis, Grünberg
Paclitaxel	Teva, Mörfelden-Walldorf
Histone deacetylase inhibitors	
Apicidin	Alexis, Grünberg
MS-275	Alexis, Grünberg
NaB	Sigma, Deisenhofen
TSA	Alexis, Grünberg
Vorinostat	Alexis, Grünberg
NF-κB inhibitors	
BAY 11-7082	Alexis, Grünberg
Caffeic acid phenylethyl ester	Alexis, Grünberg
Flavopiridol (indirect)	Alexis, Grünberg
LY294002 (indirect)	Alexis, Grünberg
Roscovitin (indirect)	Alexis, Grünberg
Other inhibitors	
AEBSF (Serine protease inhibitor)	Alexis, Grünberg
Bortezomib (Proteasome inhibitor)	LC Laboratories, Woburn, MA, USA
Caffeine (ATM/ATR inhibitor)	Alexis, Grünberg
CHK2 inhibitor II	Calbiochem, Canada, US
KU-55933 (ATM inhibitor)	KuDOS pharmaceuticals, Cambridge, UK
Nutlin-3 (MDM2 inhibitor)	Alexis, Grünberg

3.1.4 Reagent for cell viability assay

Alamar Blue	Invitrogen, Darmstadt
Trypan Blue in 0.5 % PBS	Biochrom AG, Berlin

3.1.5 Agent for mitochondrial membrane potential loss measurement

3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) Molecular Probes, Eugene, OR, USA

3.1.6 Reagents for the caspase-3 activity test

Reagent	Chemical	Concentration	Source
Lysis buffer	Tris-HCl	10 mM	Roth, Karlsruhe
	$NaH_2PO_4/NaHPO_4$	10 mM	Merck, Darmstadt
	Triton X-100	1%	Sigma, Deisenhofen
	NaCl	130 mM	Roth
	$Na_4P_2O_7$	10 mM	Sigma
Caspase-AFC- buffer	HEPES (pH 7.5)	20 mM	Sigma
	Glycerol	10%	Merck
	DTT	2 mM	Sigma
	Caspase-3 substrate	25 µg/ml	Sigma

3.1.7 Caspase substrate and inhibitors

	Reagent	Concentration	Source
Caspase-3-substrate	Ac-DEVD-AFC in PBS	0.5 mg/ml	Bachem, Heidelberg
Caspases inhibitor	z-VAD-fmk pan-caspase inhibitor	20 µg/ml	Alexis, Grünberg

3.1.8 Reagents for flow cytometry analysis

	Chemical	Concentration	Source
Cell cycle analysis	PBS with 1% glucose		PAA, Cölbe
	RNase A	50 µg/ml	Roche, Mannheim
	Propidium iodide	50 µg/ml	Sigma, Deisenhofen
Cell death analysis	Propidium iodide in PBS	2 µg/ml	Sigma

3.1.9 Reagents for Western blot analysis

Reagent	Chemical	Concentration	Source
Lysis buffer	PBS		PAA
	Tris-HCL (pH-7.4)	40 mM	Sigma
	NaCl	150 mM	Roth
	Triton X-100	1%	Sigma
	Na-desoxycholate	0.5%	Sigma
	SDS	0.1%	Roth
Protease inhibitor (Complete, Mini, EDTA-free)	Serine and cysteine proteases inhibitor cocktail		Roche
Stacking gel	Acrylamide/Bisacrylamide (37.5/1) (Rotiphorese ® Gel 30)	5%	Roth
	Tris-HCl (pH 6.8)	0.125 mM	Sigma
	SDS	10%	Roth
	H_2O		Braun
	APS	10%	Sigma
	TEMED		Bio-Rad
Running gel			
High percentage gel	Acrylamide/Bisacrylamide (37.5/1)	15%	Roth
	Tris-HCl (pH 8.8)	0.38 mM	Sigma
	SDS	10%	Roth
	H2O		Braun
	APS	10%	Sigma
	TEMED		Bio-Rad
Low percentage gel	Acrylamide/Bisacrylamide (80/1)	8%	Roth
	Tris-HCl (pH 6.8)	0.125 mM	Sigma
	SDS	10%	Roth

	H_2O		Braun
	APS	10%	Sigma
	TEMED		Bio-Rad
Loading buffer	Tris-HCl (pH 6.8)	0.625 M	Sigma
	Glycerol	5 ml	Merck
	SDS	10%	Roth
	β-Mercaptoethanol	0.5 ml	Sigma
	Bromophenolblue (w/v)	1%	Sigma
	Ethanol		Roth
	H_2O		Braun
Electrophoresis buffer (10x)	Tris-HCl (pH 8.3)	25 mM	Sigma
	Glycine	192 mM	MP Biomedicals, Illkrich, France
	SDS	0.2%	Roth
Electro transfer buffer	Tris-HCl (pH 8.3)	5 mM	Sigma
	Glycine	38 mM	MP Biomedicals
	SDS	0.2%	Roth
	Methanol (w/v)	20%	J. T. Baker, Deventer, The Netherlands
Wash buffer	Tris-HCl (pH 6.7)	62.5 mM	Sigma
	β-Mercaptoethanol	100 mM	Sigma
TBST buffer	Tris-HCl (pH 7.5)	4 mM	Sigma
	NaCl	100 mM	Roth
	Tween 20 (v/v)	0.05%	Merck
Blocking buffer	Non fat dry milk, 5% in TBST buffer		
Incubation buffer	Non fat dry milk, 5% in TBST buffer		

Miscellaneous	Acid sodium salt dihydrate	Roth
	99 % Glucose ($C_6H_{12}O_6$)	
	Albumin fraction V (Bovine serum albumin)	Roth
	B Rotiphorese gel 2% gel	Roth
	BCA^{TM} protein assay kit	Thermo Scientific
	Calcium chloride ($CaCl_2$)	Fluka Analytica
	Disodium hydrogen phosphate (Na_2HPO_4)	Merck
	Distilled water	Braun
	Dithiothreithol (DTT)	Sigma
	Ethylenediaminetetraacetic	Sigma
	H_2O_2	Sigma
	HCl	Sigma
	HEPES	Sigma
	(2 - [4 - (2-hydroxyethyl)-	
	1-piperazinyl]-ethanesulfonic acid)	
	Isopropanol	Merck
	Luminol	Sigma
	Milk powder	Roth
	p-Coumaric acid	Sigma
	Sodium dihydrogen phosphate	Merck
	monohydrate (NaH_2PO x H_2O)	
	Tris (tris [hydroxymethyl] aminomethane)	Roth
	Hyper filmTM	Amersham, Braunschweig
	PVDF membrane	Amersham

3.1.10 Antibodies for Western blot

Antibody	Type	Dilution	Source	Cat.No
Primary antibodies				
Anti-acetyl-p53 (Lys373/382)	Rabbit polyclonal	1:500	Upstate Temecula, CA, USA	06-758

Anti-acetyl histone-H3	Rabbit polyclonal	1:25,000	Upstate	06-599
p53 (DO-2)	Mouse monoclonal	1:500	Santa Cruz Heidelberg	sc-43394
Anti-GAPDH	Mouse monoclonal	1:100,000	Biodesign International	H86504M
Anti-phospho-ATM (Ser1981)	Mouse monoclonal	1:1000	Upstate	05-740
Anti-phospho-H2AX (Ser139)	Rabbit polyclonal	1:1000	Upstate	07-164
Phospho-p53 (Ser15)	Mouse monoclonal	1:1000	Cell Signaling	9286
pBRCA1 (Ser1524)	Rabbit polyclonal	1:1000	Cell Signaling	9009S
pFANCD2 (Ser222)	Rabbit polyclonal	1:1000	Cell Signaling	4945S
p95/NBS1 (Ser343)	Rabbit polyclonal	1:1000	Cell Signaling	3001S
pCHK1 (Ser345)	Rabbit monoclonal	1:1000	Cell Signaling	2348
pCHK2 (Thr68)	Rabbit polyclonal	1:1000	Cell Signaling	2661
pAkt (Ser473)	Rabbit monoclonal	1:1000	Cell Signaling	4058
pMDM2 (Ser166)	Rabbit polyclonal	1:1000	Cell Signaling	3521
pRad-17 (Ser645)	Rabbit polyclonal	1:1000	Cell Signaling	3421

Secondary antibodies		
Peroxidase-conjugated goat anti-rabbit IgG	1:25,000	Dianova, Hamburg
Peroxidase-conjugated goat anti-mouse IgG	1:25,000	Bio-Rad, München

3.1.11 List of qRT-PCR primers for amplification of the genes used

Gene	Source
MDM2: Hs99999008_m1	Applied Biosystem, Darmstadt
MDM4: Hs00159092_m1	Applied Biosystem
p21: Hs00355782_m1	Applied Biosystem
PUMA: Hs00248075_m1	Applied Biosystem
β-2-microglobulin: Hs00187842_m1	Applied Biosystem

3.1.12 HDAC activity assay

Reagent	Chemical	Concentration
Lysis/ developing buffer	Tris-HCl, pH 8.0	50 mM
	NaCl	137 mM
	KCl	2.7 mM
	$MgCl_2$	1 mM
	Triton X-100	1 %
	Trypsine	2 mg/ml
Activity buffer	Tris-HCl, pH 8.0,	50 mM
	NaCl	137 mM
	KCl	2.7 mM
	$MgCl_2$	1 mM
	BSA	1 mg/ml

3.1.13 Experiment kits

Kit	Source	Cat.No
Protein assay kit Bicinchonic assay (BCA)	Pierce, Rockford, IL, USA	23225
Mycoplasma detection kit Mycoplasma PCR ELISA	Applichem, Darmstadt	A3744, 0020
Total RNA kit Peqgold total RNA isolation	Peqlab, Erlangen	12-6834-01
cDNA kit Omniscript	Qiagen, Hilden	205113

3.1.14 *List of equipments used*

Equipment	Source
Water bath / circulating MP-1	Julabo Labortechnik, Seelbach
Bio-Rad Mini-Protean system	Bio-Rad, München
CELL STAR culture flask (5, 20 ml)	Greiner, Frickenhausen
CELL STAR sterile pipettes (1, 2, 5, 10, 25 ml)	Greiner, Frickenhausen
Heraeus CO_2 incubator BBD® 6220	Kendro Laboratory products, Hanau
FACS Canto II® flow cytometer	Becton Dickinson, Heidelberg
TRA-14 automatic freezer	Cryoson, Schöllkrippen
Fiber pads	Bio-Rad, München
Gel electrophoresis chambers	Bio-Rad, München
Culture plate 6, 12, 96 well	Greiner, Frickenhausen
Phase contrast microscope CKX41	Olympus, Hamburg
Magnetic stirrer	Hei-Mix S Heidolph, Schwalbach
Carl Zeiss microscope	Carl Zeiss, Jena
Mini Trans-Blot electrophoresis	Bio-Rad, München
Multipette ® plus	Eppendorf, Hamburg
Neubauer counting chamber (Improved)	Fine Optics, Bad Blankenburg
Pasteur pipettes, sterile	COPAN, Murrieta, CA, USA
pH meter	Mettler -Toledo, Giessen
Accu-jet® pro pipette	Brand, Wertheim
Power supply	Bio-Rad, München
Spectrophotometer SLT spectra	Tecan, Männedorf, Switzerland
Spectrophotometer FLUROstar Omega	BMG Labtech, Offenburg
Clean bench Heraeus® Herasafe	Kendro Laboratory products, Hanau
Thermo compact mixer	Eppendorf, Hamburg
Centrifuge 5415 R	Eppendorf, Hamburg
Emmi® ultrasonic cleaner 20 HC	EMAG Technologies, Mörfelden-Walldorf
Scientific Industries vortex	Genie 2, NY, USA
Weighing balance	Sartorius, Göttingen
Hettich-centrifuge	Rotanta /TR-4400, Tuttlingen
7900 HT Fast Real-Time PCR system	Applied Biosystems, Darmstadt

All the unspecified reagents were purchased from Germany.

3.2 Methods

3.2.1 Cell lines and culture conditions

A549 cells (1.5 x 10^6) were cultured in Ham's F-12 medium and SKOV-3 cells were cultured (2 x 10^6) in McCoy's 5A medium. A2780 (1.5 x 10^6), MCF-7, PC-3 cells and Ewing's sarcoma cells (WE-68, VH-64, CADO-ES-1 and SK-ES-1) (1.5 x 10^6) were all cultured in RPMI-1640 medium, supplemented with 10 % foetal calf serum, 100 unit/ml penicillin G sodium, 100 µg/ml streptomycin sulphate and 2 mM L- glutamine. All cells were cultivated at 37°C in a humidified 5% CO_2 incubator. For ES cells collagen coated culture flask were used. The cells were allowed to grow a confluence of 70% to 80% and subsequently split by trypsination. Trypsin-EDTA treatment was performed by removing the medium from the cells, followed by washing the cells once with PBS and adding adequate amount of trypsin-EDTA. After 5 to10 min incubation at 37°C, cells were resuspended in fresh medium and centrifuged at 2500 rpm for 5 min (to remove trypsin-EDTA), and reseeded with fresh medium at the required density. The cells were routinely passaged. Cell viability was determined by the trypan blue dye exclusion test or by Alamar Blue assay. Cells were regularly inspected to be free of mycoplasma with mycoplasma detection reagents. To prepare cells for long time storage, logarithmically growing cells were trypsinised, harvested by addition of medium, and centrifuged at 2500 rpm for 5 min. The cell pellet was washed once with PBS at 2500 rpm for 5 min, the cells were resuspended in 75% Medium, 20% FCS and 5% DMSO and transferred in to cryo-vials. Cells were transferred into vials and freezed using TRA-14 automatic freezer before finally being stored in liquid nitrogen. To revive the cells, the cryo-vials were removed from the liquid nitrogen and thawed at 37°C. The cells were then mixed with 15 ml of fresh medium, centrifuged at 1500 rpm (to remove DMSO), and seeded in a culture flask with fresh medium.

3.2.2 Treatment of cells

Cells were seeded at a density of 1.5 x 10^5 or 2 x 10^5 cells/well in 6-well plates or 2000 cells/well in 96-well plates for 24 h prior to starting an experiment. Cells were treated either with the inhibitors caffeine, KU-55933, bortezomib, NF-κB inhibitors, CHK1, CHK2 inhibitor II, nutlin-3, AEBSF, z-VAD-fmk as indicated concentration for 1 h or left untreated before application of HDACi or paclitaxel or doxorubicin. The latter were added directly to the culture medium containing inhibitor without a medium change. Cells were then exposed to HDACi or paclitaxel for additional 24 h (caspase-3 activity, immunoblotting, quantitative PCR), 48 h (flow cytometric analyses) or 72 h (Alamar Blue assay).

3.2.3 Inhibitor experiments

3.2.3.1 Caspase inhibitor

For inhibition of caspase activity, approximately 2 x 10^5 cells were incubated with the irreversible, cell permeable pan caspase inhibitor z-VAD-fmk (20 μM) for an hour before treatment with the agents.

3.2.3.2 Serine protease inhibitor

For inhibition of serine protease activity, approximately 2 x 10^5 cells were incubated with serine protease inhibitor AEBSF for an hour before treatment with the agents. Different concentrations 50 μM, 100 μM and 200 μM of AEBSF were used.

3.2.3.3 MDM2 inhibitor

For inhibition of MDM2, cells were incubated with the MDM2 inhibitor nutlin-3 for an hour before treatment with the agents. Three different concentrations 2 μM, 3 μM and 10 μM of nutlin-3 were used.

3.2.4 Cytofluorometric analysis of cell death

To determine the cell death induced by HDACi in A549, MCF-7, A2780, PC-3 and ES cells, cytofluorometric analysis of propidium iodide (PI) uptake was used in this study; PI crosses the plasma membrane only after loss of its integrity (Bezvenyuk et al., 2000). Cells were seeded at 1 x 10^5 to 2 x 10^5 cells/well in 2 ml medium in 6 well culture dishes. After allowing cells to attach for 24 h, cells were treated with the indicated concentrations of HDACi (vorinostat, MS-275, NaB and apicidin). To determine cell death, cells were harvested using trypsin-EDTA and centrifuged for 10 min at 3000 rpm. Briefly after discarding the medium the cells were resuspended in 300 μl phosphate buffered saline (PBS), followed by 5 min incubation in 2 μg/ml PI at 4^{o}C. PI uptake was assessed by flow cytometry analysis on a FACS Canto II using DIVA software. 10,000 cells were analysed in each sample; data were gated to exclude debris. Each experiment was performed a minimum of three times.

3.2.5 Quantification of apoptotic cells

3.2.5.1 Cell cycle analysis

The effect of chemotherapeutic agents on cell proliferation was evaluated by measuring the distribution of cells in different phases of the cell cycle by flow cytometry. This determination was based on the measurement of the DNA content of nuclei labeled with PI (Vindelov and Christensen, 1990). Internucleosomal DNA fragmentation is a typical sign of apoptotic cell death. A549, MCF-7, A2780 and PC-3 cells were seeded at 1 x 10^5 to 2 x 10^5 cells/well in 2 ml medium in 6-well culture plates. After growing cells for 24 h, cells were treated with the indicated concentrations of HDACi (vorinostat or MS-275 or NaB or apicidin), or cytostatics. After treatment the cells were harvested using trypsin-EDTA and centrifuged for 10 min at 3000 rpm. Cells were washed twice with PBS to remove left over culture medium. Briefly after discarding the washing solution the cell pellet was resuspended in the left over PBS. Ice-cold 70% ethanol was added dropwise while vortexing, in order to avoid cell clumping. Cells were fixed overnight at 4^{o}C. After fixing, the cells were centrifuged for 10 min at 3000 rpm and ethanol was removed completely. Cells were resuspended in 500 µl assay buffer containing phosphate buffered saline with 1% glucose, 50 µg/ml RNase A, and 50 µg/ml PI. The cells were incubated for 30 min at room temperature. The DNA content of the cells was determined on a FACS Canto II flow cytometer. Data were accumulated by the DIVA software from BD Biosciences. Percentage of cells in different phases of the cell cycle was calculated as area under the distribution curve. Percentage of hypo-diploid cells, the cells which are undergoing death was assessed by quantitating the sub-G_1 peak. In all experiments, 20,000 cells were collected; data were gated to exclude debris. Each experiment was performed a minimum of three times.

3.2.5.2 Caspase activity

In addition to internucleosomal DNA fragementation, other characteristic events typical of apoptosis can be used as end points. Activation of caspases is an early marker of the cells undergoing apoptosis. Measurement of caspase activity is one of the crucial parameters in analysis of apoptotic cell death. Here we chose to study caspase-3 activities. All known caspases possess an active site cysteine and cleave a specific amino acid sequence after aspartic acid residues (D). The synthetic substrates used for caspase assay were Ac-DEVD-AFC Trifluoromethylcoumarin (AFC) is a fluorescent compound, but fluorescence is blocked until the compound is cleaved off from the rest of the substrate. Cleavage of this substrate is cleaved mainly by the specific caspases.

Caspase-3 activities were measured 24 or 48 h after treatment with agents using the synthetic fluorogenic substrate Ac-DEVD-AFC for determining caspase-3 activities. A549, MCF-7, A2780 and PC-3 cells were seeded at 1.5 x 10^5 to 2 x 10^5 cells/well in 2 ml medium in 6-well culture plates. After 24 h, cells were treated with the indicated concentrations of HDACi (vorinostat or MS-275 or NaB or apicidin), or cytostatics. After 24 h or 48 h treatment the cells were harvested using trypin-EDTA and centrifuged for 10 min at 3000 rpm. Cells were washed twice with PBS to remove left over culture medium. Once medium was completely removed, the cell pellet was lysed in 100 µl of ice cold lysis buffer on ice for 30 min. Once the cells were completely lysed, the activity of caspase in cytosolic extracts was assayed in the caspase buffer. After incubation at 37^{o}C for 2 h, the released AFC was analysed on Omega fluorometer using an excitation and emission wavelength of 390/510 nm. Relative caspase activities were calculated as a ratio of emission of treated cells to untreated cells.

3.2.5.3 Cytofluorometric analysis of mitochondrial membrane potential loss

Mitochondrial membrane potential ($\Delta\psi_m$) loss was determined by assessing the accumulation of the cationic lipohilic flurochrome 3, 3'-dihexyloxacarbocyanine-iodide ($DiOC_6(3)$) in the mitochondrial matrix. $DiOC_6(3)$ is a positively charged molecule that permeates through the plasma membrane. At low concentrations, it accumulates in mitochondria due to their large negative membrane potential and is retained inside the mitochondria. If the mitochondrial membrane is disrupted it can no longer retain $DiOC_6(3)$.

The cells were seeded at 1 x 10^5 or 2 x 10^5 cells/well in 2 ml medium in 6-well culture dishes. After 48 h of treatment the cells were incubated with 10 µg/ml $DiOC_6(3)$ in complete medium for 30 min at 37^{o}C in a humidified atmosphere of 5% CO_2 in the dark. At the end of the incubation period the cells were harvested and centrifuged for 10 min at 3000 rpm. Cells were resuspended in 400 µl ice-cold PBS and analysed immediately by flow cytometry. Data were collected on a FACS Canto II and quantification was performed using the DIVA software. In all experiments, 20,000 cells were analysed; data were gated to exclude debris. Each experiment was performed a minimum of three times.

3.2.6 Western blot analysis

Cell fractionation and Western blotting

Cells were treated with respective agents in a cell culture flask and harvested after indicated period of incubation. In all the experiments presented, cell lysates were prepared from floating

dead cells and adherent cells harvested together. Cells were centrifuged at 3000 rpm for 10 min, and washed twice with PBS. After washing, cell lysis was achieved by addition of lysis buffer, incubated for 15 min on ice, and then subjected to vigorous vortexing followed by brief sonication. Debris was spun down at 13,000 rpm for 10 min and the supernatant was collected and stored at -20^{o}C. The obtained supernatant was considered as crude cell lysate. The cell pellets and protein solutions were handled at 4^{o}C to avoid protein degradation during the protein preparation. Protein concentration was determined using the BCA assay kit according to the manufacturer's instructions. 5 µl samples were diluted in 200 µl BCA solution in an ELISA plate and incubated for 30 min at 37^{o}C in the dark. Absorption was measured using an ELISA reader at 590 nm. BSA was used as standard and the protein concentration was calculated on the basis of the derived standard curve.

Proteins were denatured by heating at 90^{o}C for 4 min immediately prior to loading. For immunoblotting, 30 µg to 60 µg of total cellular protein per lane were separated by standard SDS-PAGE on 8% to 15% gel (% of the gel used based on size of the proteins) and electrophoretically transferred to PVDF (polyvinylidine difluoride) membranes. To reduce non specific binding of antibodies, PVDF sheets were saturated for 1 hour in TBST blocking buffer containing 5% dry milk, and 0.05% Tween 20. After one hour, membranes were washed three times for 10 min each with TBST buffer. All washing steps were performed at room temperature. After washing, membranes were incubated overnight at 4^{o}C with p53 (DO-2), phospho-p53 (Ser15), pATM (Ser1981), pH2AX (Ser139), pBRCA1 (Ser1524), p95/NBS1 (Ser343), pRad-17 (Ser645), pFANCD2 (Ser222), pAkt (Ser473), pMDM2 (ser166) and anti-actyl-p53 (Lys373/382).

At the end of incubation, membranes were washed 3 times for 10 min each with TBST buffer. The membranes were then incubated for 3 h at room temperature with peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs (dilution 1:25,000) in TBST buffer containing 5% milk. Once again membranes were washed 3 times (10 min each) with TBST buffer. The specific protein signals were visualised using chemiluminescent peroxidase substrate for 5 min and exposing the membranes to the high performance chemiluminescene film for detection. Protein loading was verified by detection of GAPDH using mouse anti-GAPDH monoclonal antibody.

3.2.7 Cell proliferation assay

3.2.7.1 Alamar Blue assay

2000 cells/well were seeded in triplicate in 96-well plates. At the end of the treatment period, 1/10 volume of Alamar Blue (Biosource, Solingen, Germany) solution was added and cells incubated at 37°C for an additional 3 h. The fluorescence was measured on a fluorescence spectrophotometer, BMG Labtech (Offenburg, Germany) FLUOstar Omega using an excitation/emission wave length of 544/590 nm. Results are expressed as a percentage of fluorescence of untreated control cells.

3.2.7.2 Viable cell count

Approximately 1 x 10^5 to 2 x 10^5 cells/well were seeded in 6-well plates. After the treatment period, cells were harvested and counted under a microscope. Cell viability was assessed by trypan blue dye exclusion.

3.2.8 Quantitative real-time RT-PCR

3.2.8.1 RNA and cDNA preparation

1.5 x 10^5 cells were harvested from a 6-well growing culture plates and centrifuged at 3000 rpm for 5 minutes to pellet the cells. The supernatant was discarded and cell pellet was used for RNA preparation using the Peqgold total RNA kit including DNase digestion (Peqlab, Erlangen, Germany). Total RNA was prepared according to the manufacturer's protocol.

RNA was quantified and checked for quality based on 260:280 ratios and 260:230 ratios. 0.5 µg RNA was used directly for preparation for cDNA. RNA was transcribed into cDNA using Omniscript (Qiagen, Hilden, Germany) according to manufacturer's instructions. The cDNA sample obtained after the preparation was diluted and a final amount of 5 ng of cDNA was used for the real-time PCR.

3.2.8.2 Real-time PCR

The primers for amplification of the genes MDM2, MDM4, p21 and PUMA were purchased from Qiagen (QuantiTect® Primer assay). Quantitative PCR for MDM2, MDM4, p21 and PUMA was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Expression levels were normalised to β-2-microglobulin. Reactions were

done in duplicate using Applied Biosystems Taqman Gene Expression Assays (MDM2: Hs99999008_m1; MDM4: Hs00159092_m1; p21: Hs00355782_m1; PUMA: Hs00248075_m1; β-2-microglobulin: Hs00187842_m1) and Universal PCR Master Mix. All procedures were carried out according to the manufacturer's protocol. The relative MDM2, p21 and PUMA expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method (Schmittgen and Livak, 2008).

3.2.9 Cellular senescence assay

Cellular senescence was determined by assessing senescence-associated β-galactosidase (SA-β-Gal) activity at pH 6.0. At the end of the treatment period, cells were washed with PBS, fixed for 5 min with 1% glutaraldehyde, washed with PBS, and incubated at 37°C for 18 h in fresh staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside [Peqlab], 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6$, 150 mM NaCl and 2 mM $MgCl_2$ in 40 mM citric acid/sodium phosphate at pH 6.0. After washing with PBS, cells were viewed with an Olympus (Hamburg, Germany) CKX41 phase contrast microscope.

3.2.10 HDAC activity assay

HDAC activity using Boc-Lys(Ac)-AMC

Approximately 2 x 10^5 cells in 90 µl per well were seeded in 96-well plates and were incubated overnight at 37^{o}C. Cells were treated with TSA, vorinostat and NaB with or without AEBSF and incubated for an hour. Then 10 µl of 2 mM Boc-Lys(Ac)-AMC (Bachem AG, Switzerland) per well were added and further incubated for 3 h. Furthermore, cells were incubated for 3 h with 100 µl lysis/developing buffer per well. Finally, the fluorescence was measured on a fluorescence spectrophotometer, BMG Labtech FLUOstar Omega using an excitation/emission wave length of 355/460 nm. Results are expressed as a percentage of fluorescence of untreated control cells.

Recombinant HDAC1 activity using Boc-Lys(Ac)-AMC

Forty microlitres of activity buffer were used per reaction and TSA or AEBSF or TSA/AEBSF was added to the activity buffer in the corresponding reactions. Then, 5 µl of HDAC1 solution (0.1 µg/µl) and 5 µl of Boc-Lys(Ac)-AMC (2 mM) solution were added per reaction and incubated for an hour. One reaction without HDAC1 solution was used for blank sample. Further, 50 µl of trypsin solution (2 mg/ml) per reaction were added and incubated for an hour. Lastly, the fluorescence was measured on a fluorescence spectrophotometer, BMG Labtech FLUOstar Omega using an excitation/emission wave length of 355/460 nm.

3.2.11 Synergistic effect

Around 1.5 x 10^5 to 2 x 10^5 cells were plated in 6-well plates and incubated for 24 h as described in the cell culture section. Cells were pretreated with 3 µM or 10 µM nutlin-3 for 1 h and cotreated further with HDACi for 48 h. The cells were then incubated for appropriate time period in order to determine the cell viability, cell cycle, cell death, mitochondrial membrane potential loss and caspase activities.

3.2.12 Combination index method

Cells were pretreated with 3 µM or 10 µM nutlin-3 for 1 h and cotreated further with different concentration of HDACi or paclitaxel for 48 h in A549 cells. Likewise, 10 µM or 25 µM CAPE was administrated to VH-64 cells, and cells were exposed to various concentration of nutlin-3 for another 48 h. Cell death was determined by flow cytometric analyses of PI uptake. Cell death data were used to study the combination index (CI) values. CI theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. CI values for nutlin-3 plus HDACi vorinostat or MS-275 or apicidin or NaB or paclitaxel in A549 cells and nutlin-3 plus CAPE in VH-64 cells were calculated using the Chou-Talalay method (Chou, 2010).

4 RESULTS

4.1 HDACi-induced effect on cell cycle in cancer cells

4.1.1 Vorinostat induces cell cycle arrest in the presence of the pan-caspase inhibitor z-VAD-fmk in A549 cells

As mentioned in the earlier studies, the induction of apoptosis by vorinostat in A549 and PC-3 cells was observed (Sonnemann et al., 2006a). On treatment with the broad-spectrum irreversible pan-caspase inhibitor z-VAD-fmk, vorinostat-induced apoptosis was considerably reduced, as assessed by determining the sub-G_1 population of cells. Interestingly, a G_2/M cell cycle arrest was observed in parallel (Figure 6C). These results lead to the idea that HDACi could induce cell cycle arrest in the absence of caspase activity. In the current study, we confirmed the result in A549 cells. Apoptosis was determined through quantifying DNA fragmentation during cell cycle progression by staining the nuclei of ethanol-fixed cells with PI and quantifying the percentage of each cell cycle phase by flow cytometry. A time course analysis revealed that 20 μM of vorinostat showed a strong increase in the percentage of apoptotic cells (sub-G_1 phase) and at the same time cells in the G_1 and G_2/M phase decreased (Figure 6). When the cells were pretreated with 20 μM z-VAD-fmk prior to the addition of vorinostat, vorinostat-induced apoptosis was prevented in a highly significant manner. After inhibition of caspases by z-VAD-fmk, a G_2/M arrest, previously concealed after exposure to vorinostat alone, became clearly visible (Figure 6C). This confirmed the previous findings from our group (Sonnemann et al., 2006) and provided evidence that vorinostat is capable of eliciting tumour growth arrest when its apoptosis-inducing activity is blocked.

4.1.2 Vorinostat induces cell cycle arrest in MCF-7 cells

This finding prompted us to investigate how HDACi induce a G_2/M arrest. To facilitate these investigations, we employed MCF-7 breast cancer cells, a cell line deficient in caspase-3 expression where we could study HDACi-induced cell cycle arrest without z-VAD-fmk treatment (Janicke et al., 1998). We hypothesised that MCF-7 cells would respond to HDACi treatment by undergoing a G_2/M arrest rather than apoptosis due to the lack of caspase-3 activity.

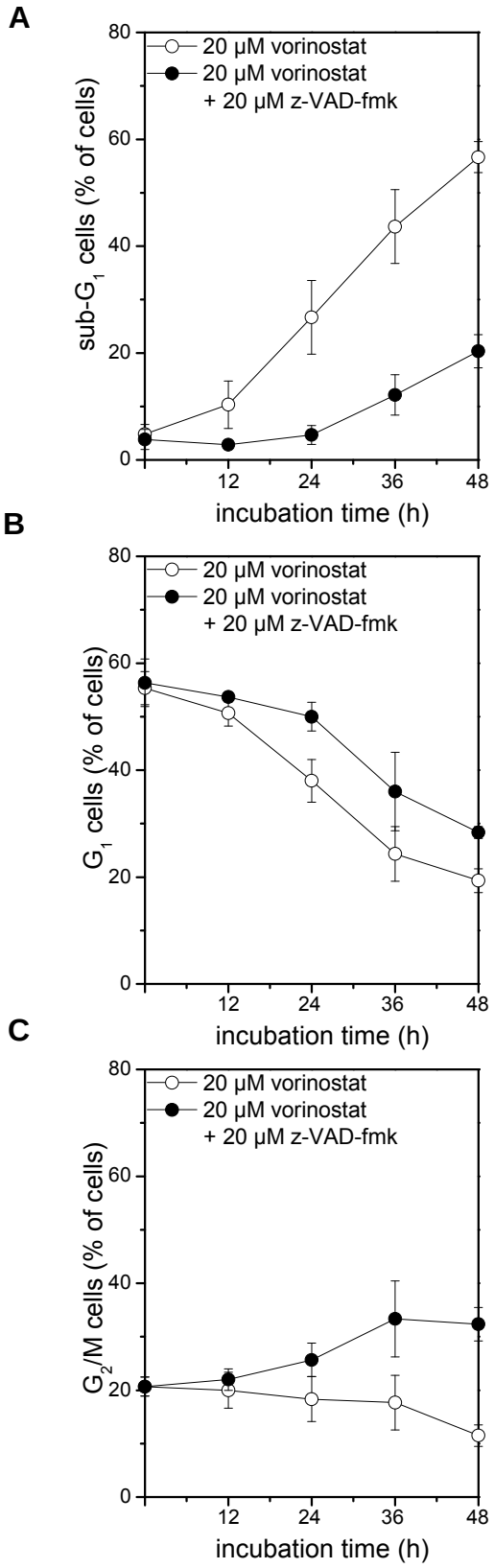

Figure 6 z-VAD-fmk inhibits HDACi-induced apoptosis in A549 cells.

*Progression of cell cycle in **A)** sub-G_1 cells **B)** G_1 cells **C)** G_2/M cells treated with only vorinostat and vorinostat along with z-VAD-fmk: 20 μM z-VAD-fmk was applied 1 h before treatment with 20 μM vorinostat, and then incubated for 48 h. Cell cycle profiles were analysed by flow cytometry. Means of 3 separate experiments are shown.*

Cell cycle analysis was performed as described in the previous section 4.1.1 to assess the effect of vorinostat on MCF-7 cells. Briefly, cell cycle distribution was determined by staining the nuclei of ethanol-fixed cells with PI and quantifying the percentage of cells in each cell cycle phase by flow cytometry. This method can be used to determine the percentage of cells present in the particular stage of cell cycle. MCF-7 cells were treated with two different concentrations of vorinostat (1 µM and 10 µM) for 48 h. A time course analysis revealed that 1 µM and 10 µM of vorinostat showed strong increase of cells in the G_1 and G_2/M phase, respectively (Figure 7). Surprisingly, low and high concentrations of vorinostat induced two different effects on cell cycle progression. The cells treated with a low concentration of 1 µM of vorinostat showed G_1 cell cycle arrest (Figure 7A). At the high concentration of 10 µM, vorinostat caused a G_2/M cell cycle arrest (Figure 7B). We did not observe any marked increase in the sub-G_1 population of cells, probably due to the lack of caspase-3 in MCF-7 cells (data not shown).

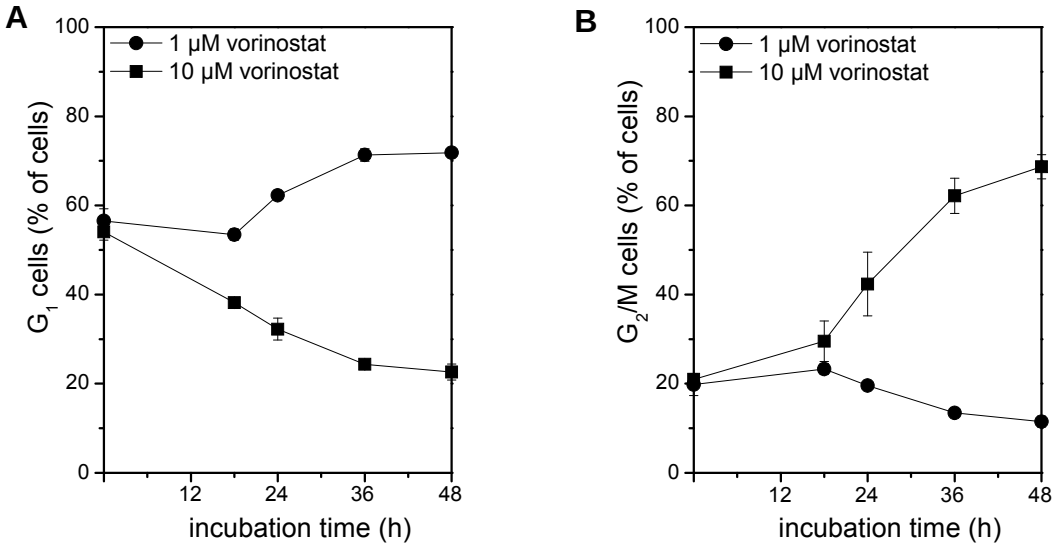

Figure 7 Vorinostat induces cell cycle arrest in MCF-7 cells.

*Cell cycle progression in **A)** G_1 cells **B)** G_2/M cells were analysed by flow cytometry. Cells were treated with 1 µM and 10 µM of vorinostat, and then incubated for 48 h. Means of 3 separate experiments are shown.*

4.1.3 HDACi (vorinostat, MS-275) induce phosphorylation of ATM at Ser1981 in MCF-7 cells

Typically, DNA damage leads to cell cycle arrest through activation of ATM kinase by autophosphorylation at serine 1981 (Ser1981). This is considered to be an important biochemical event during the DNA damage response (Bakkenist and Kastan, 2003). Since, vorinostat had shown to elicit cell cycle arrest in MCF-7 cells, further insight into the underlying mechanisms

might be given by answering the question, if the vorinostat-induced cell cycle arrest is generated by the activation of ATM. For this, MCF-7 cells were exposed to 10 µM of vorinostat for the

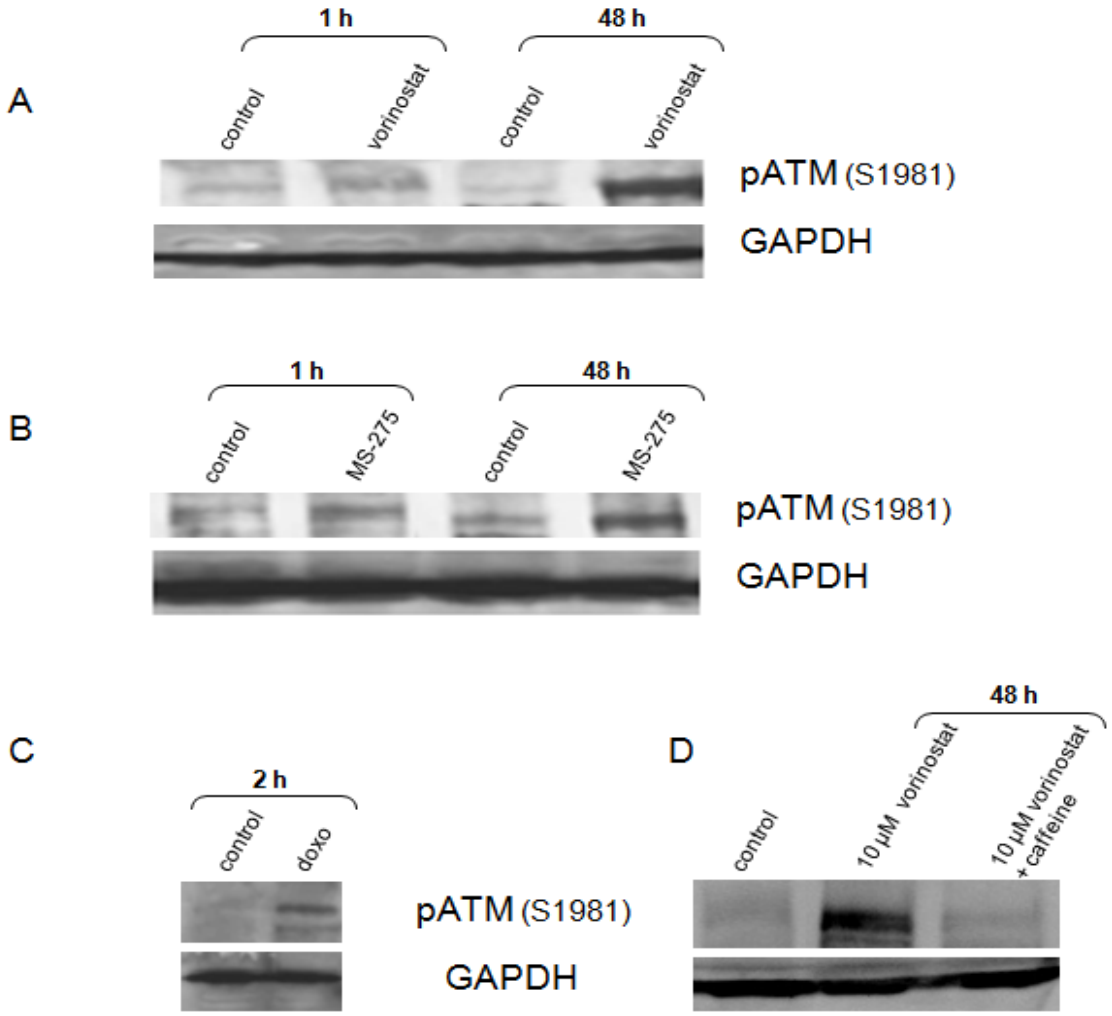

Figure 8 Effect of vorinostat and MS-275 on pATM in MCF-7 cells.

*Cells were treated with **A)** 10 µM vorinostat or **B)** 10 µM MS-275 or **C)** 1 µM doxorubicin and **D)** 2 mM caffeine 1 h prior to the treatment of vorinostat and harvested at the indicated times, followed by cells were subjected to Western blot analysis using special gradient resolving gel (upper - 8% with an acrylamid/bisacrylamid ratio 80:1; lower - 15% with an acrylamid/bisacrylamid ratio 37.5:1). pATM was observed after 1 h and 48 h by vorinostat (A) and MS-275 (B) treatment. Caffeine inhibits vorinostat-induced pATM (D). Doxorubicin used as a positive control for pATM (C).*

indicated time points as shown in Figure 8A, and Western blot was performed with a phospho-specific ATM-Ser1981 antibody. Exposure to 10 µM of vorinostat resulted in the induction of a marked phosphorylation of ATM (pATM) at Ser1981 in a time-dependent manner. Treatment with vorinostat resulted in moderate pATM after 1 h and strong pATM after 48 h. Similar ATM phosphorylation was also observed by another class of HDACi, MS-275 (Figure 8B), in MCF-7 cells. Further, to assess whether the HDACi effect on pATM at Ser1981 is a general effect, another cell line, ovarian cancer SKOV-3 cells which in contrast to MCF-7 cells expresses

caspase-3, was used (Figure 9). Therefore, MS-275-induced phosphorylation of ATM at Ser1981 was investigated in SKOV-3 cells. When SKOV-3 cells were incubated with 20 µM of MS-275, phosphorylation of ATM was observed after 30 min. Doxorubicin, an established DNA damage-inducing agent, served as positive control for ATM phosphorylation (Figure 8C) (Lai et al., 2009). To further substantiate this finding, MCF-7 cells were pretreated with 2 mM caffeine, an inhibitor of ATM. Caffeine abolished vorinostat-induced ATM phosphorylation (Figure 8D). These findings suggest that both vorinostat and MS-275 could inhibit cell cycle progression through activation of ATM.

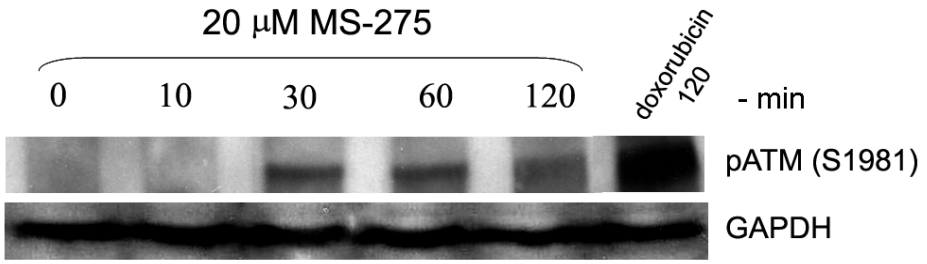

Figure 9 Effect of MS-275 on pATM in SKOV-3 cells.

Cells were incubated with 20 µM MS-275 for various times (0, 10, 30, 60, 120 min) followed by cells were harvested and subjected to Western blot analysis using anti-ATM phospho Ser1981 and anti-GAPDH antibodies. For doxorubicin the incubation time was 120 min.

4.1.4 HDACi (vorinostat, MS-275) induce phosphorylation of H2AX in MCF-7 and SKOV-3 cells

Phosphorylated H2AX is a marker of DNA damage, it is phosphorylated at the Ser139 residue in response to DNA double-strand breaks (DSB) (Burma et al., 2001). It is known as gamma H2AX (γH2AX). PI3K (Phosphatidyl-inositol-3-kinase) family of proteins, ATM, DNA-protein kinase and ATR (ATM and RAD3-related) are the responsible kinases to mediate this phosphorylation event (Tang et al., 2010). To examine whether vorinostat induces H2AX phosphorylation in MCF-7 cells, cells were treated with 10 µM of vorinostat and harvested after the indicated time points, and H2AX phosphorylation was analysed by Western blotting with anti-γH2AX antibody. Figure 10 shows significant and modest H2AX phosphorylation after treatment with vorinostat at 45 and 75 min, respectively. Pretreament with 2mM caffeine inhibited vorinostat-induced H2AX phosphorylation. Significant phosphorylation of H2AX was also observed after 2 h treatment of cells with the DNA damage-inducing agent doxorubicin, used as positive control. This finding suggests that vorinostat induces DNA damage.

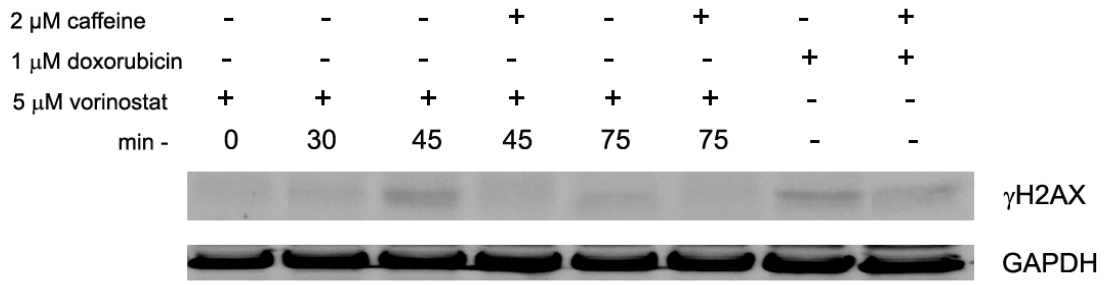

Figure 10 Effect of vorinostat on γH2AX in MCF-7 cells.

One hour prior the cells were treated with 2 mM caffeine and cotreated with 5 μM vorinostat at various time point, followed by cells were subjected to Western blot analysis using anti-GAPDH and anti-γH2AX antibodies. For doxorubicin the incubation time was 120 min.

To assess whether the HDACi effect on γH2AX is a general one, vorinostat-induced γH2AX was investigated in another cancer cell line, SKOV-3. SKOV-3 cells were treated with 5 μM of vorinostat and Western blot analysis showed minor phosphorylation of γH2AX at 60 min

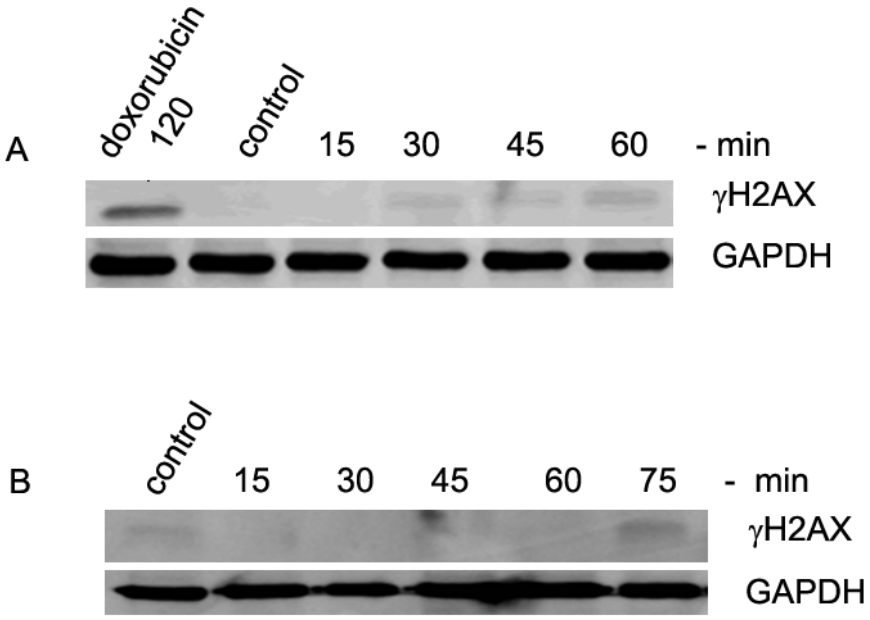

Figure 11 Effect of vorinostat and MS-275 on γH2AX in SKOV-3 cells.

*Cells were incubated with **A)** 5 μM vorinostat **B)** 5 μM MS-275 at various time point (0, 15, 30, 45, 60, 75 min) and 1 μM doxorubicin for 120 min, followed by cells were harvested and subjected to Western blot analysis using anti-γH2AX and anti-GAPDH antibodies.*

(Figure 11A). SKOV-3 cells treated with 5 μM of MS-275 showed modest γH2AX at 75 min (Figure 11B). Doxorubicin, a positive control, induced γH2AX at 2 h. These results indicate that HDACi induce phosphorylation of H2AX through DNA damage in both MCF-7 and SKOV-3 cells.

4.1.5 Effect of vorinostat on phosphorylation of ATM substrate proteins in MCF-7 cells

Several proteins such as p53, p95/NBS1, MDM2, CHK2, BRCA1, CtIP, 4E-BP1 and CHK1 have been identified as substrates for ATM (Kastan and Lim, 2000;Zhao and Piwnica-Worms, 2001). These proteins play important roles in the regulation of apoptosis and cell cycle progression. Therefore, we investigated the effect of vorinostat on the phosphorylation of some of the ATM substrate proteins, such as phospho-BRCA1, -FANCD2, -p53, -Akt, -p95/NBS1, -RAD17, -CHK1, -CHK2. The MCF-7 cells were treated with vorinostat for the same time points as in the previous experiments.

The phosphorylation of different ATM substrate proteins were determined by immunoblot using specific antibodies of phospho-BRCA1, -FANCD2, -p53, -Akt, -NBSC1, -RAD17, -CHK1, -CHK2.

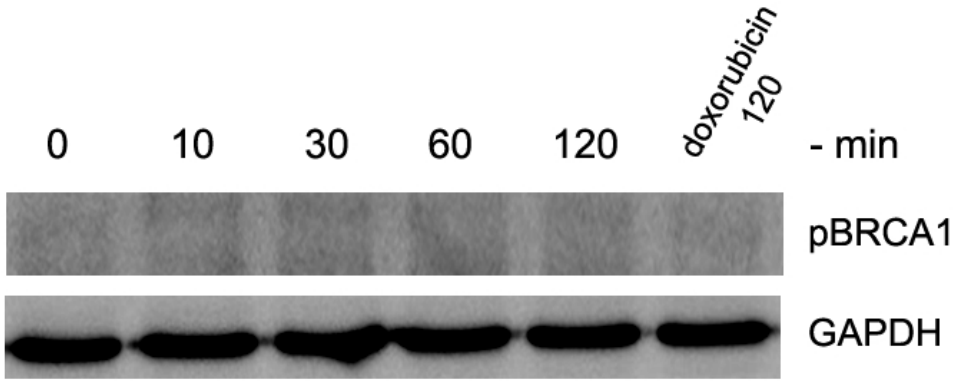

Figure 12 Effect of vorinostat on pBRCA1 in MCF-7 cells.

Cells were incubated with 10 µM vorinostat at various time points (0, 10, 30, 60, 120 min), followed by cells were harvested and subjected to Western blot analysis using phospho-BRCA1 and GAPDH antibodies.

None of these ATM substrate proteins showed phosphorylation by vorinostat in the earlier time points. For example, in Figure 12, immunoblot analysis revealed that the ATM substrate BRCA1 was not phosphorylated after treatment with vorinostat. Similar effects were observed for other ATM substrate proteins (data not shown).

4.1.6 Effect of ATM kinase inhibitors on vorinostat-induced cell cycle arrest in MCF-7 cells

As caffeine inhibited HDACi-induced ATM phosphorylation in MCF-7 cells, caffeine and the specific ATM inhibitor KU-55933 were used to evaluate the role of ATM in vorinostat-induced

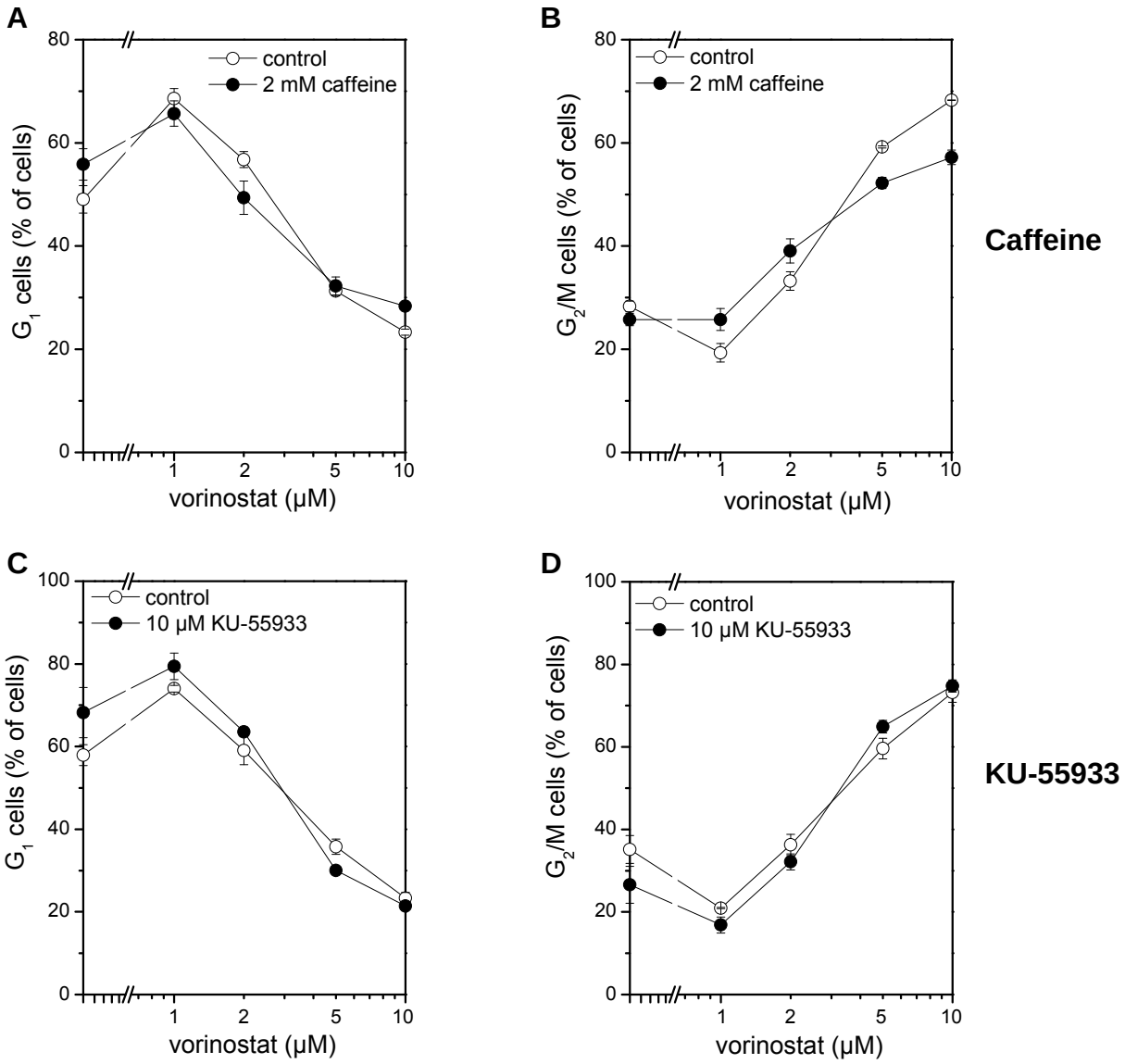

Figure 13 Effect of ATM inhibitors on vorinostat-induced cell cycle arrest in MCF-7 cells.

*Cells were treated either with vorinostat alone or vorinostat and caffeine (**A** and **B**) or vorinostat and KU-55933 (**C** and **D**), both caffeine or KU-55933 were applied 1 h prior to the treatment with vorinostat and cells were then incubated for 48 h, followed by cell cycle progression in G_1 cells (**A** and **C**) G_2/M cells (**B** and **D**) were analysed by flow cytometry. Means of 3 separate experiments are shown.*

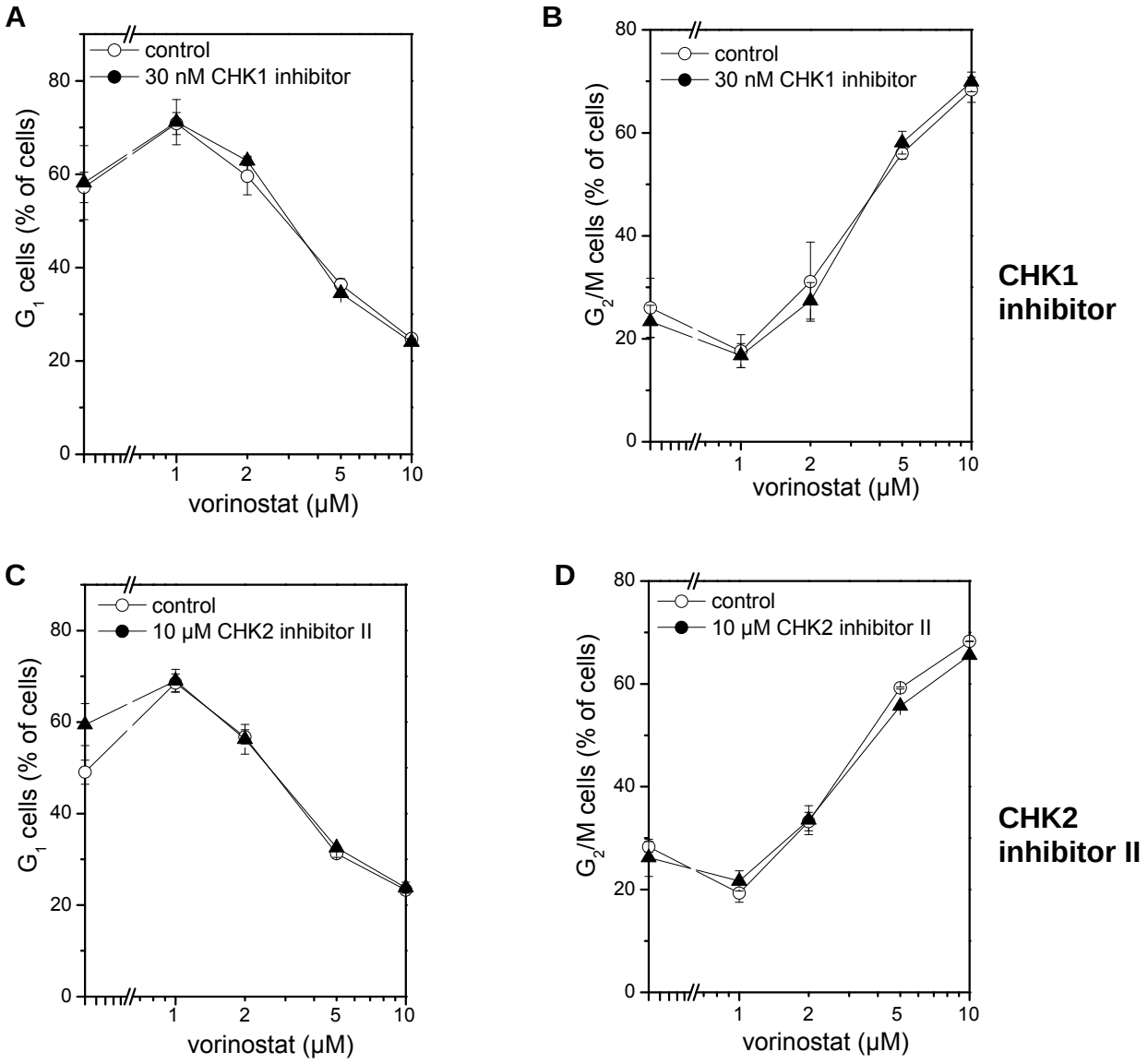

Figure 14 Effect of CHK1 and CHK2 inhibitors on vorinostat-induced cell cycle arrest in MCF-7 cells.

*Cells were treated either with vorinostat alone or vorinostat and CHK1 inhibitor (**A** and **B**) or vorinostat and CHK2 inhibitor II (**C** and **D**), both inhibitors were applied 1 h prior to the treatment with vorinostat and cells were then incubated for 48 h, followed by cell cycle progression in G_1 cells (**A** and **C**) G_2/M cells (**B** and **D**) were analysed by flow cytometry. Means of 3 separate experiments are shown.*

cell cycle arrest in MCF-7 cells. Cells were preincubated with 2 mM caffeine for 1 h, and incubated with various concentrations of vorinostat for 48 h and analysed by flow cytometry. As shown in Figure 13, pretreatment of cells with either caffeine or KU-55933 did not show any significant inhibitory effect on vorinostat-induced cell cycle arrest (Figure 13).

In addition, the role of CHK1 and CHK2 in cell cycle progression was evaluated using CHK1 and CHK2 inhibitors. CHK1 and CHK2 are activated by pATM in response to double-strand

DNA breaks (DSBs). CHK1 and CHK2 regulate fundamental cellular functions such as DNA replication, cell cycle progression, chromatin reformation, and apoptosis (Bartek et al., 2004). For this reason, we examined the effect of CHK1 inhibitor and CHK2 inhibitor II on vorinostat in MCF-7 cells by cell cycle analysis. As shown in Figure 14, both 30 nM of CHK1 inhibitor and 10 μM of CHK2 inhibitor II had no effect on vorinostat-induced G_1 and G_2/M arrest. This indicates that CHK1 and CHK2 do not have any significant effect in vorinostat-induced cell cycle arrest.

4.1.7 Effect of bortezomib on vorinostat-induced cell cycle arrest in MCF-7 cells

The degradation of regulatory proteins by the proteasome has an important role in the DNA damage response in tumour and normal cells. Proteasome's target proteins such as: $p21^{WAF}$, $p27^{KIP}$, p53, RAD51, cyclins (D, E, B), PARP, and nuclear-factor kappa B (NF-κB), regulate cell-cycle progression, DNA repair, and cell death (Choudhury et al., 2008). Cyclins are important regulatory proteins for cell cycle progression, activated by CDK-mediated phosphorylation. The level of cyclins is critically controlled by the ubiquitin-proteasome system (Fasanaro et al., 2010). Thus, the proteasome could be a specific target by chemical inhibitors to halt cell cycle progression and induce cell cycle arrest along with ATM activation in cancer cells. Bortezomib (PS-341, Velcade^{TM}) is a prominent proteasome inhibitor. It has been approved by the FDA for the treatment of multiple myeloma and is now under clinical trials for other types of cancer (Adams, 2002;Voorhees et al., 2003;Adams, 2004).

To elucidate whether the proteasome is involved in HDACi-induced G_2/M arrest, the effect of bortezomib on vorinostat-induced cell cycle arrest was examined. MCF-7 cells were pretreated with two different concentrations of bortezomib (10 nM and 100 nM) and incubated for 48 h with different concentrations of vorinostat. The cells were analysed by flow cytometry. The cells pretreated with 10 nM bortezomib significantly increased G_2/M arrest induced by vorinostat. As shown in Figure 15, it is observed that the cell cycle arrest starts at a concentration as low as 1 μM and further increases as the vorinostat concentration increases. In parallel, the G_1 arrest induced by low concentrations of vorinostat was abrogated when the cells were pretreated with bortezomib (Figure 15A). The result further substantiates that the proteasome has a role in vorinostat-induced cell cycle arrest in MCF-7 cells.

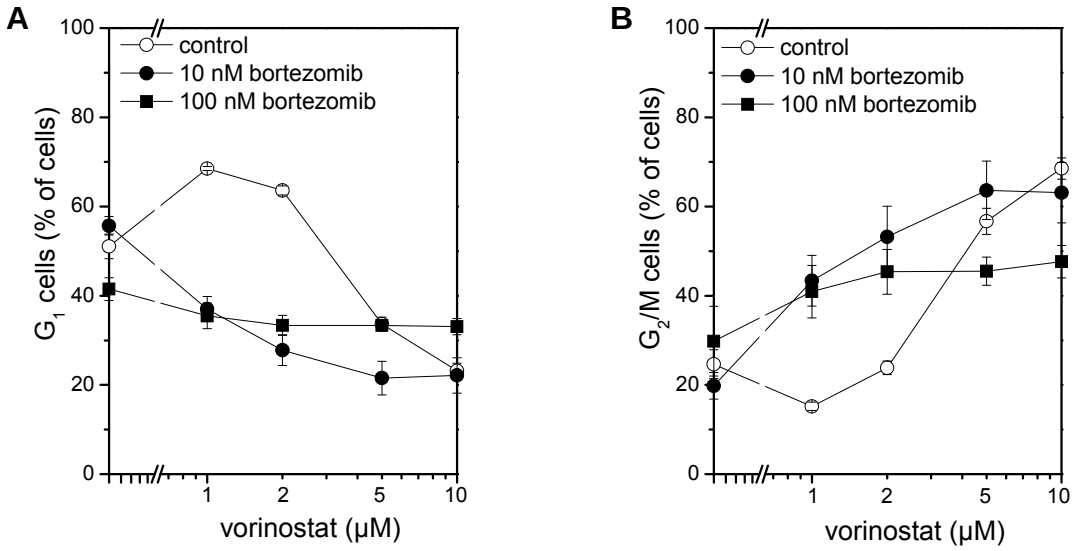

Figure 15 Effect of bortezomib on vorinostat-induced cell cycle arrest in MCF-7 cells.

Cells were pretreated for 1 h with 10 nM or 100 nM bortezomib and cotreated with different concentrations of vorinostat, were then incubated for 48 h, followed by cell cycle progression in ***A)*** *G_1 cells* ***B)*** *G_2/M cells were analysed by flow cytometry. Means of 3 separate experiments are shown.*

4.1.8 Effect of NF-κB inhibitors on vorinostat-induced cell cycle arrest in MCF-7 cells

NF-κB is a substrate of the proteasome and is activated by proteasome-mediated IκB degradation (Sethi and Tergaonkar, 2009). The inhibition of the proteasome by bortezomib leads to NF-κB inhibition, promoting antitumoural effects (Dolcet et al., 2006). It has been shown that NF-κB is activated by HDACi (Denlinger et al., 2004). Thus, we were interested to know whether the effect observed by bortezomib is brought through NF-κB inhibition. For this, specific NF-κB inhibitors were used to determine the role of NF-κB in HDACi-induced G_2/M arrest.

4.1.8.1 Effect of the NF-κB inhibitor CAPE on vorinostat-induced cell cycle arrest

Caffeic acid phenethyl ester (CAPE) is a potent inhibitor of activation of nuclear transcription factor NF-κB (Natarajan et al., 1996). We determined the effect of CAPE on vorinostat-induced cell cycle arrest in MCF-7 cells. Cells were pretreated 1 h before with 10 µM and 50 µM

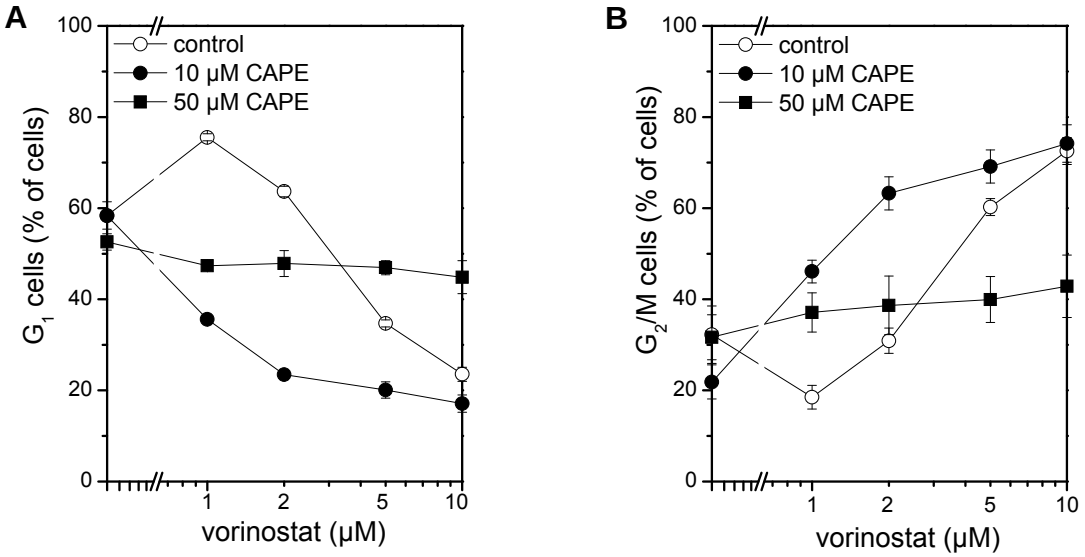

Figure 16 Effect of CAPE on vorinostat-induced cell cycle arrest in MCF-7 cells.

Cells were pretreated for 1 h with 10 µM or 50 µM CAPE and cotreated with different concentrations of vorinostat, were then incubated for 48 h, followed by cell cycle progression in ***A)*** *G_1 cells* ***B)*** *G_2/M cells were assessed by flow cytometry. Means of 3 separate experiments are shown.*

CAPE, respectively, further incubated with vorinostat for 48 h and cell cycle distribution was assessed by using flow cytometry. Interestingly, the effects of CAPE mimicked the effects of the proteasome inhibitor bortezomib. Combination of 10 µM of CAPE with vorinostat showed an increased G_2/M arrest. However combination of 50 µM of CAPE with vorinostat neutralised the effect attained by the vorinostat (Figure 16). This result suggests that the effect of the proteasome inhibition on vorinostat-induced cell cycle arrest is the result of NF-κB inhibition.

4.1.8.2 Effect of other NF-κB inhibitors BAY 11-7082, flavopiridol and roscovitin on vorinostat-induced cell cycle arrest

To confirm the results of CAPE on vorinostat-induced G_2/M arrest, other NF-κB inhibitors, such as BAY 11-7082, flavopiridol and roscovitin, with different modes of action were used: BAY 11-7082 is a synthetic NF-κB inhibitor that downregulates IκBα phosphorylation and leads to the inhibition of NF-κB activation. It has been found that BAY 11-7082 inhibits constitutive activity of NF-κB, leading to cell cycle arrest in G_1 phase and rapid induction of apoptosis in multidrug-resistant cancers (Garcia et al., 2005;Kim et al., 2005). The semisynthetic flavone flavopiridol (NSC 649890) and roscovitin inhibit the activation of NF-κB (Takada and Aggarwal, 2004). Therefore, we investigated the effect of BAY 11-7082, flavopiridol and roscovitin on vorinostat-induced G_2/M arrest in MCF-7 cells.

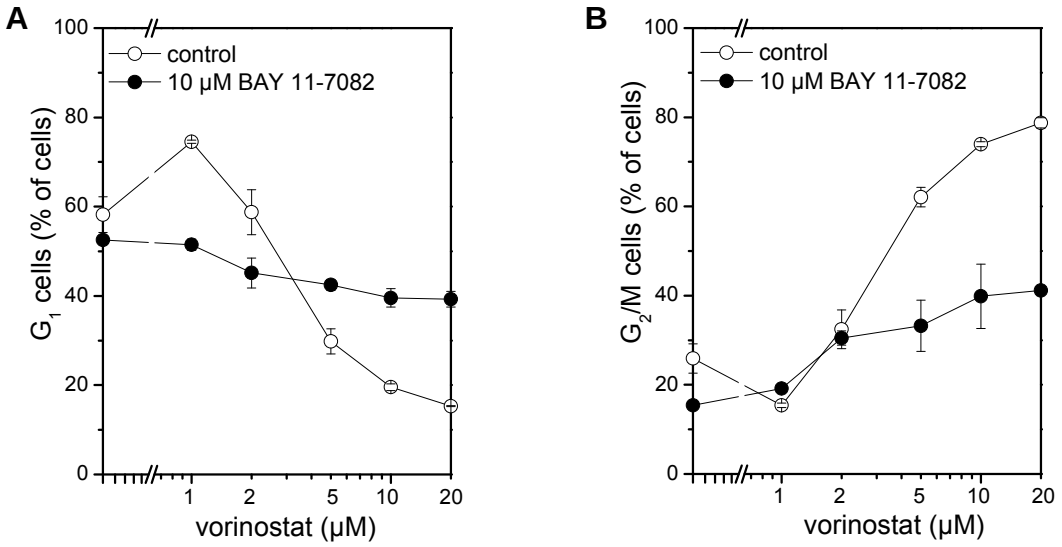

Figure 17 Effect of BAY 11-7082 on vorinostat-induced cell cycle arrest in MCF-7 cells.

Cells were pretreated with 10 µM BAY 11-7082 for 1 h prior to the treatment with different concentrations of vorinostat. Cells were then incubated for 48 h, followed by cell cycle progression in ***A)*** *G_1 cells* ***B)*** *G_2/M cells were assessed by flow cytometry. Means of 3 separate experiments are shown.*

For this, MCF-7 cells were preincubated with NF-κB inhibitors, and further incubated with various concentrations of vorinostat for 48 h and analysed by using flow cytometry. As shown in Figure 17, Figure 18 (AB) and Figure 18 (CD), BAY 11-7082, roscovitin and flavopiridol had similar effects on vorinostat-induced G_2/M arrest as CAPE, indicating that indeed NF-κB has a role in HDACi-induced G_2/M arrest.

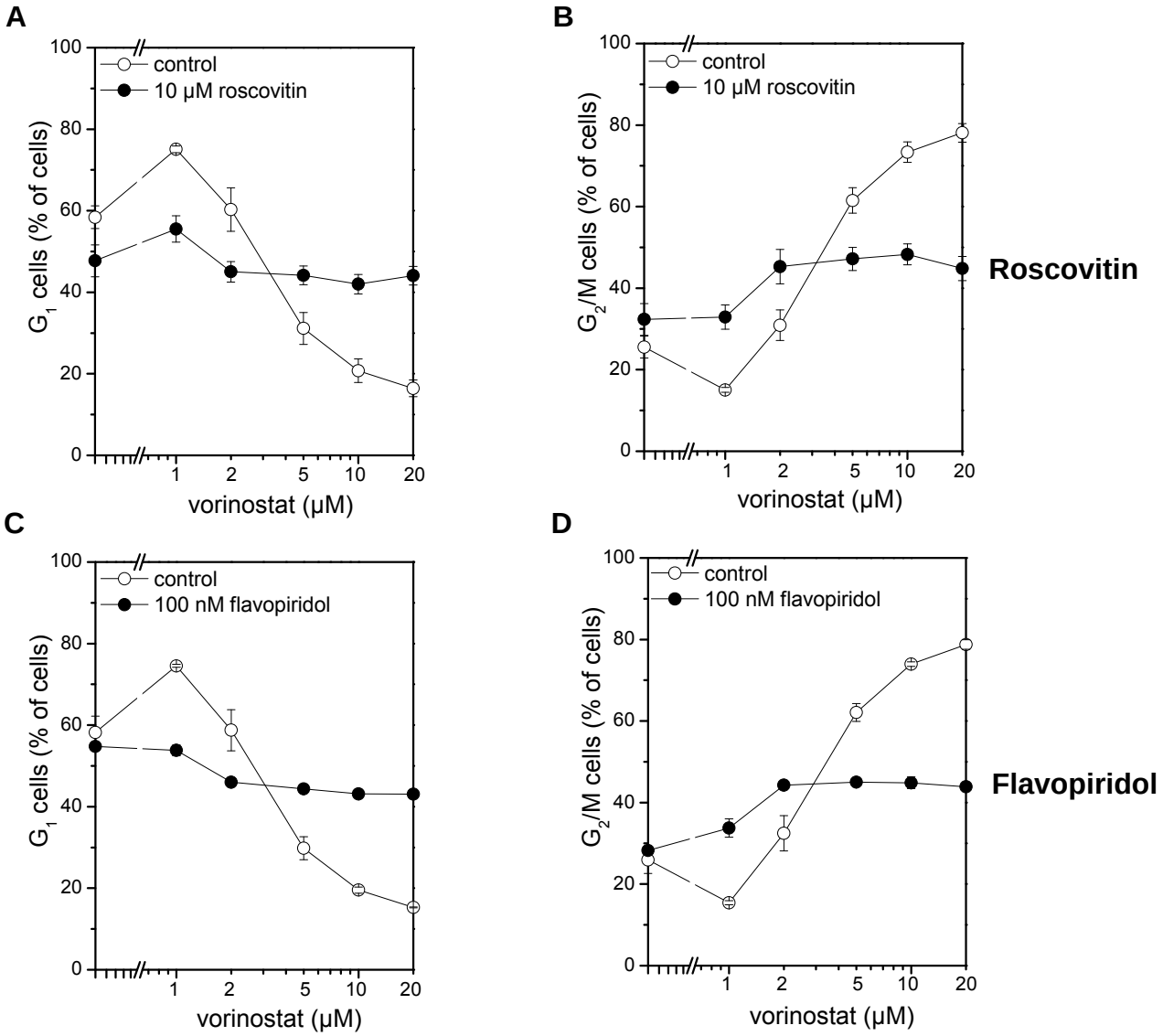

Figure 18 Effect of roscovitin and flavopiridol on vorinostat-induced cell arrest in MCF-7 cells.

*Cells were treated either with vorinostat alone or vorinostat and roscovitin (**A** and **B**) or vorinostat and flavopiridol (**C** and **D**), inhibitors were applied 1 h prior to the treatment of vorinostat and cells were then incubated for 48 h, followed by cell cycle progression in G_1 cells (**A** and **C**) G_2/M cells (**B** and **D**) were analysed by flow cytometry. Means of 3 separate experiments are shown.*

4.1.9 Effect of LY294002 on vorinostat-induced cell cycle arrest in MCF-7 cells

HDACi have been shown to activate NF-κB through activating PI3K/Akt pathway (Denlinger et al., 2005). Thus we analysed whether inhibition of PI3K could mimic NF-κB inhibition. The PI3K inhibitor LY294002 strongly inhibits both PI3K activation and NF-κB-dependent gene expression (Shah et al., 2001). To address the hypothesis of pharmacologic inhibition of the PI3K/Akt pathway could inhibit NF-κB and sensitise cancer cells to cell cycle arrest by HDAC

inhibition, effect of LY294002 on vorinostat was analysed in MCF-7 cells. For this, cells were preincubated with 25 µM of LY294002 and cotreated with vorinostat for 48 h and then cell cycle analysis was done by flow cytometry. Combined treatment with vorinostat and LY294002 abolished G_1 arrest and increased G_2/M arrest at lower concentrations of vorinostat (1 µM and 2 µM) (Figure 19).

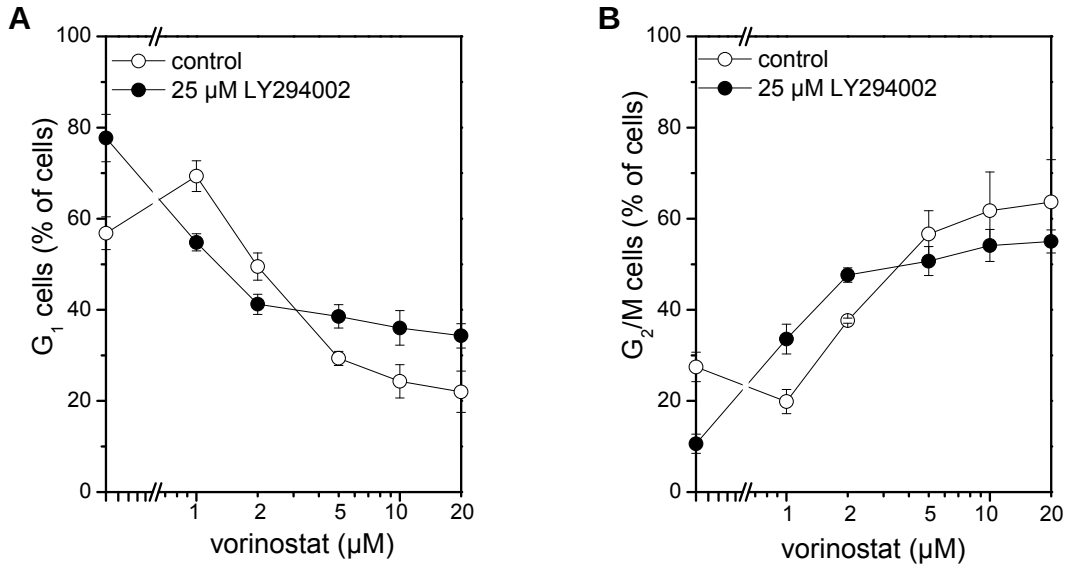

Figure 19 Effect of LY294002 on vorinostat-induced cell cycle arrest in MCF-7 cells.

Cells were pretreated with 25 µM LY294002 for 1 h prior to the treatment with different concentrations of vorinostat. Cells were then incubated for 48 h, followed by cell cycle progression in ***A)*** *G_1 cells* ***B)*** *G_2/M cells were assessed by flow cytometry. Means of 3 separate experiments are shown.*

4.1.10 Effect of nutlin-3 on vorinostat-induced cell cycle arrest

The results presented suggest that CHK1 and CHK2 do not contribute to HDACi-induced cell cycle arrest, whereas the use of NF-κB inhibitors indicates that NF-κB has a role in HDACi-induced G_2/M arrest in MCF-7 cells. Since p53 is an ATM substrate and plays an important role in the regulation of cell cycle progression, we investigated whether p53 participates in vorinostat-induced G_2/M arrest in MCF-7 cells. For this, nutlin-3, a small molecule inhibitor which restores p53 function by inhibiting the p53 suppressor protein murine double minute 2 (MDM2), was used in combination with vorinostat in MCF-7 cells. As shown in Figure 20C, nutlin-3 treatment prevented the vorinostat-induced G_2/M arrest. This shows that p53 has a role in vorinostat-induced G_2/M arrest in MCF-7 cells. To assess whether the nutlin-3 effect on vorinostat is a general one, the effect of nutlin-3 on vorinostat-induced G_2/M arrest was also

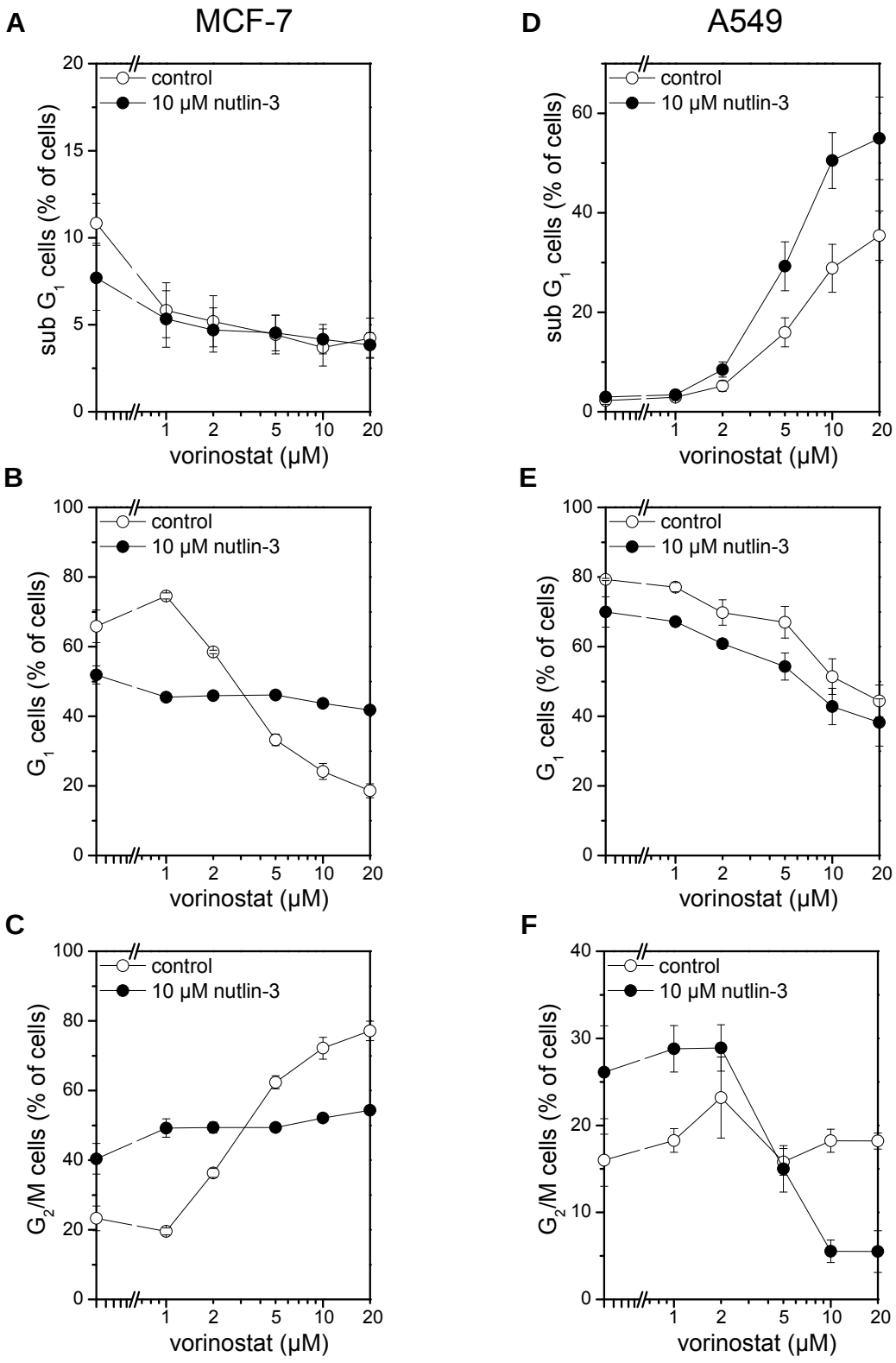

Figure 20 Effect of nutlin-3 on vorinostat-induced cell cycle arrest and apoptosis in MCF-7 and A549 cells.

Progression of cell cycle in cells treated with either vorinostat or vorinostat and nutlin-3. Cells were pretreated with 10 μM nutlin-3 for 1 h to the treatment with different concentrations of vorinostat, and further incubated for 48 h. Cell cycle profiles were analysed by flow cytometry. ***A)*** *sub-G_1 cells* ***B)*** *G_1 cells* ***C)*** *G_2/M cells in MCF-7 cells,* ***D)*** *sub-G_1 cells* ***E)*** *G_1 cells* ***F)*** *G_2/M cells in A549 cells. Means of 3 separate experiments are shown.*

analysed in p53 wild-type A549 cancer cells (Figure 20F). When vorinostat was combined with nutlin-3 in A549 cells, we observed enhanced apoptosis (Figure 20D). This interesting effect stimulated us to study the combination of nutlin-3 with HDACi in more detail.

4.2 Synergistic activity of HDACi with nutlin-3 in p53 wild-type cancer cell lines

Of the fifty percent of human tumours with wt-p53, many are thought to have compromised p53 function due to increased MDM2 levels (Brown et al., 2009). Hence, restoration of p53 function through blocking the p53-MDM2 interaction appears to be an attractive strategy for the treatment of p53 wild-type cancers. Therefore, activation of the p53 pathway by antagonising its negative regulator MDM2 might offer a new therapeutic strategy for the great majority of tumour malignancies.

The combination of nutlin-3 and HDACi had not shown significant apoptosis in MCF-7 cells, likely due to the lack of caspase-3 activity (Figure 20A). However, the apoptotic effect was synergistically enhanced when vorinostat was combined with nutlin-3 in A549 cells (Figure 20D). Hence, similar investigations were conducted to determine the effect of nutlin-3 in combination with HDACi belonging to four different structural classes - vorinostat, MS-275, sodium butyrate (NaB) and apicidin - in p53 wild-type A549 and A2780 cells and p53 null PC-3 cells.

4.2.1 Nutlin-3 and HDACi synergise to induce cell death in p53 wild-type A549 cells

To assess a possible favourable interaction between nutlin-3 and HDACi, we initially monitored cell viability in the p53 wild-type lung cancer cell line A549 using Alamar Blue assay. Figure 21 shows that the four HDACi tested (vorinostat, NaB, MS-275, apicidin) reduced cell viability in a concentration-dependent manner. In contrast, cells were only marginally sensitive to nutlin-3 alone, in concordance with previous reports on its effects on A549 cells (Tovar et al., 2006;Tokalov and Abolmaali, 2010). However, in conjunction with HDACi, nutlin-3 caused a marked additional decrease in cell viability. For example, 5 μM vorinostat reduced cell viability by 41% and 10 μM nutlin-3 reduced cell viability by 10%, while the combination of both compounds reduced cell viability by 74% (Figure 21A).

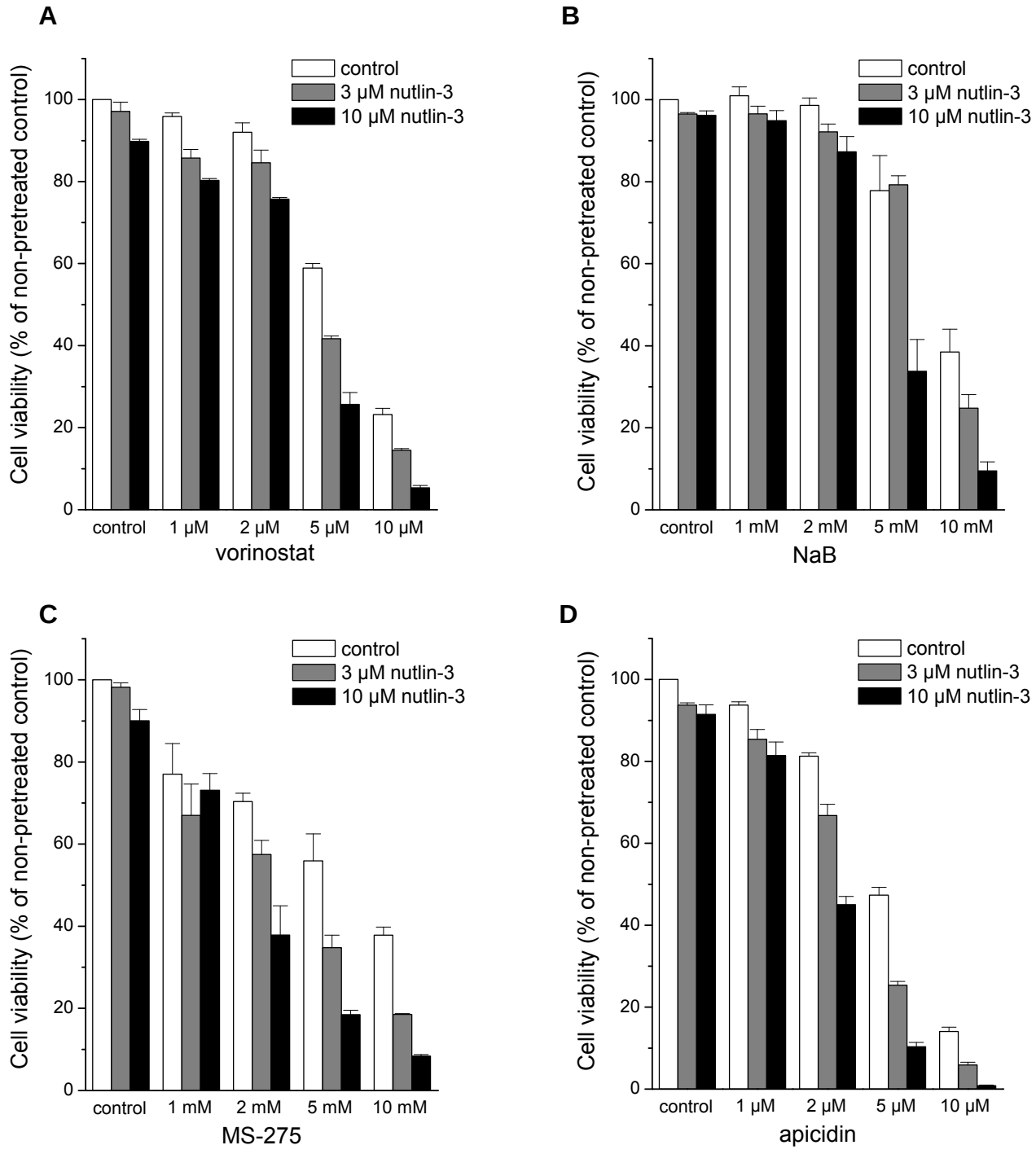

Figure 21 Nutlin-3 and HDACi cooperate in affecting cell viability in A549 cells.

One hour after administration of nutlin-3, cells were further exposed to HDACi ***A)*** *vorinostat,* ***B)*** *NaB (sodium butyrate),* ***C)*** *MS-275, and* ***D)*** *apicidin, for 72 h. Cell viability was determined by Alamar Blue assay. Means ± SEM of 3 separate experiments are shown.*

The Alamar Blue assay is a convenient method to indirectly measure the number of viable cells, but it is not capable of distinguishing between effects on proliferation and cell death. Thus, to establish whether nutlin-3 and HDACi cooperated in eliciting cell death, we determined the latter by flow cytometric analysis of PI uptake. These measurements revealed a cooperative induction of cell death after combined treatment with nutlin-3 and HDACi (Figure 22). For example, when administered individually, 10 μM nutlin-3 and 5 μM vorinostat elicited cell death in 15% or 19% of cells, respectively. When applied together, the agents evoked cell death in 49% of cells. To test for synergy, we analysed these data by the combination index (CI) method (CI < 1 is indicative for a synergistic interaction (Chou, 2010)). The calculated CI values indicate synergism for the combinations of nutlin-3 with vorinostat or apicidin at most concentrations, and for the combinations of nutlin-3 with NaB or MS-275 at all concentrations applied (Table 3 to Table 6).

p53 is a potent inducer of apoptosis and as such it predominantly triggers the mitochondrial pathway of apoptosis (Vousden and Lane, 2007). The latter is also the major pathway for HDACi to elicit cell death (Xu et al., 2007). We therefore assessed whether nutlin-3 and HDACi could interact at the mitochondrial level. Since this apoptotic pathway involves a perturbation of mitochondrial potential loss ($\Delta\psi_m$), we determined $\Delta\psi_m$ dissipation by flow cytometric analysis of $DiOC_6(3)$ staining. As presented in Figure 23, the results reflect those of the cell death assay: nutlin-3 cooperated with all HDACi applied to induce decay of $\Delta\psi_m$.

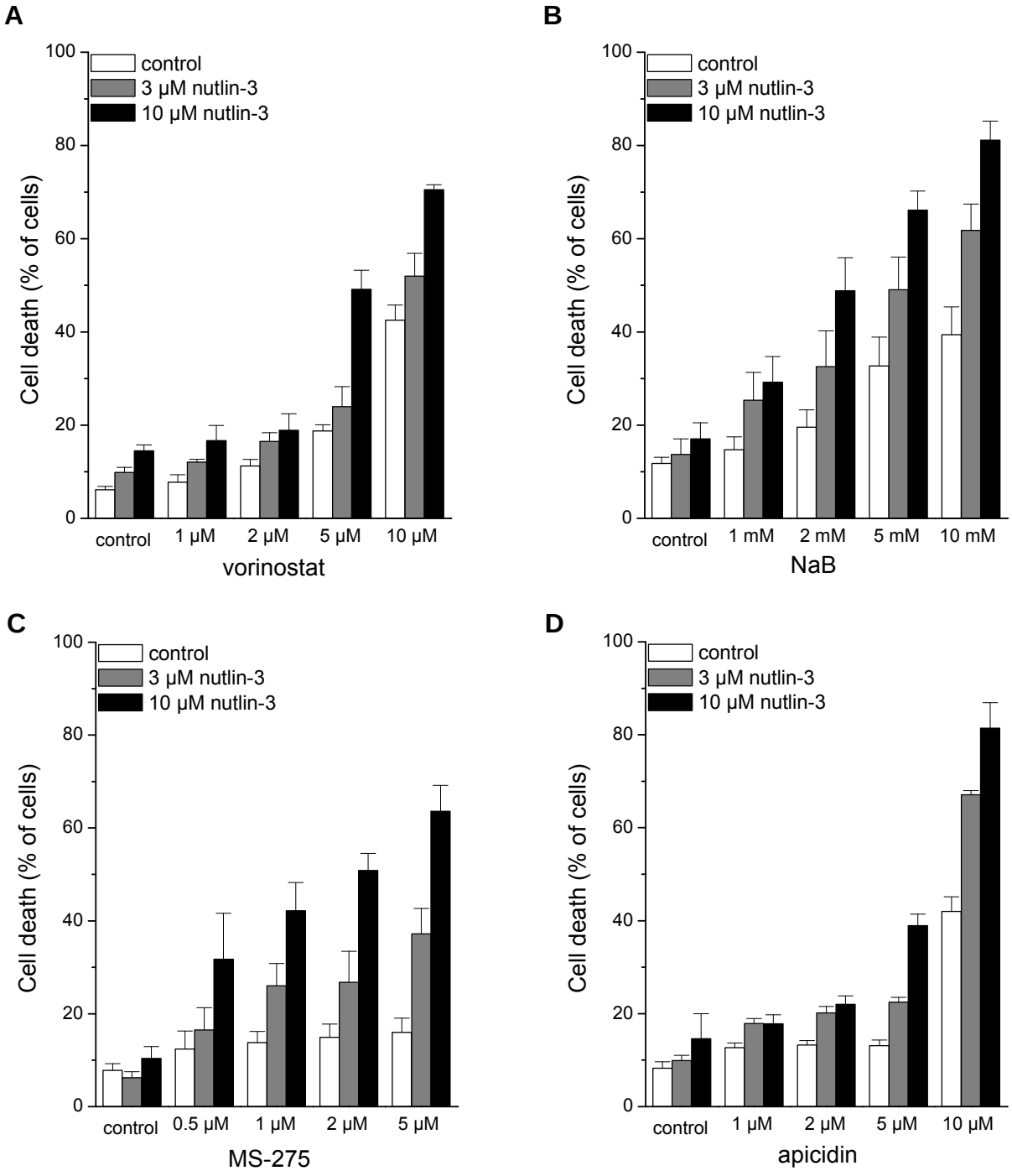

Figure 22 Nutlin-3 and HDACi cooperate in inducing cell death in A549 cells.

One hour after administration of nutlin-3, cells were exposed to HDACi ***A)*** *vorinostat,* ***B)*** *NaB,* ***C)*** *MS-275, and* ***D)*** *apicidin, for another 48 h. Cell death was determined by flow cytometric analysis of PI uptake. Means ± SEM of 3 separate experiments are shown.*

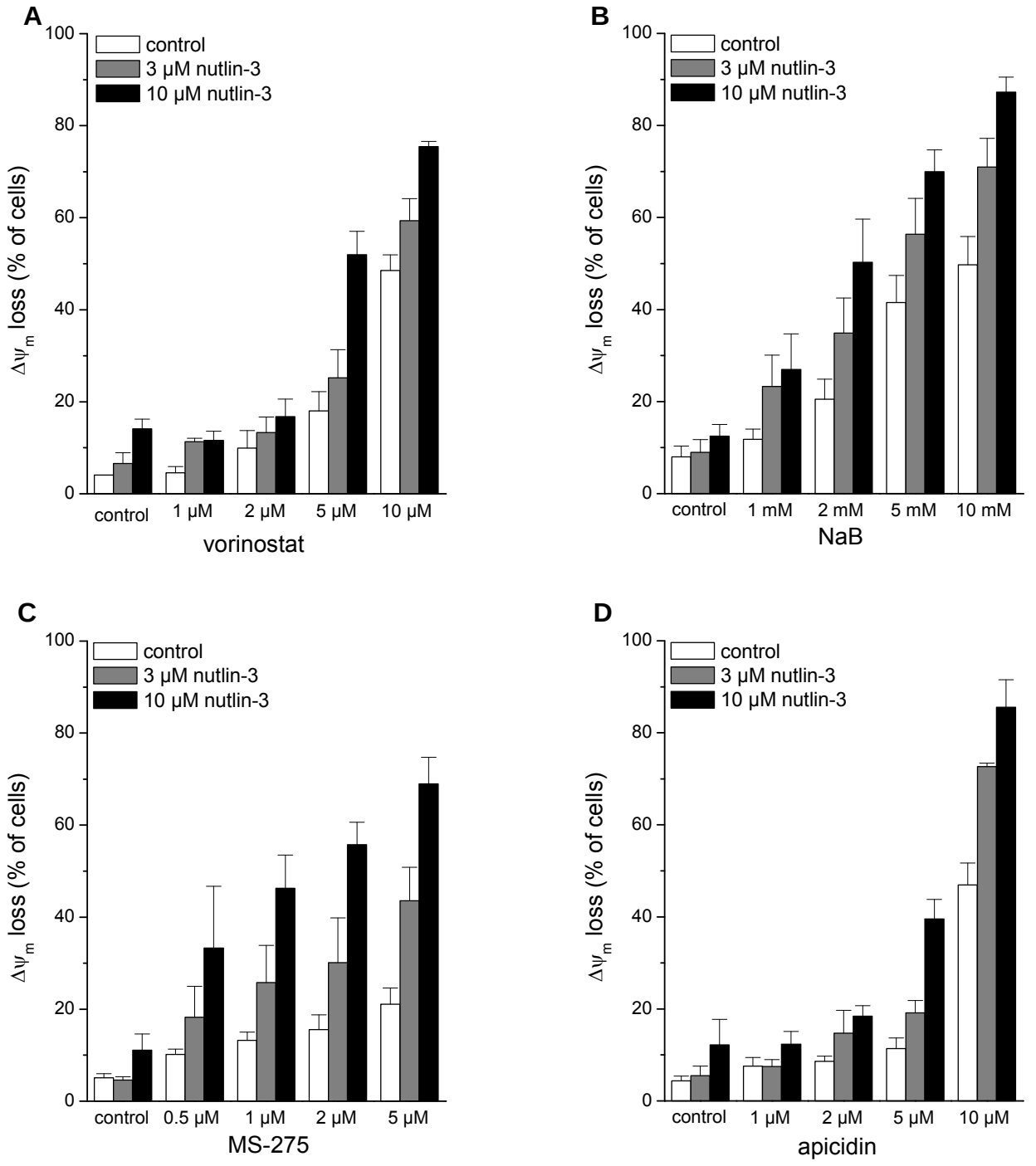

Figure 23 Nutlin-3 and HDACi cooperate in inducing $\Delta\psi_m$ loss in A549 cells.

*One hour after administration of nutlin-3, cells were exposed to HDACi **A)** vorinostat, **B)** NaB, **C)** MS-275, and **D)** apicidin for another 48 h. $\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of 3 separate experiments are shown.*

Table 3 Combination index values for nutlin-3 plus vorinostat in A549 cells.

Nutlin-3 (µM)	Vorinostat (µM)	CI
3	1	1.030
3	2	0.858
3	5	1.045
3	10	0.509
10	1	0.956
10	2	0.968
10	5	0.295
10	10	0.212

Based on data from Figure 22, CI values were calculated using the Chou-Talalay method.

Table 4 Combination index values for nutlin-3 plus NaB in A549 cells.

Nutlin-3 (µM)	NaB (mM)	CI
3	1	0.344
3	2	0.357
3	5	0.279
3	10	0.236
10	1	0.267
10	2	0.114
10	5	0.086
10	10	0.046

Based on data from Figure 22, CI values were calculated using the Chou-Talalay method.

Table 5 Combination index values for nutlin-3 plus MS-275 in A549 cells.

Nutlin-3 (µM)	MS-275 (µM)	CI
3	0.5	0.178
3	1	0.030
3	2	0.028
3	5	0.009
10	0.5	0.052
10	1	0.020
10	2	0.009
10	5	0.003

Based on data from Figure 22, CI values were calculated using the Chou-Talalay method.

Table 6 Combination index values for nutlin-3 plus apicidin in A549 cells.

Nutlin-3 (µM)	Apicidin (µM)	CI
3	1	0.484
3	2	0.615
3	5	1.087
3	10	0.079
10	1	0.854
10	2	0.682
10	5	0.301
10	10	0.022

Based on data from Figure 22, CI values were calculated using the Chou-Talalay method.

4.2.2 Nutlin-3 and vorinostat synergise to induce cell death in p53 wild-type A2780 cells

To confirm the synergistic activity of nutlin-3 and HDACi in another cell line with wt-p53, we

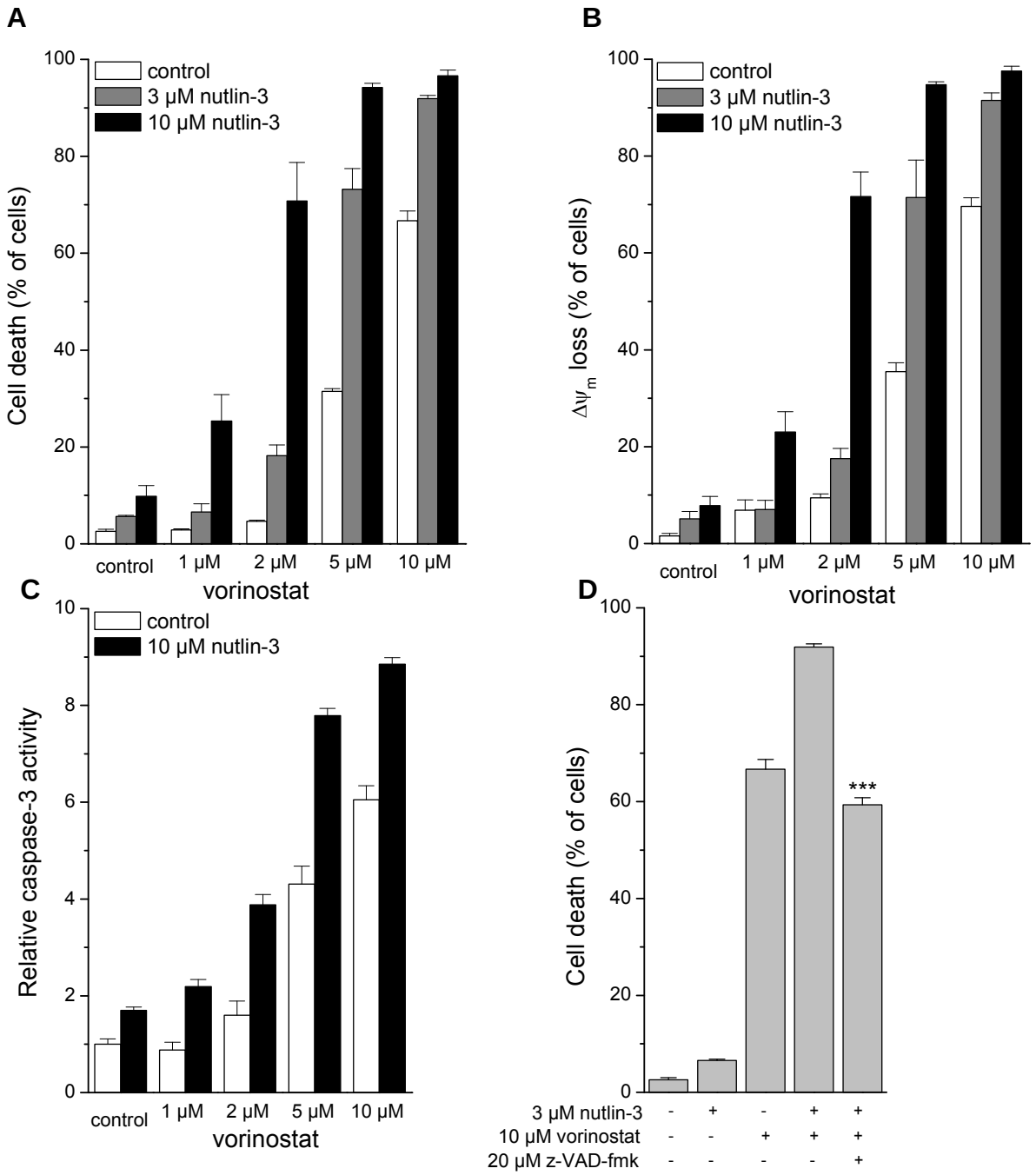

Figure 24 Nutlin-3 and vorinostat cooperate in inducing cell death, $\Delta\psi_m$ loss and caspase-3 activity in A2780 cells.

One hour after administration of nutlin-3, cells were exposed to vorinostat for another 24 h (caspase-3 assay) or 48 h (flow cytometric analyses). ***A)*** *Cell death was determined by flow cytometric analysis of PI uptake,* ***B)*** *$\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining.* ***C)*** *Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AFC; relative caspase-3 activities are the ratio of treated cells to untreated cells.* ***D)*** *Cell death was assessed by flow cytometric analysis, z-VAD-fmk was applied 1 h before treatment with nutlin-3. Means ± SEM of 3 separate experiments are shown (***$p < 0.005$).*

engaged A2780 ovarian cancer cells. Figure 24A and B shows that nutlin-3 and the HDACi representatively used, vorinostat, also exerted a cooperative cytotoxic activity in A2780 cells, as judged by assessing cell death and $\Delta\psi_m$ dissipation. The cell death data were analysed by the CI method, evidencing a synergistic effect at all concentrations except one (Table 7). To explore whether the synergistic action of nutlin-3 and HDACi involved caspases, we determined the activity of caspase-3 and we applied the pan-caspase inhibitor z-VAD-fmk in the PI uptake analysis. As presented in Figure 24C, nutlin-3 and vorinostat cooperated in triggering caspase-3 activity. In line with this result, z-VAD-fmk reduced cell death induced by the combination of nutlin-3 and vorinostat (Figure 24D).

Table 7 Combination index values for nutlin-3 plus vorinostat in A2780 cells.

Nutlin-3 (µM)	Vorinostat (µM)	CI
3	1	1.254
3	2	0.656
3	5	0.395
3	10	0.375
10	1	0.338
10	2	0.170
10	5	0.155
10	10	0.231

Based on data from Figure 24A, CI values were calculated using the Chou-Talalay method.

4.2.3 Nutlin-3 and vorinostat do not synergise in p53 null PC-3 cells

Moreover, to validate the observed synergistic effect of the nutlin-3/HDACi combination was p53-dependent, we employed p53 null PC-3 prostate cancer cells. Figure 25A and B shows that vorinostat-induced both cell death and $\Delta\psi_m$ decay in a dose-dependent fashion. In contrast, nutlin-3 had no effect, and the cytotoxicity of vorinostat in combination with nutlin-3 did not exceed that of vorinostat alone.

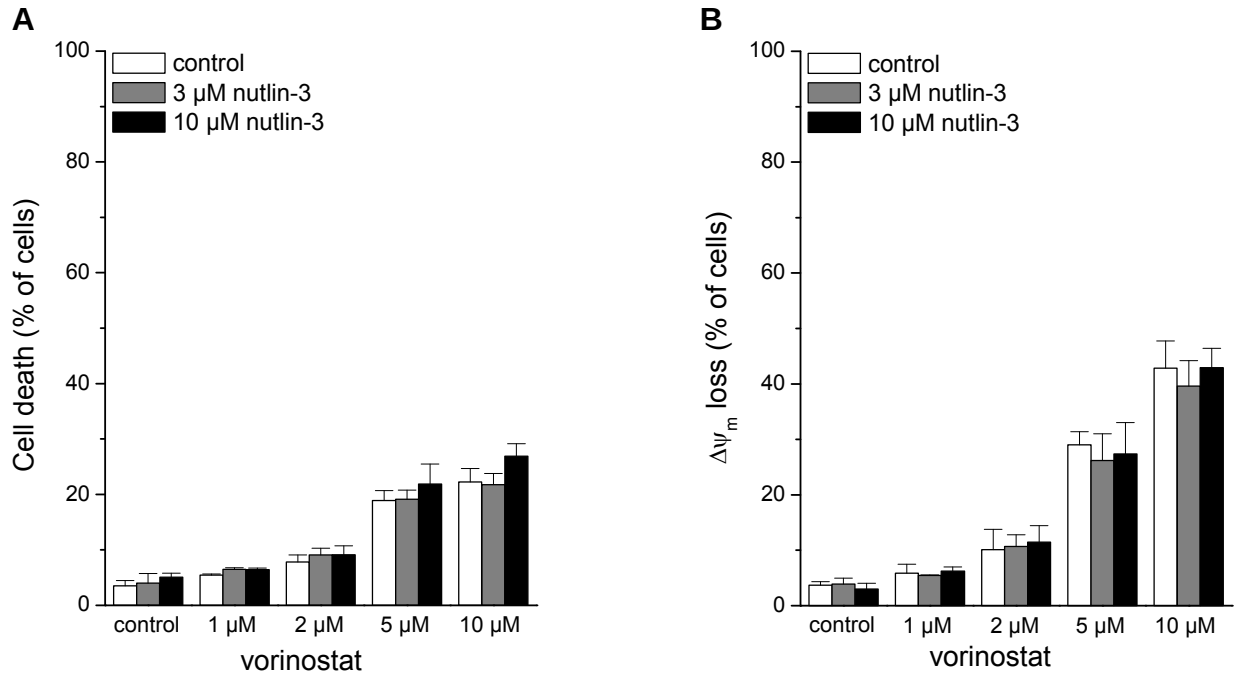

Figure 25 Nutlin-3 and vorinostat do not cooperate in PC-3 cells.

One hour after administration of nutlin-3, cells were exposed to vorinostat for another 48 h. ***A)*** *Cell death was determined by flow cytometric analysis of PI uptake, and* ***B)*** *$\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of 3 separate experiments are shown.*

4.2.4 Nutlin-3 protects A549 cells from paclitaxel-induced cytotoxic effects

Nutlin-3 has been reported to confer protection against paclitaxel (Carvajal et al., 2005;Tokalov et al., 2010). For comparison to the observed synergistic interaction of nutlin-3 and HDACi, we thus examined the combination of nutlin-3 and paclitaxel in A549 cells and determined cell death by PI uptake and $\Delta\psi_m$ loss by $DiOC_6(3)$ staining. Both cell death and mitochondrial membrane potential loss assays revealed that nutlin-3 indeed protected the cells from the cytotoxicity of paclitaxel (Figure 26). The CI analysis of the cell death results demonstrated a strong antagonism for this drug combination (Table 8).

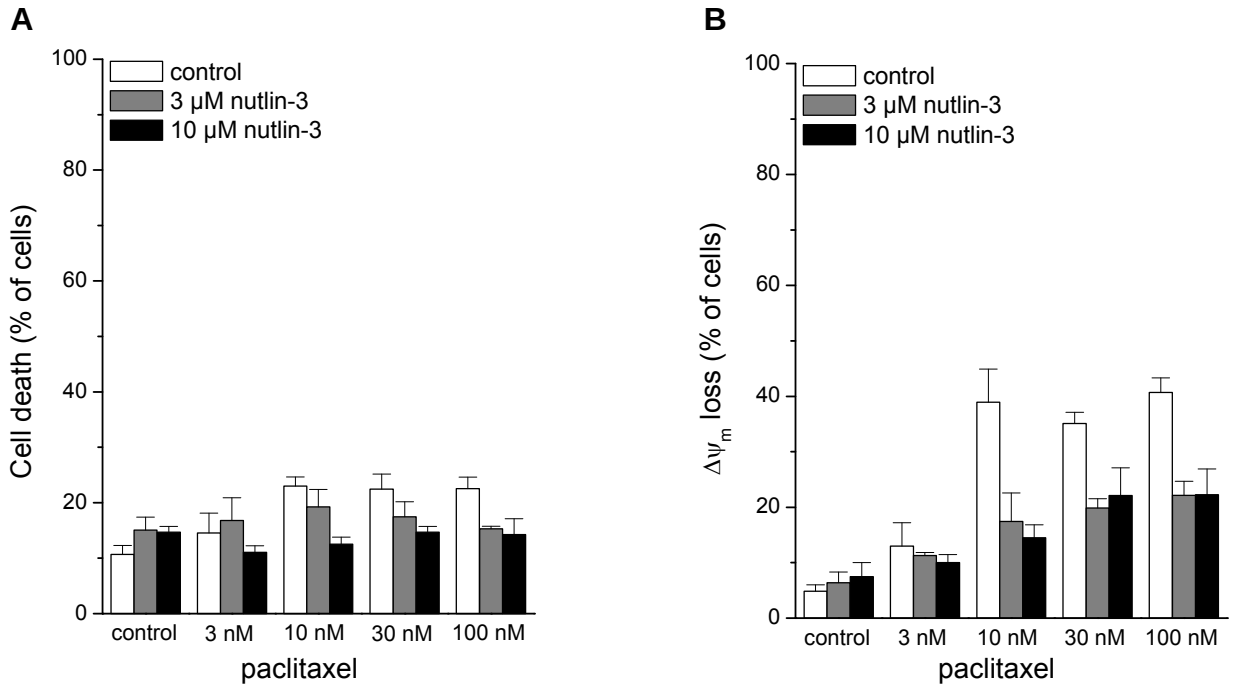

Figure 26 Nutlin-3 protects A549 cells from cytotoxicity of paclitaxel.

One hour after administration of nutlin-3, cells were exposed to paclitaxel for another 48 h. ***A)*** *Cell death was determined by flow cytometric analysis of PI uptake, and* ***B)*** *$\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of 3 separate experiments are shown.*

Table 8 Combination index values for nutlin-3 plus paclitaxel in A549 cells.

Nutlin-3 (µM)	Paclitaxel (nM)	CI
3	3	242
3	10	227,000
3	30	1701
3	100	77
10	3	33
10	10	40
10	30	33
10	100	136

Based on data from Figure 26A, CI values were calculated using the Chou-Talalay method.

4.2.5 Vorinostat induces p53 hyperacetylation

Because HDACi have been observed to activate p53 by acetylation (Luo et al., 2000;Terui et al., 2003;Zhao et al., 2006;Carlisi et al., 2008;Condorelli et al., 2008), we tested whether the synergistic activity of nutlin-3/HDACi was associated with an increase in acetyl-p53. To determine the acetylation status of p53, we used an antibody specific for p53 acetylation at lysine residues 373 and 382. For comparison, we also assessed the abundance of total p53. As expected, nutlin-3 treatment raised total p53, both in the absence and in the presence of vorinostat (Figure 27A). With respect to acetyl-p53 in A549 cells, we made the remarkable observation that nutlin-3 treatment - though enhancing total p53 - did not alter the abundance of

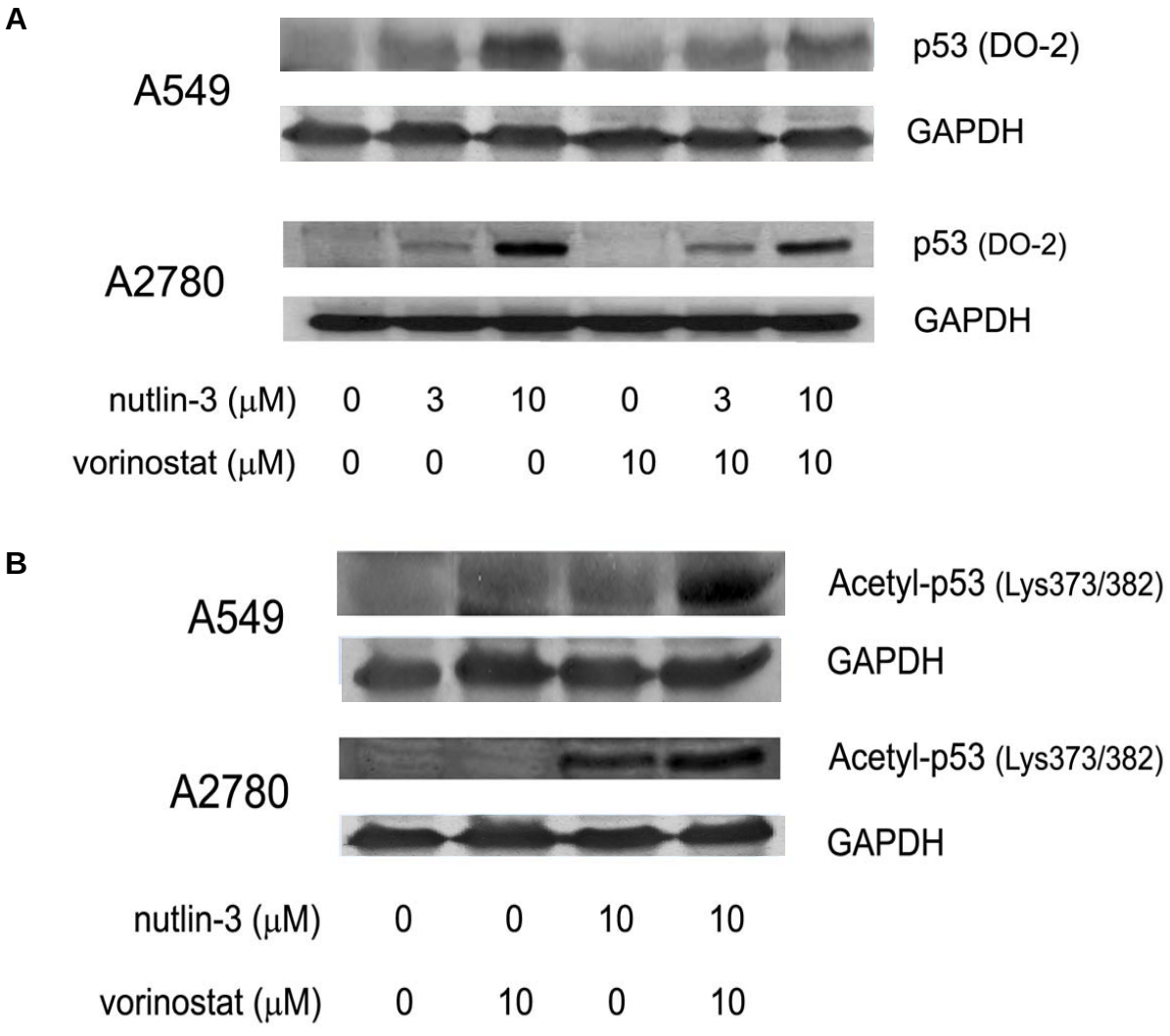

Figure 27 Vorinostat induces p53 hyperacetylation.

One hour after administration of nutlin-3, cells were exposed to vorinostat for another 24 h. ***A)*** *The expression of total p53 was determined using the anti-p53 (DO-2) antibody.* ***B)*** *The acetylation status of p53 was analysed by immunoblotting using an anti-acetyl-p53 (Lys373/382) antibody.*

acetylated p53. However, cotreatment with vorinostat resulted in a considerable increase in acetyl-p53 (Figure 27B). In A2780 cells, nutlin-3 enhanced acetyl-p53, which was further enhanced by cotreatment with vorinostat.

4.2.6 Vorinostat induces downregulation of MDM2 and MDM4 gene expression

MDM2 and MDM4 (also known as MDMX) are the main negative regulators of p53 function (Brown et al., 2009). We thus wondered whether the enhancement of nutlin-3-induced apoptosis by HDACi could be correlated with an effect of HDACi on MDM2 and/or MDM4 gene expression. The regulation of MDM2 and MDM4 expression differs in that the former is induced by p53 - that way producing a negative-feedback loop - while the latter is not (Brown et al., 2009). In consistence, we found nutlin-3 to induce the gene expression of MDM2, but not of MDM4, as determined by real-time RT-PCR (Figure 28). Interestingly, vorinostat significantly reduced the constitutive gene expression of both MDM2 and MDM4 as well as the nutlin-3-elevated gene expression of MDM2. These results suggest that vorinostat could block both the MDM2 and MDM4 expression as well nutlin-3 induced MDM2 expression.

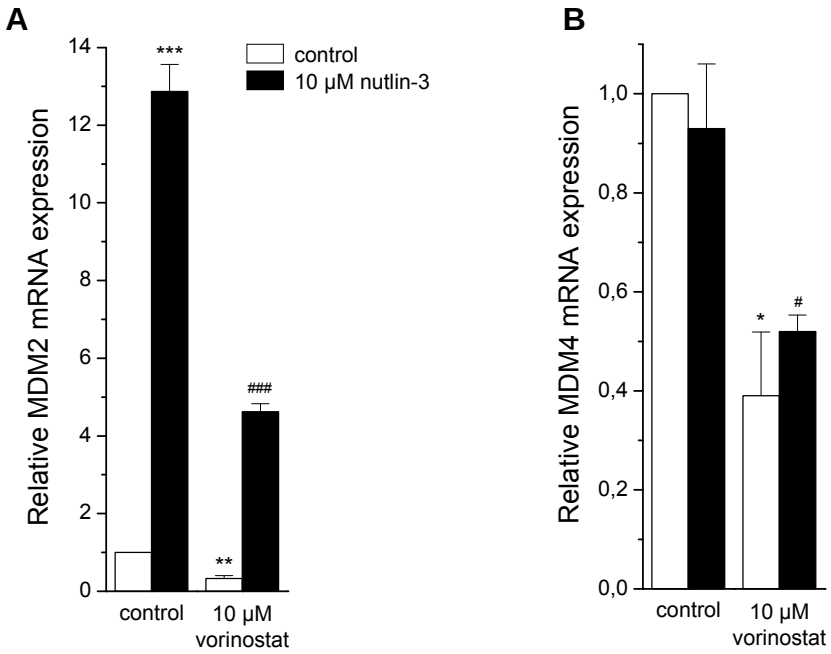

Figure 28 Vorinostat induces downregulation of MDM2 and MDM4 gene expression.

One hour after administration of nutlin-3, cells were exposed to vorinostat for another 24 h. ***A)*** *MDM2 and* ***B)*** *MDM4 mRNA expression levels were determined by real-time RT-PCR and normalised to β-2-microglobulin mRNA levels. Means ± SEM of 3 separate experiments are shown (nutlin-3- or vorinostat-treated vs. untreated: *p < 0.05, **p < 0.01, ***p < 0.005; nutlin-3/vorinostat-treated vs. nutlin-3-treated: #p < 0.05, ###p < 0.005).*

4.3 Anticancer effects of the p53 activator nutlin-3 in Ewing's sarcoma cells

Fifty percent of adult patients have tumours with mt-p53 and are, thus, unlikely to benefit from nutlin-3 therapy. However, alterations in the p53 gene are much less common in childhood malignancies. For instance, among children with Ewing's sarcoma (ES), only about 10% have been found with p53 alterations (Kovar et al., 1993;Huang et al., 2005), suggesting that targeting and activation of p53 may be an effective therapeutic strategy for ES.

Nutlin-3 has already been shown to exert anticancer effects in childhood tumour models with wt-p53, e.g. in neuroblastoma (Barbieri et al., 2006;Van Maerken et al., 2006;Van Maerken et al., 2009), in retinoblastoma (Elison et al., 2006;Laurie et al., 2006), in acute lymphoblastic leukaemia (Gu et al., 2008) and in rhabdomyosarcoma (Miyachi et al., 2009). Although 90% of ES patients with wt-p53 are potentially amenable to nutlin-3 treatment, however, it has not yet been tested for antineoplastic activity against ES.

Initially, ES cells were employed to investigate the combination effect of HDACi and nutlin-3. However no significant effect was observed in combination of HDACi with nutlin-3. But interestingly, we observed an effect of nutlin-3 as a single agent at the low concentration of 0.5 μM (Figure 29). This observation prompted us to investigate the potential anticancer action of nutlin-3 on ES cells in more depth.

4.3.1 Nutlin-3-induced effects on p53 in ES cells

To establish that the action of nutlin-3 is dependent on the p53 gene status in ES cells, we studied its effects in three ES cell lines with wt-p53 (WE-68, VH-64 and CADO-ES-1) and in one ES cell line with mt-p53 (SK-ES-1) (Kovar et al., 1993;Ottaviano et al., 2010). Initially, we assessed the impact of nutlin-3 on p53 abundance. ES cells were treated with 2 μM or 10 μM nutlin-3, and p53 abundance was determined by immunoblot analysis using the anti-p53 (DO-2) antibody.

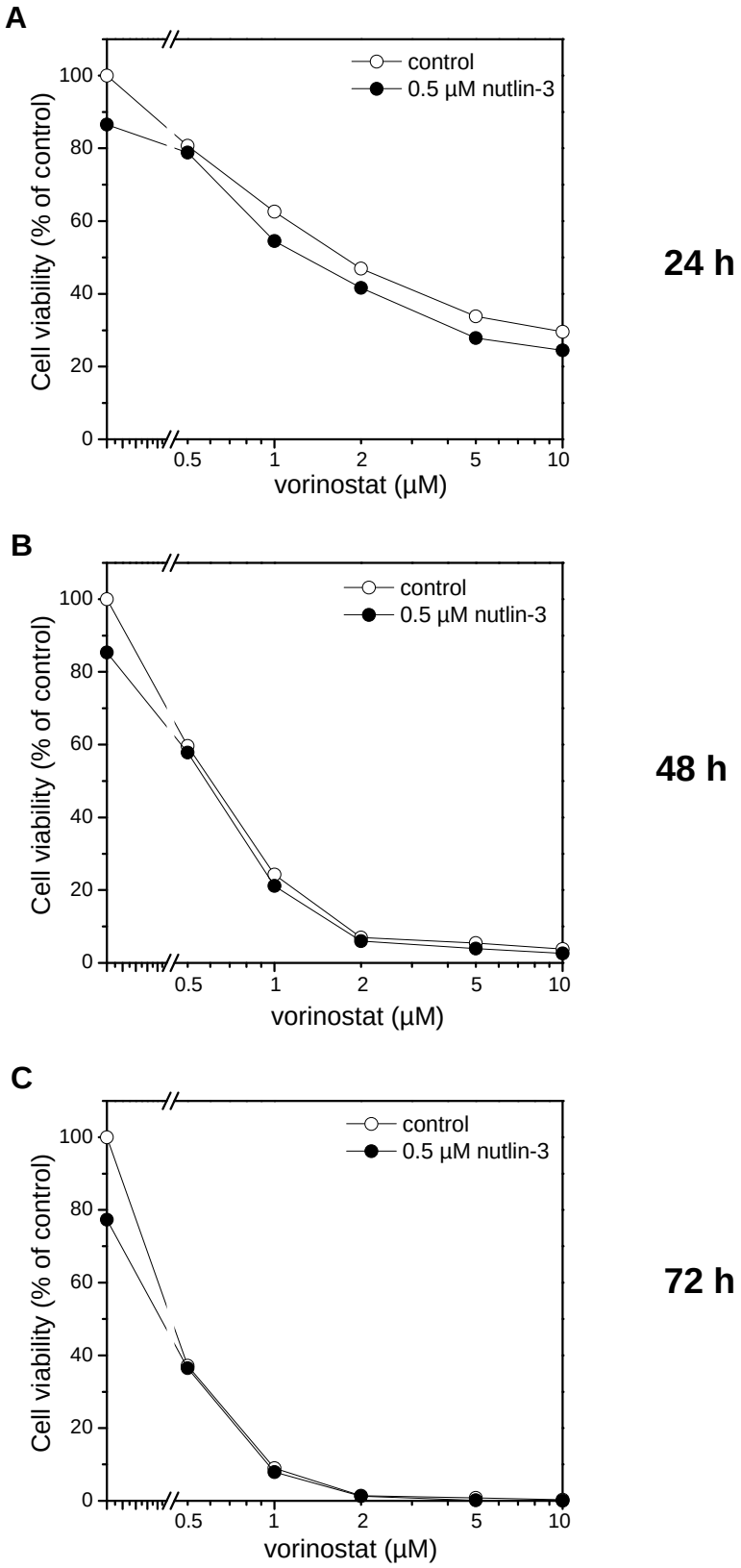

Figure 29 Effect of nutlin-3 on vorinostat-affected cell viability in WE-68 cells.

*Cells were pretreated with 0.5 µM of nutlin-3 for 1 h, and then incubated with various concentrations of vorinostat for **A)** 24 h, **B)** 48 h and **C)** 72 h. Cell viability was determined by Alamar Blue assay.*

Immunoblot detection revealed a rise of p53 after nutlin-3 exposure in the ES cell lines with wt-p53 (Figure 30). In consistence with the high expression of mt-p53 observed in a wide range of tumours (Bartek et al., 1991), mt-p53 SK-ES-1 cells displayed a much stronger constitutive expression of p53, which, however, was not enhanced by nutlin-3 treatment.

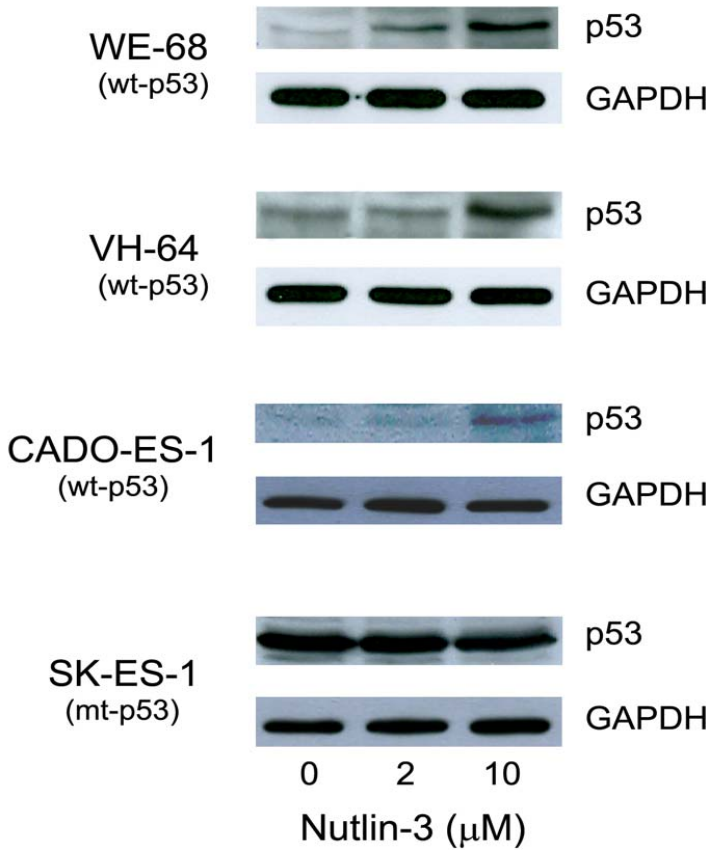

Figure 30 Nutlin-3 increases p53 level in ES cells with wt-p53.

Cells were exposed to nutlin-3 for 24 h. The abundance of p53 was determined by immunoblotting using the anti-p53 (DO-2) antibody and anti-GAPDH.

4.3.2 Nutlin-3-induced effects on p53 targets in ES cells

MDM2, p21 and PUMA are three key transcriptional targets of p53 (Vousden et al., 2009). To confirm the effect of nutlin-3 on p53, we determined the expression of these three genes by using quantitative real-time RT-PCR. As shown in Figure 31, nutlin-3-induced the mRNA levels of MDM2, p21 and PUMA in a concentration-dependent manner in the wt-p53 (WE-68, VH-64 and CADO-ES-1) cell lines, However, nutlin-3 did not induce mRNA levels of MDM2, p21 and PUMA in the mt-p53 SK-ES-1 cell line.

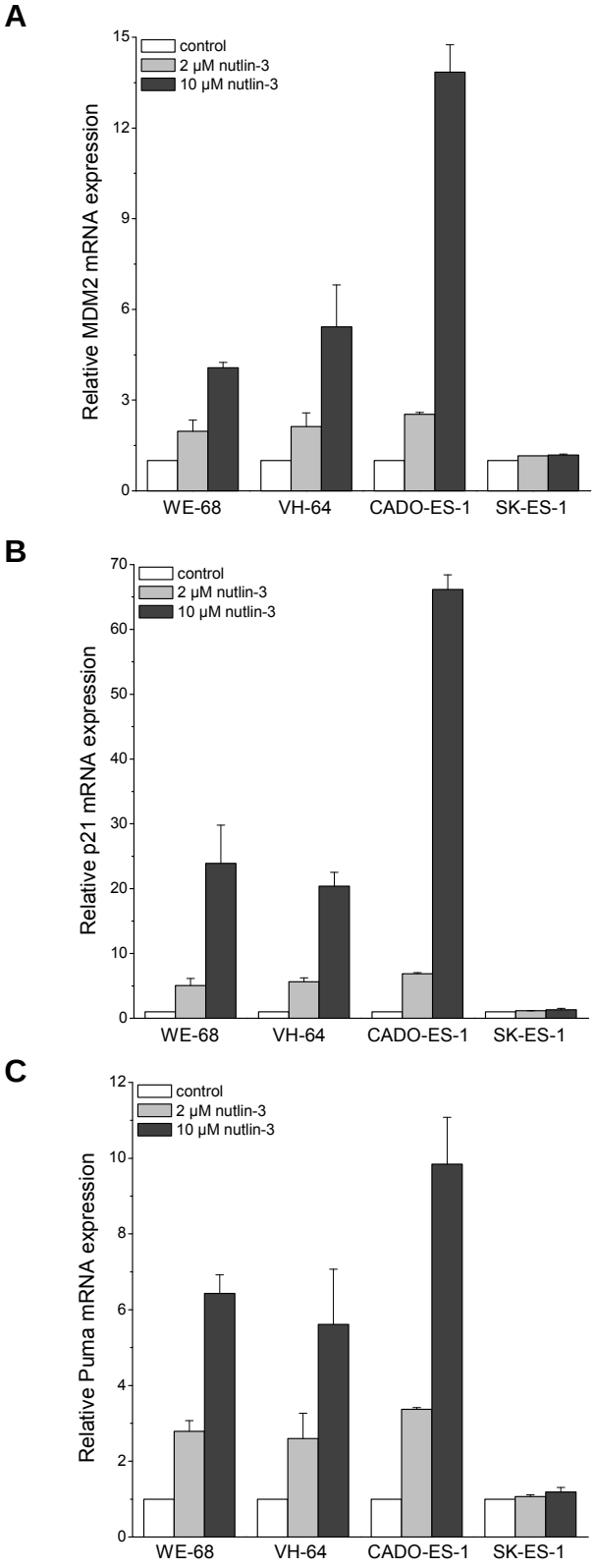

Figure 31 Nutlin-3 induces expression of p53 target genes in ES cells with wt-p53, but not in cells with mt-p53.

Cells were exposed to nutlin-3 for 24 h. ***A)*** *MDM2,* ***B)*** *p21 and* ***C)*** *PUMA mRNA expression levels were determined by real-time RT-PCR and normalised to β-2-microglobulin mRNA levels. Means ± SEM of 2 separate measurements are shown.*

4.3.3 Nutlin-3-induced antineoplastic effects in ES cells

4.3.3.1 Nutlin-3 affects cell viability in wt-p53 ES cells

To investigate the effect of nutlin-3 on cell growth and cell death in wt-p53 ES cells, we determined differential cell counts of total and viable cells. Figure 32 shows that nutlin-3 affected cell growth in a dose-dependent manner. At 72 h after treatment with 10 μM nutlin-3, the total number of cells (open symbols) was reduced by 77% to 87% in the wt-p53 cells and by 35% in the mt-p53 cells. More significantly, the number of viable cells (solid symbols), as determined by trypan blue exclusion cell count, was decreased by 93% to 95% in the wt-p53 cells and by 42% in the mt-p53 cells. The calculation of fractions of viable cells at each drug concentration revealed a decline in cell viability of 60% to 75% in the wt-p53 cells and of 11% in the mt-53 cells (inset diagrams). These findings show that nutlin-3 inhibits cell growth to a greater extent in wt-p53 cells and lesser extent in mt-p53 cells, but induces significant cell death only in wt-p53 cells.

4.3.3.2 Nutlin-3 induces cell death through apoptosis in wt-p53 ES cells

To gain further insight into nutlin-3-elicited cell death and, more specifically, apoptosis, we analysed the effects of nutlin-3 by a number of read-outs. To begin with, cell death was assessed by flow cytometric analysis of PI uptake. As shown in Figure 33A, treatment with nutlin-3 resulted in a concentration-dependent induction of cell death in cells with wt-p53, but not in cells with mt-p53.

p53 is a potent inducer of apoptosis and as such it predominantly triggers the mitochondrial pathway of apoptosis (Vousden et al., 2009). We therefore examined whether nutlin-3-mediated cell death involved apoptosis. The same was evaluated by measuring $\Delta\psi_m$ dissipation, caspase-3 activity and DNA fragmentation. First, we determined $\Delta\psi_m$ loss by flow cytometric analysis of $DiOC_6(3)$ staining. As shown in Figure 33B, the results reflect those of the cell death assay: nutlin-3 induced decay of $\Delta\psi_m$ in the wt-p53 cells, but not in the mt-p53 cells. Second, we measured caspase-3 activity. In consistence with the other read-outs, nutlin-3 caused caspase-3 activation in the wt-p53 cells, but not in the mt-p53 cells (Figure 34A). Third, we assessed cells harbouring wt-p53 for apoptosis by staining the nuclei of ethanol-fixed cells with PI and determining the DNA content by flow cytometry. Figure 34B demonstrates that nutlin-3 promoted DNA fragmentation in a dose-dependent fashion in the three wt-p53 cell lines.

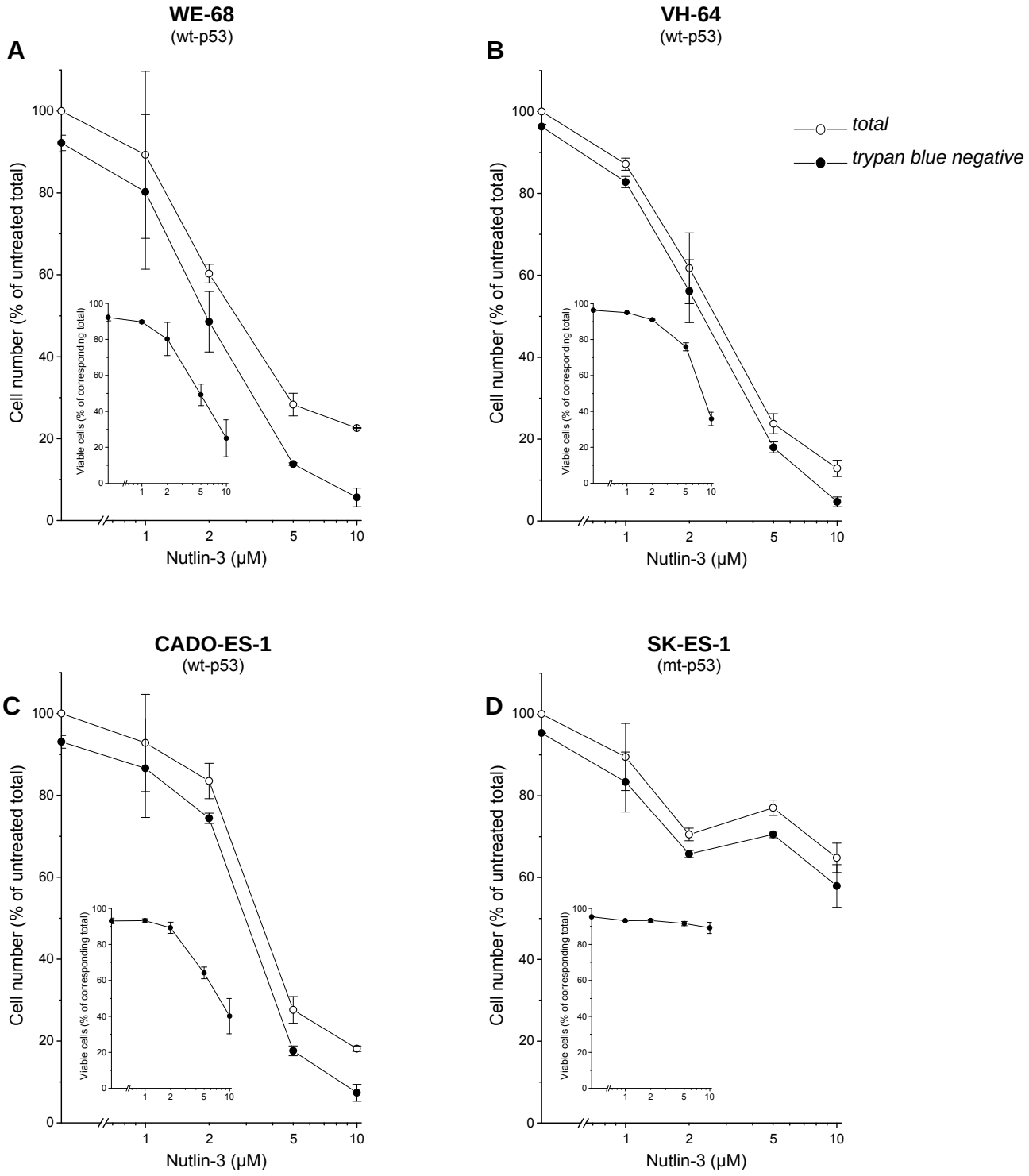

Figure 32 Nutlin-3 inhibits cell growth and induces cell death in ES cells.

Exponentially growing cells were exposed to nutlin-3 for 72 h and cell growth and viability were determined by trypan blue exclusion cell count. The insets show the percentage of viable cells at each drug concentration. ***A)*** *WE-68,* ***B)*** *VH-64,* ***C)*** *CADO-ES-1, and* ***D)*** *SK-ES-1 cells. Means ± SEM of 2 separate measurements are shown.*

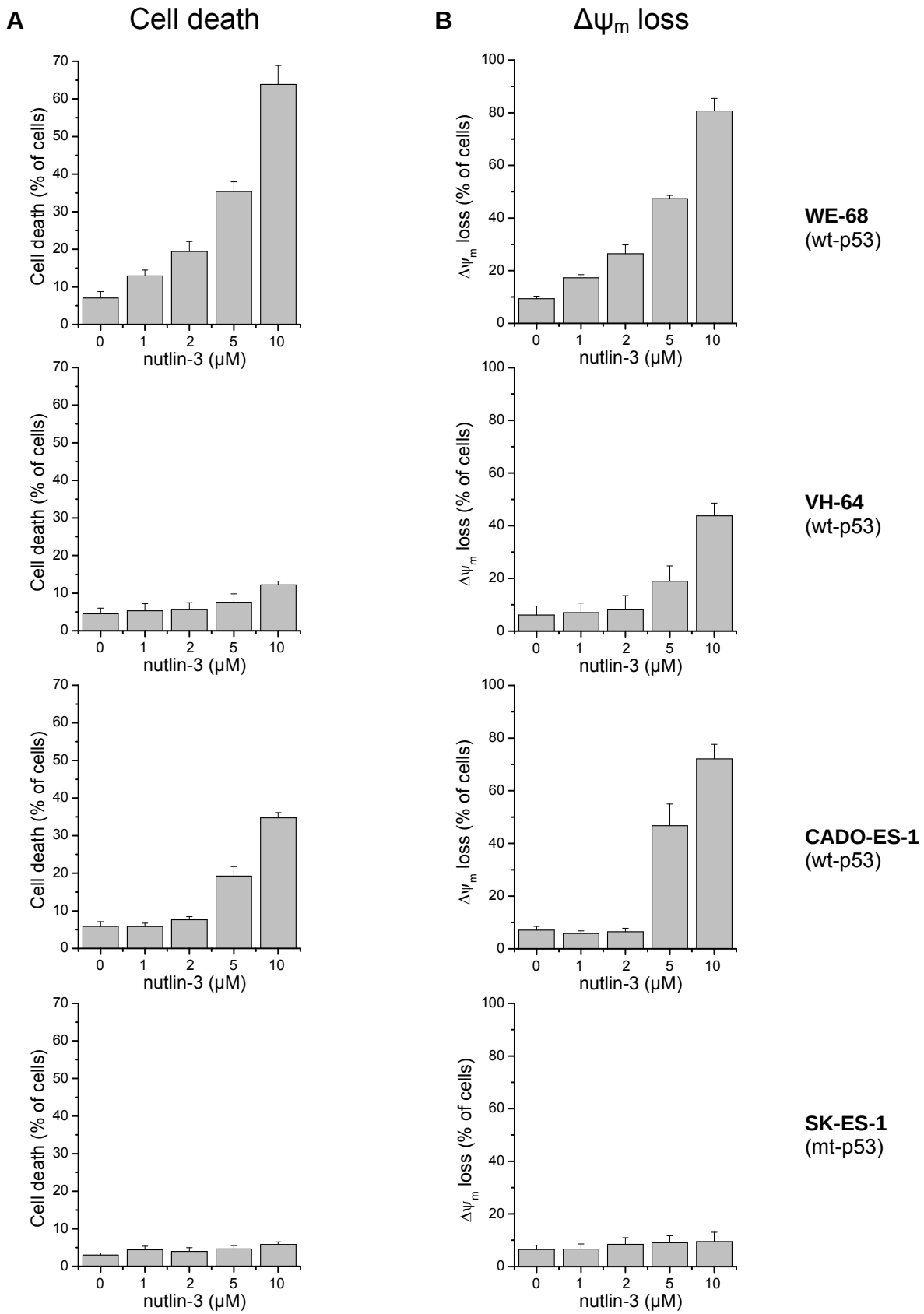

Figure 33 Nutlin-3 induces apoptosis in ES cells with wt-p53, but not in cells with mt-p53.

Cells were exposed to nutlin-3 for 48 h. ***A)*** *Cell death was determined by flow cytometric analysis of PI uptake.* ***B)*** *$\Delta\psi_m$ loss was determined by flow cytometric analysis of $DiOC_6(3)$ staining.*

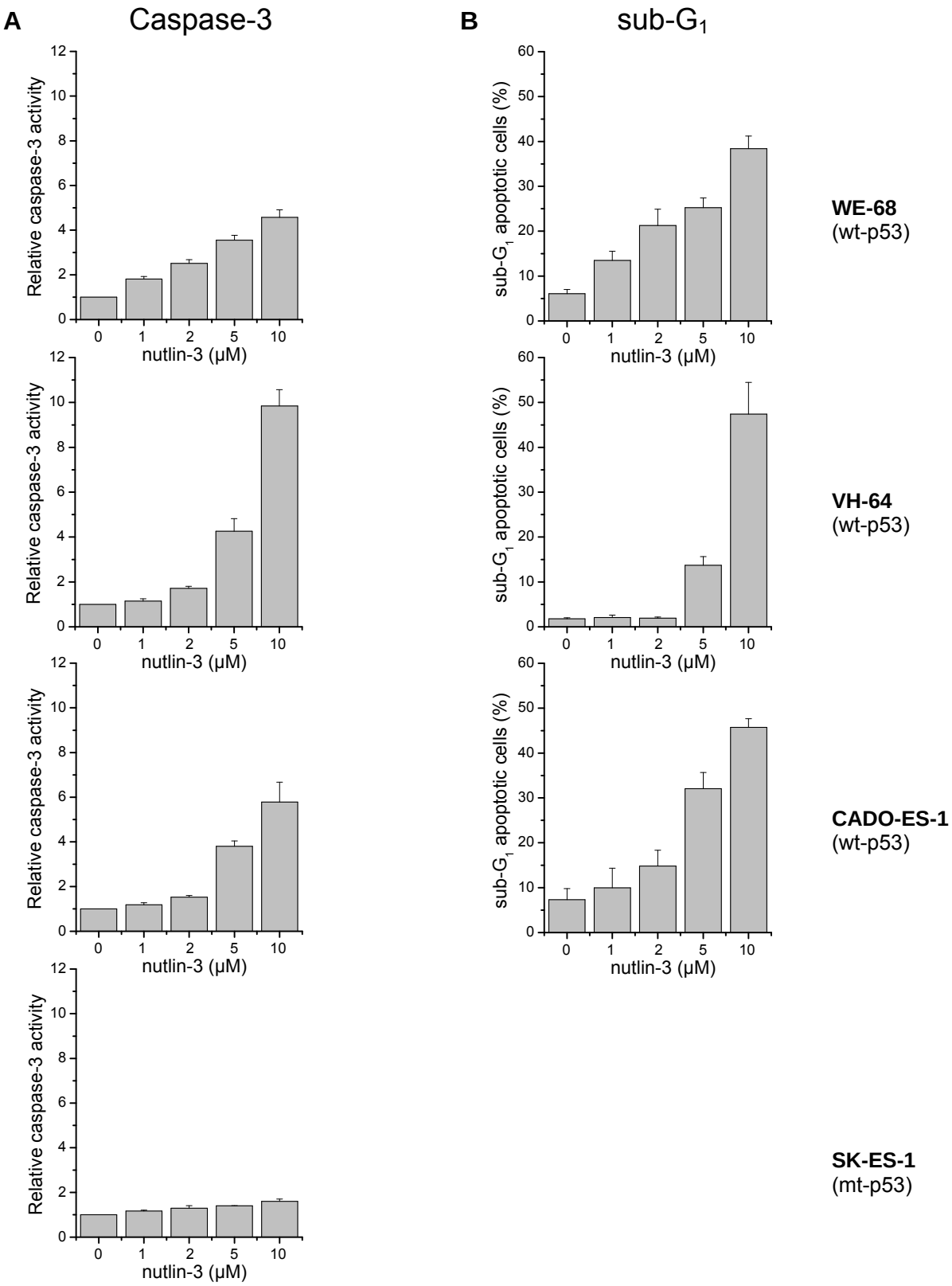

Figure 34 Nutlin-3 induces apoptosis in ES cells with wt-p53.

Cells were exposed to nutlin-3 for 24 h (A) or 48 h (B). ***A)*** *Caspase-3 activity was determined using the fluorogenic substrate Ac-DEVD-AFC; relative caspase-3 activities are the ratio of treated cells to untreated cells.* ***B)*** *DNA fragmentation was determined by flow cytometric cell cycle analysis; apoptotic cells were detected as sub-G_1 fraction. Means ± SEM of 3 separate measurements are shown.*

4.3.4 Induction of cellular senescence by nutlin-3

In addition to apoptosis, p53 can also trigger cellular senescence (Vazquez et al., 2008). Accordingly, nutlin-3 has been reported to induce a senescence response (Van Maerken et al., 2006). We thus addressed the question of whether nutlin-3 could promote cellular senescence in ES cells. CADO-ES-1 cells were treated with nutlin-3 at a non-toxic dose (2 µM). After a 4-day exposure to nutlin-3, cells were analysed for SA-β-Gal activity by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, the most widely used marker for cellular senescence. Nutlin-3-treated cells showed clear signs of senescence, whereas untreated cells did not show senescence (Figure 35).

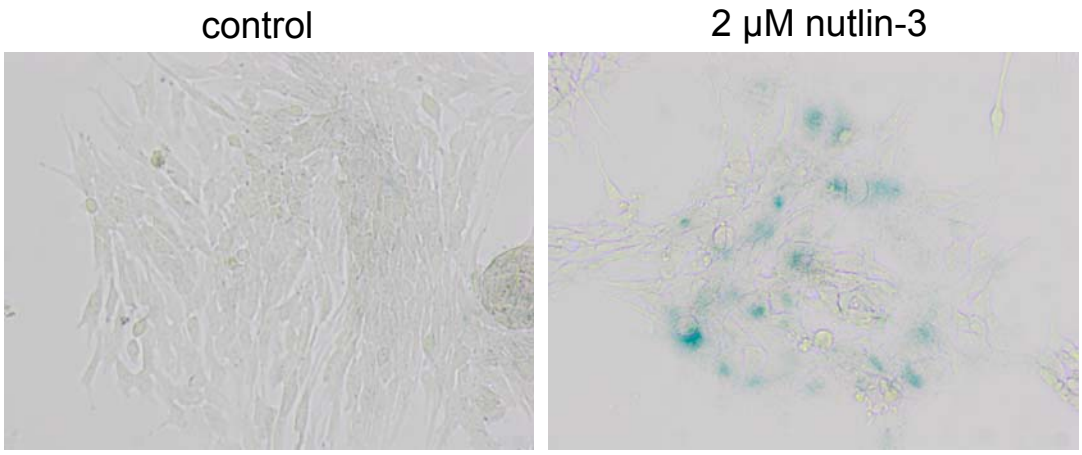

Figure 35 Nutlin-3 induces cellular senescence in CADO-ES-1 cells.
After a 96 h exposure to nutlin-3 or vehicle control, cellular senescence was detected by staining for SA-β-Gal activity.

4.3.5 Synergistic effect of nutlin-3 with the NF-κB inhibitor CAPE

The combination of nutlin-3 with other anticancer agents has been shown in vitro to have synergistic effects on tumour cells (Shangary et al., 2009). However, though the simultaneous targeting of p53 and NF-κB is considered a promising antineoplastic strategy (Dey et al., 2008), a possible favourable interaction between nutlin-3 and NF-κB inhibitors has not yet been studied. To explore this issue, we examined whether nutlin-3 and the NF-κB inhibitor CAPE would cooperate in exerting antitumour activity against wt-p53 ES cells. For this purpose, VH-64 cells were chosen because they had exhibited the weakest response to nutlin-3 treatment, as judged by PI uptake and $DiOC_6(3)$ staining analyses (see Figure 33A and B). As shown in Figure 36, nutlin-3 and CAPE cooperated both in eliciting cell death and mitochondrial depolarisation. To test for synergy, we analysed the cell death data by the CI method (CI < 1 is indicative for a synergistic interaction; (Chou, 2010)). The calculated CI values indicated

synergism for the combinations of 10 µM CAPE with 5 µM or 10 µM nutlin-3 and for 25 µM CAPE with nutlin-3 at all concentrations (Table 9).

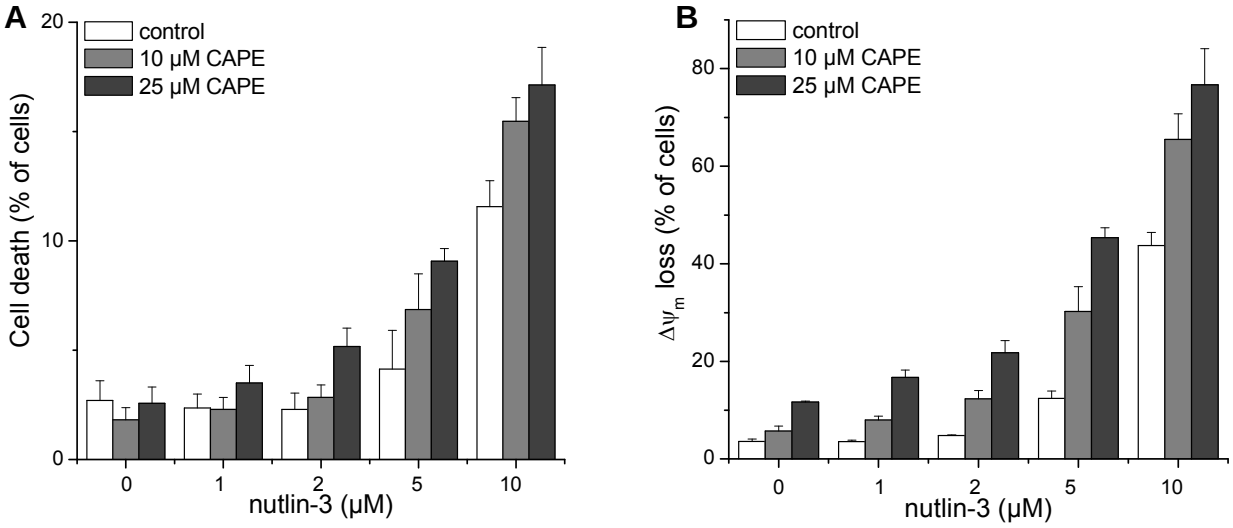

Figure 36 Nutlin-3 and the NF-κB inhibitor CAPE cooperate in inducing cell death and $\Delta\psi_m$ loss in VH-64 cells.

One hour after administration of CAPE, cells were exposed to nutlin-3 for another 48 h. ***A)*** *Cell death and* ***B)*** *$\Delta\psi_m$ were determined by flow cytometric analyses of PI uptake and $DiOC_6(3)$ staining, respectively. Means ± SEM of 3 separate measurements are shown.*

Table 9 Combination index values for CAPE plus nutlin-3 in VH-64 cells.

CAPE (µM)	Nutlin-3 (µM)	CI
10	1	1.251
10	2	1.372
10	5	0.761
10	10	0.419
25	1	0.829
25	2	0.598
25	5	0.514
25	10	0.356

Based on data from Figure 36A, CI values were calculated using the Chou-Talalay method.

4.4 Involvement of serine proteases in HDACi-induced cell death

Results from initial experiments employing the serine protease inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) suggested a role for serine protease activity in HDACi-induced apoptosis in MCF-7 cells. This was initially found by Michael Sigler from our group. To reproduce this finding, cells were treated with AEBSF and cotreated with varying concentrations of vorinostat, and cells were analysed by cell cycle analysis. It was again observed that the vorinostat-induced apoptosis was reduced by AEBSF (Figure 37).

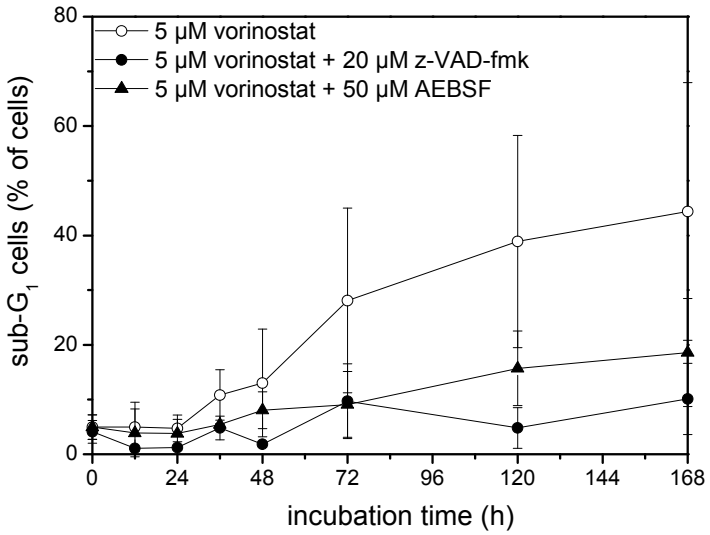

Figure 37 Effect of z-VAD-fmk and AEBSF on vorinostat in MCF-7 cells.

Cells were pretreated with 20 µM of z-VAD-fmk or 50 µM of AEBSF one hour prior to the application of vorinostat, then incubated for 168 h. Cell cycle analysis was assessed by using flow cytometry. Means of 3 separate experiments are shown.

4.4.1 AEBSF prevents HDACi-mediated apoptosis in SKOV-3 cells

To extend this finding, we also employed SKOV-3 cells to assess serine protease involvement in HDACi-elicited apoptosis by using AEBSF. Cells were pretreated for 1 h with AEBSF or z-VAD-fmk and further incubated with HDACi (TSA, vorinostat, NaB and MS-275) for 48 h. Cell death was determined by PI uptake using flow cytometry. TSA- and vorinostat-induced cell death was 45% and 37%, respectively. In the presence of AEBSF, TSA- and vorinostat-induced cell death was reduced to 3% and 5%, respectively. In contrast, AEBSF had no effect on cell death triggered by NaB and MS-275. For comparison, the pan-caspase inhibitor z-VAD-fmk reduced cell death mediated by all four HDACi (Figure 38).

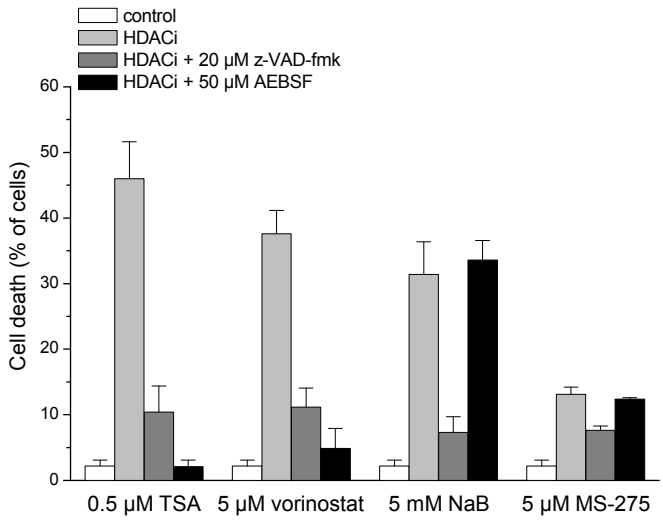

Figure 38 AEBSF protects against HDACi-mediated cell death in SKOV-3 cells.

An hour after administration of AEBSF, cells were further incubated with HDACi for 48 h. Cell death was determined by PI uptake using flow cytometry. Means of 3 separate experiments are shown.

4.4.2 AEBSF reduces TSA-mediated cytotoxic effect in SKOV-3 cells

To validate the protecting effect of AEBSF on TSA-induced cytotoxicity, cells were pretreated with AEBSF and cotreated with 1 µM of TSA for 10 days, and clonogenic assay was performed. Figure 39 shows that the cyctotoxic effect of TSA was fully suppressed by AEBSF under these

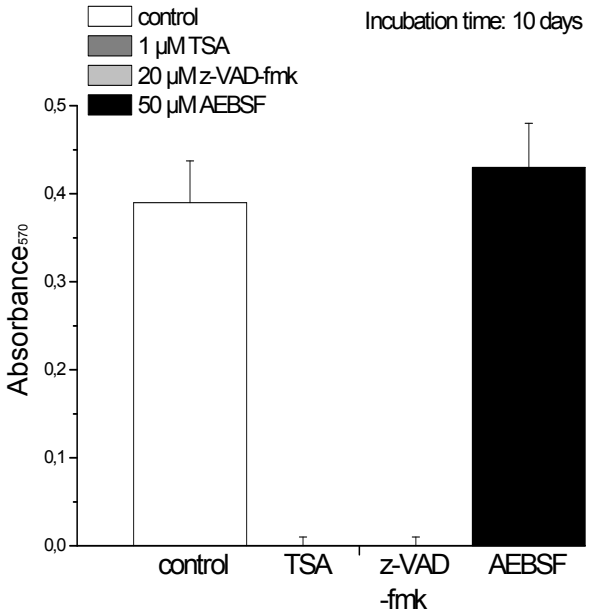

Figure 39 AEBSF protects against TSA-mediated cytotoxic effect in SKOV-3 cells.

An hour after administration of AEBSF, cells were treated with TSA and incubated for 10 days, after which a clonogenic assay was performed. Means of 3 separate experiments are shown.

extremely toxic conditions. This effect of AEBSF seemed too strong to be due to a specific inhibition of proteases and, thus, prompted speculation that it was, rather, due to a direct inactivation of TSA by AEBSF.

4.4.3 AEBSF prevents TSA-mediated HDAC inhibition in both SKOV-3 and A549 cells

To verify this speculation, it was tested whether AEBSF could impede the effect of TSA on HDAC enzymatic activity in SKOV-3 cells. HDAC activity was measured in cells pretreated with various concentration of AEBSF and cotreated with TSA, vorinostat and NaB. HDAC

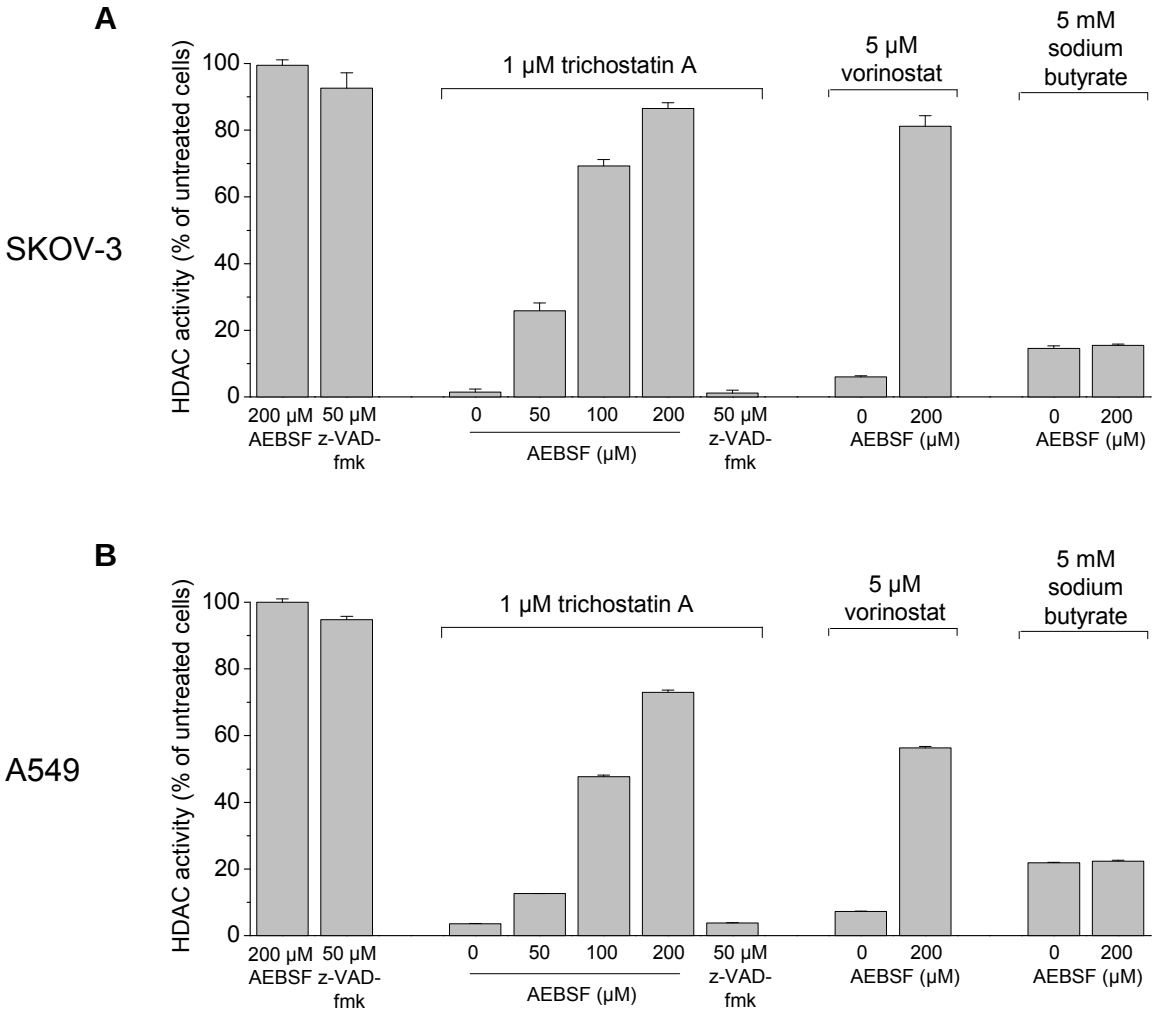

Figure 40 HDAC activity was measured using Boc-K(Ac)-AMC in cells.

A) *SKOV-3 cells,* ***B)*** *A549 cells. HDAC activity was measured using Boc-K(Ac)-AMC. Means ± SEM of 3 separate experiments are shown.*

activity was measured in intact cells as described by Beckers and coworkers (Ciossek et al., 2008). AEBSF potently impaired the HDAC inhibitory effect of TSA and vorinostat, but it left NaB action unscathed (Figure 40A). For comparison, the pan-caspase inhibitor z-VAD-fmk did not affect the inhibitory action of TSA. To confirm the effect of AEBSF on TSA-mediated HDAC inhibition, AEBSF effect on TSA was investigated in another cancer cell line, A549, yielding similar results (Figure 40B).

4.4.4 AEBSF prevents TSA-mediated HDAC inhibition in a cell-free system

As well, recombinant HDAC1 activity was measured using a cell-free assay. Figure 41 shows that 50 µM, 100 µM, and 200 µM of AEBSF also impaired the recombinant HDAC1 inhibitory effect of TSA in this system.

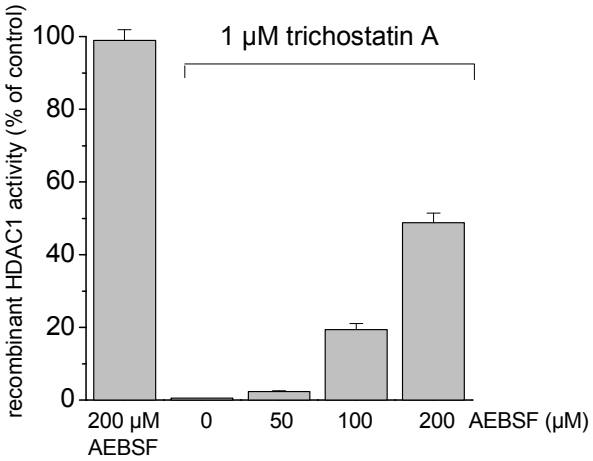

Figure 41 Recombinant HDAC1 activity was measured using Boc-K(Ac)-AMC.
Means ± SEM of 3 separate experiments are shown.

4.4.5 AEBSF reduces HDACi-induced acetylation of histone H4 in both SKOV-3 and A549 cells

In addition, SKOV-3 and A549 cell lines were used to examine AEBSF effect on HDACi-induced acetylation of histone H4 by analysing the acetylation status of H4. Cells were pretreated with AEBSF for an hour and cotreated with TSA, vorinostat and NaB, and further incubated for 24 h. Total protein was prepared and subjected to Western blot analysis (Figure 42).

Treatment with TSA and vorinostat resulted in marked accumulation of acetylated histone H4 after 24 h in both SKOV-3 and A549 cells. Pretreatment with AEBSF reduced accumulation of acetylated histone H4 in both SKOV-3 and A549 cells.

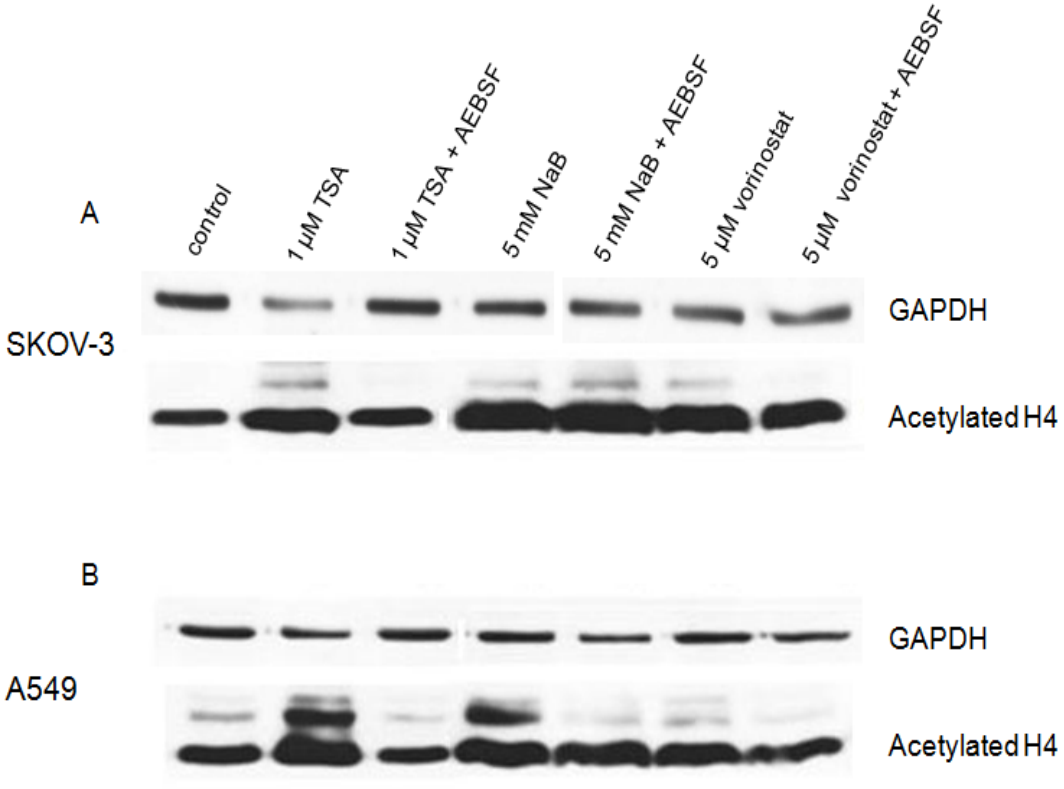

Figure 42 AEBSF prevents HDACi-induced acetylation of histone H4.

A) *SKOV cells and* ***B)*** *A549 cells. Cells were pretreated with AEBSF and cotreated with HDACi, further incubated for 24 h. Cells were harvested and Western blot analysis carried out using anti-Acetyl-histone H4 and GAPDH antibodies.*

5 DISCUSSION

5.1 HDACi vorinostat-induced G_2/M arrest in MCF-7 breast cancer cells

HDACi are potent anti cancer agents, which exert a diverse range of effects on cell growth and survival in neoplastic cells. They have been shown to induce transformed cell death by inducing growth arrest, extrinsic and intrinsic apoptosis pathway, senescence, mitotic cell death, autophagic cell death and antiangiogenesis through transcription-dependent and transcription-independent mechanisms (Minucci et al., 2006;Xu et al., 2007). As well, studies have revealed that HDACi-induced G_1 and G_2/M cell cycle arrest, cell differentiation and cell death are more pronounced in cancer cells than in normal cells (Ruefli et al., 2001;Minucci et al., 2006;Oh et al., 2010).

HDACi-induced G_1 cell cycle arrest is accomplished by induction of $p21^{WAF1}$ expression in different cancers (Richon et al., 2000;Huang et al., 2000;Gui et al., 2004;Ocker et al., 2007;Noro et al., 2010;Hrzenjak et al., 2010). HDACi-induced G_2/M cell cycle arrest has been reported in a few studies (Qiu et al., 2000;Roy et al., 2005;Strait et al., 2005;Takai et al., 2006;Yang et al., 2009). However, little is known how the G_2/M arrest is brought about. Therefore, in the present study we investigated the mechanism of HDACi-induced G_2/M cell cycle arrest. In an earlier study, we demonstrated that HDACi require activated caspases to induce apoptosis in A549 and PC-3 cells. Concomitantly, we found that HDACi induced a strong G_2/M cell cycle arrest in both the cell lines, when apoptosis was blocked through the inhibition of caspase activity (Sonnemann et al., 2006).

To facilitate this study, we employed caspase-3-deficient MCF-7 breast cancer cells, a cell line which responds to HDACi treatment by undergoing cell cycle arrest rather than apoptosis. Hence, it allows studying HDACi-induced cell cycle effects without inhibiting caspase activity. The data presented here indeed show that HDACi inhibited cell proliferation through inducing cell cycle arrest in G_2/M phase in MCF-7 cells. In addition, this study also shows the involvement of the proteasome and NF-κB signalling in HDACi-induced cell cycle arrest.

Vorinostat is a main member of HDACi and a potent inhibitor of tumour cell growth both in culture (Richon et al., 1998) and in tumour-bearing animal models (Cohen et al., 1999;Butler et al., 2000). It has been shown to induce cell cycle arrest in several human transformed cell lines,

including prostate carcinoma, bladder carcinoma, myeloma, breast carcinoma, and murine erythroleukemia cells (Richon et al., 1996;Huang and Pardee, 2000;Richon et al., 2000;Butler et al., 2000;Butler et al., 2002). In this study, we found vorinostat to inhibit cell proliferation in a dose-dependent manner by inducing a weak G_1 arrest at low concentrations and a strong G_2/M arrest at higher concentrations. By which mechanism may HDACi induce G_2/M arrest? Typically, DNA damage leads to cell cycle arrest (Bunz et al., 1998;Xiao et al., 2006;Wei et al., 2010;Nam et al., 2010). We, thus, hypothesised that HDACi treatment induces a DNA damage response. In fact, HDACi have been reported to cause DNA damage (Gaymes et al., 2006;Munshi et al., 2006;Zhang et al., 2006;Lee et al., 2010). In order to evaluate whether HDACi induce DNA damage in tumour cells, we analysed proteins typically involved in the DNA damage response. We found that vorinostat induced phosphorylation of H2AX (γH2AX) at Ser139, which is a marker for DNA damage (Burma et al., 2001), suggesting that HDACi treatment indeed produces DNA damage.

In addition, we checked for phosphorylation of ATM in vorinostat-treated MCF-7 cells. DNA damage, particularly induction of DNA double-strand breaks (DSBs), has been reported to activate ATM through auto- or trans-phosphorylation of Ser1981. It has also been shown that DNA damage leads to cell cycle arrest through the activation of ATM (Bakkenist et al., 2003). In accordance with the data published, we observed that vorinostat induced ATM phosphorylation. Vorinostat-induced ATM phosphorylation could be inhibited by caffeine (an inhibitor of ATM and ATR), indicating that ATM phosphorylation is brought about by autophosphorylation. These data are in line with the hypothesis that the vorinostat-induced G_2/M cell cycle arrest is the result of DNA damage and mediated by ATM. These results are also concordant with previous studies that reported a TSA- and NaB-induced DNA damage response (Ju and Muller, 2003;Kim et al., 2009). Other HDACi have also been shown to induce ATM phosphorylation in various cancer cells (Gaymes et al., 2006;Rosato et al., 2010).

As our findings indicated that vorinostat triggered cell cycle arrest by inducing DNA damage, we examined other proteins involved in the DNA damage response and/or cell cycle progression, i.e. phospho-CHK1, -CHK2, -p53, -BRCA1, -p95/NBS1, -RAD-17, -Akt (Kastan et al., 2000;Zhao et al., 2001). However, we could not detect the phophorylation of any of these proteins mediated by vorinostat treatment.

To establish the involvement of ATM in vorinostat-induced G_2/M arrest, we employed caffeine and the ATM specific inhibitor KU-55933 in the cell cycle analyses. However, although caffeine had been found to prevent vorinostat-induced ATM phosphorylation, both inhibitors had no

effect on vorinostat-induced G_2/M arrest. This finding suggests that ATM is not necessary for the G_2/M arrest mediated by vorinostat. This conclusion is further corroborated by experiments which addressed the involvement of CHK1 and CHK2 in vorinostat-induced G_2/M arrest. CHK1 and CHK2 are major effectors of ATM, linking DNA damage to cell cycle arrest (Branzei et al., 2008). As shown in Figure 14, pretreatment with inhibitors of CHK1 and CHK2 had no significant effect on vorinostat-induced G_2/M arrest. Taken together, these results suggest that the vorinostat-induced G_2/M arrest is caused by other mechanisms than activation of the ATM-CHK2 pathway.

To gain additional insight into the mechanism of vorinostat-induced G_2/M arrest, other components involved in cell cycle regulation were studied. One component with an important role in cell cycle regulation is the proteasome (Mu et al., 2007). The proteasome inhibitor bortezomib is known to disrupt this process (Murakawa et al., 2007). Bortezomib (also called PS-341, VelcadeTM) has been approved by the FDA for the treatment of multiple myeloma and is now undergoing clinical trials for many other types of cancer (Adams, 2002;Voorhees et al., 2003;Adams, 2004). Thus, as the proteasome is involved in cell cycle regulation (Richardson and Anderson, 2003;Jackson et al., 2005;Sterz et al., 2008), we studied its role in vorinostat-induced G_2/M arrest in MCF-7 cells by using bortezomib. Our result shows that the vorinostat-induced G_1 arrest was abrogated by pretreatment with bortezomib at lower concentration. A low dose of bortezomib turned the vorinostat-induced G_1 arrest to a G_2/M arrest and a high dose of bortezomib neutralised vorinostat-induced G_2/M arrest, suggesting that the proteasome has a role in vorinostat-induced cell cycle arrest. These data are in agreement with the findings in colon cancer cell lines (Pitts et al., 2009). According to this observation, we found that both vorinostat and a lower concentration of bortezomib resulted in a synergistic inhibition of proliferation in MCF-7 cells.

Bortezomib has been shown to suppress NF-κB activity by blocking IkBα degradation (Hideshima et al., 2003). We, thus, tested whether the observed effects of bortezomib on vorinostat-induced G_2/M arrest could be due to the inhibition of NF-κB activity. Therefore, we employed the NF-κB inhibitor CAPE. The results obtained by using CAPE revealed that the vorinostat-induced G_2/M arrest was increased by cotreatment with CAPE at lower concentration (10 μM), but neutralised at higher concentration (50 μM). This result perfectly reflects the one obtained with the proteasome inhibitor and suggests that NF-κB is involved in the G_2/M arrest brought about by vorinostat. In addition, another specific NF-κB inhibitor, BAY 11-7082,

reduced the vorinostat-induced G_2/M arrest, which is in concordance with the result obtained by vorinostat in combination with CAPE.

Further, we also analysed the involvement of NF-κB in vorinostat-induced G_2/M arrest using indirect NF-κB inhibitors such as flavopiridol and roscovitin. We observed similar effects as with CAPE on cell cycle arrest, confirming the involvement of NF-κB in vorinostat-induced G_2/M arrest. It has been reported that HDACi mediated the activation of NF-κB through the PI3K/Akt pathway (Denlinger et al., 2005). Therefore, we assessed whether the PI3K/Akt inhibitor LY294002 could mimic the inhibition of NF-κB. We found that the inhibition of vorinostat-induced cell cycle arrest by PI3K/Akt inhibitor LY294002 mimicked the NF-κB inhibition. This finding is compatible with the notion that vorinostat induces NF-κB through activating PI3K/Akt pathway and further confirms the contribution of NF-κB in HDACi-induced cell cycle arrest.

Summing up, our experimental data demonstrate that vorinostat inhibits cell proliferation by inducing G_2/M cell cycle arrest in MCF-7 cells. We present novel data on the mechanism of vorinostat-induced G_2/M cell cycle arrest in MCF-7 cells. Vorinostat was able to induce phosphorylation of ATM at Ser1981 and γH2AX at Ser139. Though these two proteins were not found to be directly involved in vorinostat-induced cell cycle arrest, the induced phosphorylations of these proteins provide evidence that vorinostat-induced a DNA damage response. Vorinostat-induced G_2/M cell cycle arrest is affected by the proteasome and NF-κB pathway.

Since p53, a powerful tumour suppressor protein protecting cells from neoplastic transformation by mediating apoptosis and cell cycle arrest (Vousden et al., 2007), is a target of ATM, we wondered whether p53 might have a role in the observed vorinostat-induced G_2/M arrest. Therefore, we employed the MDM2 inhibitor nutlin-3 to determine the role of p53 in vorinostat-induced G_2/M arrest in MCF-7 cells. Nutlin-3 functions by restoring the p53 protein by interrupting the p53 and MDM2 protein complex. The obtained result shows that nutlin-3 prevented the vorinostat-induced G_2/M arrest, suggesting that p53 has a role in vorinostat-induced G_2/M arrest in MCF-7 cells.

To confirm this observation in another cell line, we tested the combination of nutlin-3 and vorinostat in p53 wild-type A549 cancer cells. Interestingly, in A549 cells we observed enhanced apoptosis by combination treatment with vorinostat and nutlin-3. Hence, the combination effects of vorinostat and nutlin-3 were further investigated in detail.

5.2 Synergistic activity of HDACi with nutlin-3 in p53 wild-type cancer cells

The second part of our study was to explore the combined effect of nutlin-3 and HDACi on cancer cells. We noted that nutlin-3 synergistically acted with four compounds representative for four different structural classes of HDACi to produce cytotoxic effects in p53 wild-type (wt) A549 and A2780, but not in p53 null PC-3 carcinoma cells. We found that nutlin-3 and HDACi cooperated to trigger cell death, and combination index (CI) analysis revealed that this interaction was synergistic. These findings are in good agreement with a study on the cooperative induction of p53 target genes by the treatment of nutlin-3 in combination with knockdown of HDAC2 (Harms and Chen, 2007).

Similar to the majority of anticancer agents, nutlin-3 and HDACi cause cell death by eliciting apoptosis (Xu et al., 2007;Brown et al., 2009). Our results indicate that the combined treatment of nutlin-3 and HDACi produced a cooperative induction of apoptosis. We found that nutlin-3 in combination with HDACi cooperatively provoked loss of $\Delta\psi_m$ and caspase-3 activation, both indicative for apoptosis (Figure 23 and 24). In addition, the use of the pan-caspase inhibitor z-VAD-fmk showed that caspase activity was required to some extent for nutlin-3/HDACi-induced cell death. Nutlin-3 has been shown to trigger cell cycle arrest rather than apoptosis in ten randomly selected cancer cell lines (Tovar et al., 2006). Nutlin-3 was observed to mediate G_1 and/or G_2/M arrest in all the cell lines, but apoptosis in only a few cell lines, with A549 being hardly apoptotically responsive. In agreement with this finding, we also noted that nutlin-3 treatment alone was not effective in A549 as well as in A2780 cells. Thus, our findings suggest that the cotreatment of HDACi converts the nutlin-3 effect from cell cycle arrest into apoptosis.

To throw light on the mechanism of the observed synergistic activity of nutlin-3 and HDACi, we determined the acetylation of p53 and the gene expression of MDM2 and MDM4. Our findings revealed that i) HDACi may act together synergistically with nutlin-3 by stimulating p53 hyperacetylation. It has been reported that the acetylation of p53 is required for p53 activation (Tang et al., 2008). As expected, the abundance of total p53 was enhanced by nutlin-3 treatment but we failed to observe an increase in acetylated p53 after nutlin-3 treatment in A549 cells. Nevertheless, nutlin-3 in combination with vorinostat resulted in a strong increase of acetyl-p53 (Figure 27). These observations suggest the explanation that nutlin-3 treatment could induce total, nonacetylated and biologically low active p53; HDACi treatment subsequently induces

acetylation and hyperactivation of p53. - ii) HDACi may enhance the antitumour activity of nutlin-3 through the suppression of nutlin-3-mediated MDM2 expression. Activation of p53 is required for the upregulation of MDM2, which in turn downregulates p53 (Vousden et al., 2007). Hence, by activating the p53 target MDM2, nutlin-3 could probably limit its own efficacy. Here, we have shown that nutlin-3-induced MDM2 gene expression was reduced by vorinostat (Figure 28A). - iii) HDACi may enhance nutlin-3-induced tumour cell death by downregulating the gene expression of MDM4. MDM4 is the second main p53 negative regulator; it is structurally homologues but functionally not redundant to MDM2. Unluckily, nutlin-3 and other MDM2-targeting agents fail to successfully target MDM4 (Brown et al., 2009). Rather, overexpression of MDM4 renders tumour cells resistant to nutlin-3 (Hu et al., 2006;Patton et al., 2006;Wade et al., 2006). It has been reported that concurrent targeting of MDM2 and MDM4 produced effective apoptosis in tumour cells overexpressing MDM2 and MDM4 (Hu et al., 2007). In this study, we have found that MDM4 gene expression was reduced by vorinostat (Figure 28B).

The above explanations are not mutually exclusive, but they require that HDACi augment nutlin-3-induced apoptosis by exerting p53-dependent and p53-independent effects. It has also been observed that HDACi increase the acetylation of p53, which in turn promotes p53 to induce its target genes (Luo et al., 2000). Further, we found HDACi reduced MDM2 gene expression, indicating that HDACi may exert p53-independent effects on p53 targets. Consistently, HDACi have indeed been reported to increase the expression of the p53 target p21 in a p53-independent manner (Nakano et al., 1997;Vrana et al., 1999). Therefore, it is also possible that HDACi have a p53-independent effect on the regulation of p53 targets such as proapoptotic proteins, thus lowering the threshold for nutlin-3-mediated apoptosis.

In a clinical point of view, two main aspects should be considered when implementing the nutlin-3 combination therapy. Firstly, nutlin-3 has to clearly collaborate with the agent it is combined with. Studies have shown that nutlin-3 synergises with other agents e.g. the genotoxic cytostatics doxorubicin, chlorambucil, cisplatin, etoposide and topotecan (Barbieri et al., 2006;Coll-Mulet et al., 2006;Laurie et al., 2006), the apoptosis-inducing cytokine TRAIL (Secchiero et al., 2007a), the proteasome inhibitor bortezomib (Ooi et al., 2009) and the BCR/ABL kinase inhibitor imatinib (Kurosu et al., 2010), but nutlin-3 has also been shown to protect against paclitaxel-induced cytotoxicity (Carvajal et al., 2005;Tokalov et al., 2010), the antimetabolites gemcitabine and cytarabine (Kranz and Dobbelstein, 2006), and the polo-like kinase 1 inhibitor BI-2536 (Sur et al., 2009). Therefore, potential combination therapies of nutlin-3 have to be

cautiously assessed to ensure a successful application [of note, the protecting effect of nutlin-3 may be exploited for the treatment of patients with p53 mutant tumours, a concept known as cyclotherapy (Brown et al., 2009)]. In this study, we have shown that HDACi are a class of drugs that synergise with nutlin-3, on the other hand, it confirms that nutlin-3 prevents paclitaxel-induced cytotoxic effects. Secondly, nutlin-3 may best be combined with agents that induce tumour cell death through independent effect of p53. It has been shown that nutlin-3 is an effective p53 activator and, therefore, may put heavy selection pressure on tumour cells for loss of p53 function to generate treatment resistance. The development of nutlin-3 resistance could be prevented by cotargeting p53-independent of pathways. HDACi have been reported both in vitro and in vivo to mediate apoptosis independently of p53 (Vrana et al., 1999;Ruefli et al., 2001;Insinga et al., 2005b;Lindemann et al., 2007), and our findings presented here also reveal the susceptibility of p53 null PC-3 cells to vorinostat-mediated cell death. Recently, it has been reported that cotreatment of HDACi abolished the development of resistance against cisplatin, the EGFR kinase inhibitor erlotinib and the RAF kinase inhibitor AZ628 (Sharma et al., 2010), providing support for the general utility of HDACi in overcoming drug resistance.

Nutlin-3 is a promising agent for antitumour treatment. However, nutlin-3 treatment alone may be inadequate to produce the antitumour effect. In a report on an orthotopic retinoblastoma model, nutlin-3 failed to reduce tumour growth as a single agent, whereas in combination with topotecan delivered a significant antitumour effect (Laurie et al., 2006). Therefore, nutlin-3 may have to be combined with other treatment regimens to accomplish a successful result. In this study, we have illustrated that the antitumour efficacy of nutlin-3 can be significantly enhanced by the cotreatment of HDACi. This effect was noted with all four structurally different compounds assessed, indicating an HDACi class effect. These in vitro study results provide a support for an in vivo investigation into the therapeutic potential for this combination of drugs.

5.3 Anticancer effects of the p53 activator nutlin-3 in Ewing's sarcoma cells

Ewing's sarcoma (ES) is the second most common malignant bone tumour after osteosarcoma (one to three cases per million people/year). It mainly affects paediatric or young adult patients, and arises from mesenchymal tissues. (Bernstein et al., 2006;Ordonez et al., 2009). Since 1970, the survival of patients with ES has increased substantially. At present, the 5-year overall survival rate for localised ES is about 75% (Granowetter et al., 2009). As these tumours are

aggressive, multimodality therapy involving chemotherapy and local therapy like surgery and/or radiation is always essential.

Approximately half of all tumours have p53 mutations (Vogelstein et al., 2000). In contrast, p53 mutations are infrequent in ES (Park et al., 2001;Huang et al., 2005;Iwamoto, 2007;Schaefer et al., 2008). In consequence, the majority of ES patients are potentially amenable to p53-based targeted therapeutic strategies. Considering this, pharmacological activation of p53 was investigated using nutlin-3 as a novel approach for the treatment of ES. The direct and specific targeting of p53 provides an attractive therapeutic option for ES in view of the low prevalence of p53 mutations in this type of tumour.

Several preclinical studies have demonstrated the therapeutic potential of nutlin-3 in a variety of childhood tumours with wt-p53, i.e. in rhabdomyosarcoma (Miyachi et al., 2009), in osteosarcoma (Vassilev et al., 2004;Muller et al., 2007), in synovial sarcoma (D'Arcy et al., 2009), in neuroblastoma (Van Maerken et al., 2006), in retinoblastoma (Laurie et al., 2006), and in acute lymphoblastic leukaemia (Gu et al., 2008).

In the third part of this study, we have investigated the effects of nutlin-3 in ES cells and found nutlin-3 treatment to increase p53 protein (Figure 30) and to induce expression of p53 target genes (P21, MDM2, PUMA) in ES cells with wt-p53 (Figure 31), but not in ES cells with mt-p53. In addition, we have found that nutlin-3 elicited significant cell death only in wt-p53 ES cells (Figure 33). These results are consistent with the previous study that the functional effects of nutlin-3 treatment primarily depend on the presence of wt-p53 (Shangary et al., 2009). However, we also observed nutlin-3 to reduce cell growth in mt-p53 cells (Figure 32). This is perhaps explained by residual activity of the point-mutated [C176F (Kovar et al., 1993)] p53 in SK-ES-1 cells or by p53-independent effects of nutlin-3. The former explanation is not considered likely due to the lack of nutlin-3 effect on p53 abundance and p53 target gene expression in SK-ES-1 cells. The latter, nevertheless, is supported by recent articles in which nutlin-3 was demonstrated to reduce cell viability in mt-p53 cells (Van Maerken et al., 2006), to induce cell cycle arrest in p53-deficient cells (VanderBorght et al., 2006), and to enhance chemotherapy-mediated apoptosis in mt-p53 cells (Ambrosini et al., 2007). p53-independent nutlin-3 effects were explained by nutlin-3-mediated activation of other MDM2 binding partners, such as the transcription factor E2F1 and the p53 homologue p73 (Ambrosini et al., 2007;Secchiero et al., 2007b;Lau et al., 2008;Peirce and Findley, 2009;Tabe et al., 2009). From these observations, it is now understood that the anticancer activity of nutlin-3 does not solely depend on the existence of wt-p53. On the other hand, it should be noted that nutlin-3 treatment

does not produce a strong effect in mt-p53 SK-ES-1 cells, in accordance with the relatively lower efficacy of nutlin-3 in mt-p53 and p53-deficient cells found in the previous studies (Van Maerken et al., 2006;VanderBorght et al., 2006;Ambrosini et al., 2007;Lau et al., 2008;Michaelis et al., 2009;Tabe et al., 2009).

p53 suppresses the development and the progression of tumour by mediating cell cycle arrest and, importantly, apoptosis, but it is not fully understood how the decision is made between these outcomes in response to p53 activation (Vousden et al., 2009). Nutlin-3 treatment was found to promote cell cycle arrest rather than apoptosis in a study engaging ten randomly selected cancer cell lines (Tovar et al., 2006). In contrast, our results suggest that nutlin-3 treatment leads to the initiation of apoptosis in wt-p53 ES cells. We noted that nutlin-3 induced $\Delta\psi_m$ dissipation, caspase-3 activation and DNA fragmentation, three common features of apoptosis (Figure 33 and 34). In addition, nutlin-3 also induced gene expression of PUMA, which is an important mediator of p53-induced apoptosis (Yu and Zhang, 2008) in wt-p53 cells. However, we also observed that SK-ES-1 cells were resistant against nutlin-3-induced apoptosis, demonstrating that the observed anticancer effects of nutlin-3 in mt-p53 ES cells were mainly the result by cell growth inhibition. Very recently, a similar set of findings was published by Pishas et al. Both the results and implications of this study are in good agreement with our data (Pishas et al., 2011).

As an additional finding of the apoptosis determinations, nutlin-3-mediated apoptosis appeared to occur independent of caspase-8. We have found that nutlin-3 mediated an apoptotic response in CADO-ES-1 cells, a caspase-8-deficient cell line (Fulda et al., 2001). Caspase-8 has been shown to be necessary for p53-induced apoptosis in ES cells (Kovar et al., 2000). Yet, this was concluded from the apoptosis-inhibitory effect of the putative caspase-8-specific inhibitor z-IETD-fmk, which has recently been found to unselectively suppress the proteolytic activity of several caspases, including the effector caspases-3 and -7 (McStay et al., 2008;Pereira and Song, 2008).

In addition to eliciting apoptosis, we have noted that nutlin-3 was also capable of inducing cellular senescence in ES cells (Figure 35). The chemotherapy-mediated regression of cancers has long been ascribed to the drug's ability to mediate apoptosis, but recently it has been recognised that the induction of cellular senescence, an irreversible cell cycle arrest, can contribute to the therapeutic efficacy of anticancer agents (Ewald et al., 2010). In particular, restoration of p53 activity has been revealed to produce tumour regression by inducing senescence rather than apoptosis in a mouse liver carcinoma model (Xue et al., 2007). Our result

shows that nutlin-3 activates pleiotropic antineoplastic mechanisms in ES, indicating that nutlin-3 may be effective in the treatment of tumours with reduced apoptotic responsiveness.

A number of studies have been shown that nutlin-3 can synergistically act with other anticancer agents, e.g. various cytostatics (Barbieri et al., 2006;Coll-Mulet et al., 2006;Laurie et al., 2006;Ambrosini et al., 2007;Miyachi et al., 2009;Peirce et al., 2009;Tabe et al., 2009), the apoptosis-inducing cytokine TRAIL (Secchiero et al., 2007a), the proteasome inhibitor bortezomib (Ooi et al., 2009) and histone deacetylase inhibitors (this thesis, see Results part 2). However, nutlin-3 in combination with inhibitors of NF-κB has not been investigated until now. It has been reported that aberrant NF-κB activity is involved in cancer development and progression (Baud and Karin, 2009). Consequently, NF-κB inhibitors have been shown to be active against cancer cells as single agents and in combination with other antitumour therapies. Studies have been reported that NF-κB inhibition enhances the apoptotic activity of TNF-α (Javelaud et al., 2000) and HDACi (Sonnemann et al., 2007) in ES cells. In Figure 36, we found NF-κB inhibitor CAPE enhanced nutlin-3-mediated cell death. Therefore, this finding supports the idea of simultaneous targeting of p53 and NF-κB as anticancer strategy (Dey et al., 2008).

From a clinical point of view, nutlin-3 has the advantage that it is capable of activating p53 in a nongenotoxic manner, hence avoiding the DNA damage which is major disadvantage of cytotoxic antineoplastic agents. By this way, nutlin-3 may be particularly advantageous for the treatment of childhood cancers. Currently, the implementation of intensive chemotherapy has helped in controlling neoplasias in childhood, however, the severe long-term adverse effects of the existing genotoxic treatment regimens are major problems for survivors of childhood cancer (Armstrong et al., 2009). Specifically, survivors of ES are vulnerable to long-term posttherapy complications, mainly second primary malignancies and cardiac dysfunction (Ginsberg et al., 2010). Therefore, anticancer therapeutic strategies with less genotoxic drugs are of high priority and, thus, our findings presented here establish the potential application of nutlin-3 for treating patients with ES.

5.4 Involvement of serine proteases in HDACi-induced cell death

The aim of the last part of this thesis was to verify the involvement of serine proteases in HDACi-induced apoptosis. Previous experiments had shown that an inhibitor of serine proteases, AEBSF, protected MCF-7 cells from HDACi-induced cell death. This observation (Dissertation of Michael Sigler) suggested that serine proteases have a role in HDACi-induced apoptosis.

In line with this observation, a recent study has been shown that the hydroxamic acid HDACi TSA induces caspase-independent, but serine protease-dependent apoptosis in pancreatic cancer cells (Garcia-Morales et al., 2005). In keeping, we observed that TSA-induced apoptosis was inhibited by the treatment with AEBSF (Figure 38). In serine proteases, the serine residue in the active site is sulfonylated by AEBSF (Powers et al., 2002); in addition, AEBSF may also covalently modify other proteins (Conboy et al., 2008).

Our data show that TSA- and vorinostat-induced apoptosis was not completely blocked by the caspase inhibitor z-VAD-fmk in SKOV-3 cells. Interestingly, the serine protease inhibitor AEBSF was able to fully block apoptosis induced by TSA and vorinostat. In contrast, NaB- and MS-275-induced apoptosis, which could be inhibited by z-VAD-fmk, was not affected by AEBSF (Figure 38). These results suggested that all HDACi induce apoptosis through a caspase-dependent mechanism, but TSA and vorinostat induce apoptosis also through a serine protease-dependent mechanism. Similar results were obtained in A549 cells (data are not shown).

Furthermore, the effect of AEBSF on HDACi-mediated cell death was validated by performing clonogenic assays, that is, cells were continuously exposed to 1 μM TSA for 10 days. Even under this extremely toxic condition, we observed the complete suppression of the cytotoxic effect of TSA by AEBSF treatment (Figure 39). This AEBSF effect appeared too strong to us to believe that it may be due to a specific inhibition of proteases and, therefore, provoked the assumption that it was due to a direct inactivation of TSA by AEBSF.

If AEBSF directly inactivates the action of TSA, the HDAC inhibitory effect of TSA should be impeded by AEBSF. First, based on this hypothesis, we determined the HDAC activity in intact cells (Ciossek et al., 2008). As shown in Figure 40A, we noted that the HDAC inhibitory effect of TSA and vorinostat was strongly blocked, while the inhibitory effect of NaB remained untouched by AEBSF in SKOV-3 cells. In contrast, the effect of TSA was not affected by the treatment with the pan-caspase inhibitor z-VAD-fmk (Figure 40). Similar results were also obtained in A549 cells (Figure 40B), indicating a general effect. In connection with these findings, we also determined acetylated histone H4 by using Western blots analysis. We found that AEBSF treatment was able to block TSA- and vorinostat-induced, but not NaB-induced, histone hyperacetylation in both SKOV-3 and A549 cells (Figure 42). Second, using a cell-free assay, the recombinant HDAC1 activity was measured. Also, in this system, the effect of TSA was prevented by AEBSF treatment. Thus, our results indicate that AEBSF directly inactivates TSA and vorinostat, by this way restraining these HDACi from inhibiting HDAC activity, and,

consecutively, from inducing apoptosis. Herein, our finding not only lifts up the concern about the effect of AEBSF on HDACi, it also suggests that AEBSF might directly modify other compounds. Therefore, it puts the relevance of serine proteases for cellular processes into question, particularly in those cases in which the participation of serine proteases was concluded from the use of AEBSF as sole serine protease inhibitor, as for instance in a study that has shown that serine proteases have a significant function in etoposide-induced apoptosis (de Bruin et al., 2003). Therefore, a possible direct effect of AEBSF on small molecule compounds should be considered in future studies on serine proteases.

In conclusion, we have analysed the cell cycle-inhibitory effect of HDACi in MCF-7 breast cancer cells, and defined mechanisms involved in HDACi-induced G_2/M arrest. Subsequently, we have investigated the effects of HDACi in combination with the p53 activator nutlin-3, and we have observed that HDACi and nutlin-3 synergised to induce cell death in wt-p53 cells. In connection with this finding, we have also found that HDACi induced downregulation of MDM2 and MDM4 gene expression as well as hyperacetylation of p53. Further, we have evaluated the anticancer effect of nutlin-3 in Ewing's sarcoma cells, and found that nutlin-3 killed Ewing's sarcoma cells as a single agent. Finally, we have demonstrated that AEBSF directly inactivated TSA and vorinostat and, in this way, prevented HDACi-induced cell death.

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Acknowledgements

I would like to thank all who helped me to achieve my goal. Without their care and consideration, this thesis would likely not have full-fledged.

My very special gratitude is directed to my supervisor Prof. Dr. med. James F Beck for giving me this great opportunity to work in his group. I am extremely thankful for his constant encouragement and fruitful discussions and suggestions throughout my research. My sincere thanks for providing me valuable ideas for successful results and for his care and support that he offered throughout my stay in Germany.

I express my special gratitude to Dr. Jürgen Sonnemann, who supervised me through my work. It was a great opportunity to work with, and learn from him. I am extremely thankful for his fruitful discussions and suggestions for improving the manuscript. Without his support and guidance this work would not have been possible. Also I thank for his personal care and support throughout my stay in Germany.

I would like to thank Prof. Dr. Frank D Böhmer for helping me to use gel doc system during my initial working period for some of the experiments. Also I thank him for being my official mentor for my PhD. I am grateful to him for the scientific support that I received and his valuable suggestions in my thesis. Like him his lab and his colleagues are also very kind and helpful during those days. For that, I would like to thank Annette, Andrea and his PhD scholars for their kind help and cooperation.

My special thanks to Stiftung Alfried Krupp Kolleg, Greifswald and Wilhelm Sander-Stiftung, Neustadt/Donau for providing the support to perform my work.

I thank Susan and Sabine for their kindness and excellent technical support in the lab. I would like to thank all the member of my lab, Ina, Navina, Stephanie, Friederike, Dorothee, Jessica and other colleagues for making a good environment and memorable time in the lab. Special thanks to Ina for accompaning me always when I needed.

I express my special gratitude to Dr. Saravana Kumar for his scientific discussions and for his personal care and continous support throughout these years. I thank Sangita and Ramesh for their good advice, suggestions and for their support.

I would like to thank dear Bärbel for her kindness and being a great friend to me all these years. No word to express my gratitude for her help and for the nice evenings with Indian food, a memorable one.

I would like to thank specially Jenny for teaching me all the techniques. I thank my friends as well my colleagues Lenia, Johanna, Mathias, Michael and Isabel for their company and support. I really had a nice time and great fun during my stay with them at Peter Holtz Research Center of Pharmacology and Therapeutics, Greifswald University. Special thanks to Praveen, Sudarshan and all other Indian friends for their care and continuous support.

My special thanks to my dear friends Raj, Lakshman, Priya, Bala and Mohan for being there for me when needed, for all the fun I had in Germany and who never let me feel alone, lifted my spirits up in the bad times and gave me a feeling of home away from home. I thank for all your love and great support.

I thank all my friends Anu, Srisha, Krishna, Jasmine, Shaik for their help and support in right time. I thank Murali and Pamela for their care and continuous encouragement. Also, I wish to thank all those who spent time with me during my doctoral thesis, being with me in Germany or in India, either discussing problems regarding experimental work or personal problems.

I would like to thank my best friend Banumathi and childhood friend Devi for their continuous support and love.

My special thanks to my dear dad Palani and mom Chandra for their moral support, constant encouragement till today. There is no word that I could express my gratitude for all your priceless love and faith in me. Thanks for all Dad…

I wish to thank my dear brothers Punidhavendan, Dilip Kumar, Kalidasan and Barath Kumar, for their invaluable love and support and for their concern throughout these years.

Last but not least I would like to express my special thanks to my beloved husband Manickam for his great understanding, support and constant encouragement. I thank him for being always with endless love, patience and faith towards me.

And anybody I missed who deserves a mention.