



Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biologia
Scuola Dottorale in Biologia
Sezione di Biologia Applicata alla Salute dell'Uomo
XXII ciclo

**“The role of mitotic spindle alterations in the
induction of apoptosis”**

**“Ruolo delle alterazioni del fuso mitotico
nell’induzione di apoptosi”**

PhD student

Dr. C. Cenciarelli

Supervisor

Dr. A. Antoccia

PhD school Director: Prof. Paolo Ascenzi

SUMMARY	1
1. Italian.....	1
2. English.....	3
 INTRODUCTION.....	 6
 1. MITOTIC SPINDLE.....	 6
1.1 <i>MICROTUBULES</i>	6
1.2 <i>CENTROSOME</i>	9
1.3 <i>MITOTIC SPINDLE</i>	11
 2. MICROTUBULES AND CANCER THERAPY	 12
2.1 <i>MICROTUBULES DAMAGING AGENTS (MDA)</i>	13
2.2 <i>COMBRETASTATIN CA-4</i>	15
 3. APOPTOSIS.....	 16
3.1 <i>CASPASE-INDEPENDENT CELL DEATH (CICD)</i>	18
3.2 <i>MITOTIC CATASTROPHE</i>	19
 4. BCL-2 PROTEINS	 19
4.1 <i>BH3-ONLY protein: BIM</i>	22
 5. MECHANISMS OF CELL DEATH INDUCED BY MDA	 24
 6. P53 and its INDEPENDENT TRANSCRIPTION ACTIVITY	 25
 RATIONALE AND AIM OF THE PROJECT.....	 28

RESULT	30
1. <i>Interfering with spindle microtubule-dynamics arrests cells at metaphase stage with misaligned chromosomes</i>	<i>30</i>
2. <i>Combretastatin A-4 affects spindle organization giving rise to “star-like” mitosis.</i>	<i>31</i>
3. <i>Pericentriolar matrix components are present in CA-4-induced “star-like” structures</i>	<i>32</i>
4. <i>Combretastatin induces pericentriolar matrix dismantling and caspase-dependent mitotic catastrophe.....</i>	<i>35</i>
5. <i>Inhibition of Bim strongly reduced caspase-3 cleavage and apoptosis ..</i>	<i>37</i>
6. <i>Bim bound physically to microtubules by interaction with dynein, is released after CA-4 treatment and relocalizes to mitochondria</i>	<i>40</i>
7. <i>p53 is linked to dynein and localized on mitochondria after CA-4 treatment</i>	<i>42</i>
8. <i>Bim coimmunoprecipitated with p53 in the mitochondrial protein fraction</i>	<i>42</i>
DISCUSSION	43
CONCLUSION	48
REFERENCE	50

SUMMARY

1. Italiano

I microtubuli, insieme con l'actina e i filamenti intermedi sono i principali componenti del citoscheletro delle cellule eucariotiche e svolgono un ruolo fondamentale in molti processi cellulari: divisione, motilità, "trafficking" e mantenimento della forma cellulare. Il principale Centro Organizzatore dei Microtubuli (MTOC) nelle cellule di mammifero è il centrosoma che è composto da una coppia di centrioli circondata da una matrice elettrondensa chiamata materiale pericentriolare (PCM). Essendo i microtubuli fortemente coinvolti nei processi di divisione cellulare, rappresentano un target preferenziale nella terapia del cancro. Gli agenti che danneggiano i microtubuli (MDA) rappresentano una importante classe di farmaci antitumorali e benchè alcune di queste sostanze promuovano la stabilizzazione dei microtubuli e altre inducano la depolimerizzazione, tutti gli MDA presentano la caratteristica comune di sopprimere la dinamica e quindi la funzione dei microtubuli. In questo modo gli MDA causano la distruzione del fuso mitotico e un blocco nella progressione del ciclo cellulare nella transizione metafase-anafase che può portare all'attivazione dei pathways apoptotici e alla catastrofe mitotica. Quest'ultima è stata descritta recentemente come una morte cellulare che avviene durante la mitosi in seguito all'utilizzo di agenti che causano alterazioni del fuso mitotico. Recentemente abbiamo focalizzato la nostra attenzione sulla Combetastatina (CA-4), un MDA isolato dal *Combretum Caffrum*, un albero sud Africano che ha la caratteristica di non essere riconosciuta dalla pompa *Multi Drug Resistance*, una pompa cellulare che elimina rapidamente sostanze estranee inclusi molti farmaci antitumorali. Gli MDA agiscono inoltre anche sul centrosoma causando alterazioni strutturali, frammentazione e inappropriata duplicazione del centrosoma. La CA-4 è in grado di arrestare le cellule in mitosi causando, in maniera dipendente dalla concentrazione, un aumento della disorganizzazione dell'arrangiamento cromosomico e la formazione di particolari strutture chiamate "star-like".

L'analisi in microscopia a fluorescenza e confocale ha evidenziato che le "star-like" sono strutture di forma sferica formate da piccoli fasci di microtubuli connessi al cinetocore. Al centro di queste strutture sono presenti alcuni componenti della matrice pericentriolare come la γ -tubulina, la pericentrina e la nineina. Il trattamento con la CA-4, oltre a determinare alterazioni di tipo strutturale, causa morte cellulare per catastrofe mitotica caratterizzata dall'attivazione delle caspasi-3/-9 e dalla frammentazione del DNA. Il meccanismo che porta dalla depolimerizzazione dei microtubuli alla morte cellulare non è ancora completamente chiarito. Recentemente è stato dimostrato che Bim, una proteina pro-apoptotica appartenente alla famiglia Bcl-2, è associata con il motore proteico dineina e sequestrata quindi dai microtubuli. Gli MDA, attraverso l'alterazione della struttura dei microtubuli, potrebbero causare il rilascio di Bim dal citoscheletro e la sua traslocazione sul mitocondrio. Inoltre dati recenti mostrano che esistono altri meccanismi che regolano Bim come la regolazione trascrizionale. Allo stesso modo, p53, una proteina pro-apoptotica molto conosciuta è associata con i microtubuli ed utilizza il motore proteico dineina per essere trasportata nel nucleo ed agire come fattore di trascrizione. Basandoci su questi dati abbiamo valutato il coinvolgimento di Bim e di p53 nell'apoptosi indotta in seguito al trattamento con CA-4. Il trattamento con la CA-4 determina sia il rilascio dai microtubuli sia un aumento del livello trascrizionale di Bim. Inoltre il silenziamento di Bim riduce fortemente l'attivazione della caspasi-3, una delle caspasi effettrici del pathway apoptotico mitocondriale. Inoltre, recentemente è stato dimostrato che p53 può indurre apoptosi non solo agendo come fattore di trascrizione ma anche attraverso una azione indipendente dalla trascrizione grazie alla capacità di interagire direttamente con proteine della famiglia Bcl-2 presenti sul mitocondrio come Bcl-2 e Bcl-XL. Comunque l'importanza dell'attività indipendente dalla trascrizione di p53 dopo utilizzo degli MDA su cellule tumorali rimane poco compresa e studiata. Nei nostri esperimenti abbiamo verificato che il trattamento con la combretastatina causa una variazione nel legame di p53 con la dineina e parallelamente determina la presenza di tale proteina sul mitocondrio. Inoltre esperimenti di coimmunoprecipitazione rivelano che Bim e p53

interagiscono sul mitocondrio e sono probabilmente responsabili dell'attivazione del pathway mitocondriale apoptotico. I nostri dati indicano quindi che cellule H460 trattate con la combretastatina sono uccise attraverso il meccanismo della catastrofe mitotica dipendente da un prolungato arresto mitotico, attivazione delle caspasi e traslocazione mitocondriale di Bim e p53.

2. English

Microtubules, together with actin and intermediate filaments are the major components of the cytoskeleton of eukaryotic cells and play a critical role in many cellular processes, including cell division, cell motility, intracellular trafficking, and cell shape maintenance. The principal Microtubule Organizing Center (MTOC) in mammalian cells is the centrosome that is composed of a pair of centrioles surrounded by an osmiophilic matrix termed pericentriolar material (PCM). As the microtubule network is highly involved in cell proliferation, it appeared to be a preferential target for cancer therapy. Microtubule Damaging Agents (MDA) represent an important class of anticancer drugs. Although some of this agents promote microtubules stabilization and other induce microtubules disruption, all MDA share the common property of suppressing microtubules dynamics, and thereby microtubules function, leading to the disruption of the mitotic spindle and the blockade of cell cycle progression at the transition from metaphase to anaphase that leads to apoptosis or mitotic catastrophe commitment. Mitotic catastrophe has been described as cell death that occurs from metaphase of mitosis in response to agents that cause mitotic-spindle damage. Recently we focused our attention on the antineoplastic spindle poison agent combretastatin-A4 (CA-4). CA-4 is an MDA isolated from the South African tree *Combretum Caffrum* that is not recognized by the multi drug resistance pump, a cellular pump which rapidly ejects foreign molecules including many anticancer drugs. MDAs also promote several

effects on the centrosome including abnormal centrioles structure, centrosome fragmentation and inappropriate centrosome duplication. CA-4 is able to arrest cells at mitosis in a dose depending manner accompanied by an increasing disorganization of chromosomal arrangement, led to formation of particular structure named “star-like”. These structures contain pericentrosomal matrix components like γ -tubulin, pericentrin and ninein. Confocal and immunofluorescence microscopy revealed that “star like” structures are spherical structures formed by residual fragments of microtubules connected to kinetochores. At the centre of this structure are present some components of the pericentriolar matrix like γ -tubulin, pericentrin and ninein. Treatment with CA-4, which produced high frequency of “star-like” mitosis, was accompanied by mitotic catastrophe commitment characterized by activation of caspases-3/-9 and DNA fragmentation. The mechanisms that lead from microtubules depolymerization to cell death are still not completely clear. Recently it has been demonstrated that Bim, a proapoptotic Bcl-2 protein, is associated to the dynein motor complex and sequestered by microtubules. MDA, through the alteration of microtubules structure, might lead to the release of Bim from cytoskeleton and to its translocation to the mitochondria. Moreover, recent data show that further mechanisms might be at the base of Bim-induced apoptosis such as regulation of Bim transcriptional levels. In a similar way, p53 another well known pro-apoptotic molecule, is associated with cellular microtubules and is transported to the nucleus by dynein. Based on these data, we have investigated whether upon tubulin depolymerization, Bim and p53 could be involved, and eventually in which manner, in the first steps of apoptosis. CA-4 treatment induces the release of Bim from microtubules and its increase at transcriptional level. In addition, Bim silencing strongly reduced apoptotic commitment. It has recently been reported that p53 can also induce apoptosis independently of new protein synthesis however the functional importance of p53 in MDA-induced apoptosis of human cancer cells remains poorly understood. Concerning the role of p53 in CA-4-induced apoptosis, we found that treatment determined

its release from cytoskeleton and re-localization to mitochondria. Data obtained from this study showed that Combretastatin-treated H460 cells are killed via a mitotic catastrophe mechanism strictly dependent on the achievement of mitotic block, caspases activation and Bim and p53 translocation to mitochondria.

INTRODUCTION

1. MITOTIC SPINDLE

1.1 MICROTUBULES

Microtubules (MT), together with actin and intermediate filaments are the major components of the cytoskeleton of eukaryotic cells. Microtubules are long, stiff and cylindrical tubes with a diameter of about 25 nm consisting of α - and β -tubulin heterodimers that polymerize into 13 protofilaments, which form the wall of the microtubules (Fig.1)(Cassimeris, 1993;Schmidt and Bastians, 2007). Microtubules play a critical role in many cellular processes, including cell division, cell motility, intracellular trafficking, and cell shape maintenance (Bhat and Setaluri, 2007).

MTs play a particularly important role in cell organization: they pull the chromosomes apart at mitosis, act as a 'railroad system' for intracellular transport, and define the localization and structure of internal membrane systems (Rogers and Gelfand, 2000;Kline-Smith and Walczak, 2004;Gregoretti et al, 2006). They are highly dynamic polymers and their polymerization dynamics are tightly regulated both spatially and temporally (Jordan and Wilson, 2004). Two characteristics of MTs are particularly significant for these functions. First, MT nucleation is regulated, and the limitation of nucleation to an organelle near the nucleus (the centrosome) endows most cell types with a radial organization. Second, and perhaps more importantly, the cytoskeleton is dynamic: individual MTs in the same cell constantly change in length, either growing or shrinking with random transitions between these phases (Gregoretti et al, 2006). This dynamic polymerization behavior is driven by the hydrolysis of GTP, which is bound at tubulin subunits. The two ends of microtubules are distinct: one end, the plus end, is much more dynamic and can show a net growth while the other, the minus end, is anchored in the centrosome or Microtubule Organizing Center (MTOC), and is less dynamic and can show net shrinkage. Thus, at a given time, microtubules can show no change in their polymer mass, yet exhibit very high dynamics. The second important dynamic behavior of microtubules is termed treadmilling, which is a net growth at the plus end and a balanced net shortening at the minus end. Consequently, treadmilling results in a flow of tubulin subunits from the plus to the minus end of

microtubules (WatermanStorer and Salmon, 1997). Both, dynamic instability and treadmilling are important features for the function of microtubules, especially during mitosis (Schmidt and Bastians, 2007).

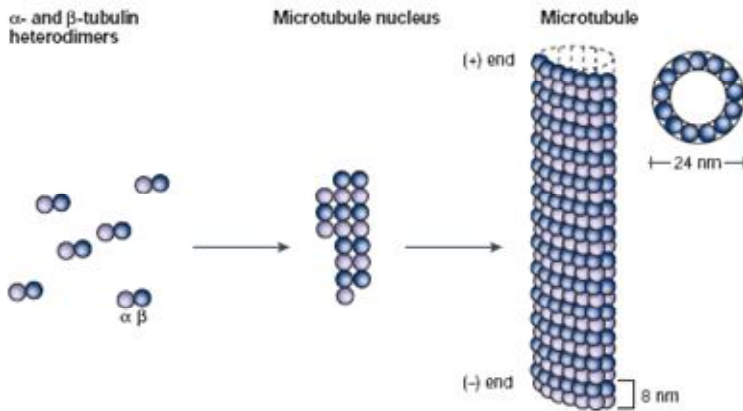


Fig.1 Polymerization of microtubules. Heterodimers of α - and β -tubulin assemble to form a microtubules. (From (Jordan and Wilson, 2004d)

The functional diversity of microtubules is achieved in several ways: through the binding of various regulatory proteins, including Microtubule Associated Proteins (MAPs), to soluble tubulin and to the microtubule surfaces and ends; by expression of different tubulin isotypes, which have different functions; and through several post-translational modifications of tubulin (Jordan and Wilson, 2004c). For example, human tubulin isotypes (6 forms of α -tubulin and 7 forms of β -tubulin) are expressed at varying levels in different cells and tissues. These tubulins can be further modified

post-translationally by polyglutamylation, polyglycylation, phosphorylation, acetylation, detyrosination/tyrosination and removal of the penultimate glutamic-acid residue of α -tubulins. There are many different MAPs, including the dynein and kinesin motor proteins, as well as many microtubule-regulatory proteins, such as survivin, stathmin, TOG, MCAK, MAP4, EB1, dynactin 1 (Fig.2)(also known as p150Glued), RAC1 and FHIT3–8 (Jordan and Wilson, 2004b). Some of these proteins move along microtubules mediating intracellular cargo transport, others have specific functions in mitosis in centrosome positioning, chromosome congression and segregation (Cassimeris and Spittle, 2001). Some of these differences predominate in certain cancer cells and some are associated with the development of drug resistance (Jordan and Wilson, 2004a).

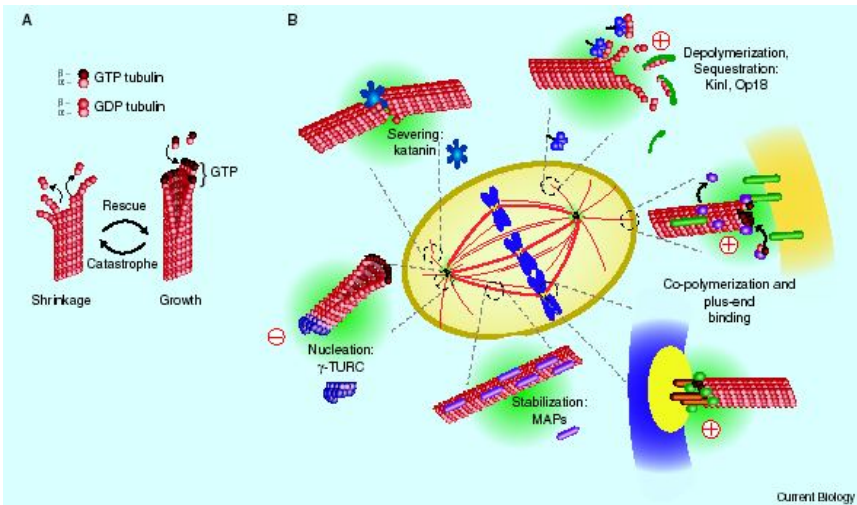


Fig. 2 Regulation of microtubules dynamic. (From(Gadde and Heald, 2004).

1.2 CENTROSOME

The centrosome of animal cells is a small non membranous organelle, and it is often associated with the nuclear membrane. It is composed of a pair of centrioles and a surrounding electron dense matrix of protein aggregates referred to as the pericentriolar material (PCM) (Fukasawa, 2002; Dutertre et al, 2002)). Each centrioles has a cylindrical shape and it is build with nine sets of triplet microtubules (Fig.3). Centrioles of the pair are different: one of them is named mother centrioles and unlike the daughter centrioles bears additional structures. It is worth noting that MT triplets of centriolar cylinders are very stable and unlike the cytoplasmic MT are not disassembled after the action of Microtubules Damaging Agents (MDA) and cold (Osborn and Weber, 1976; Alieva and Uzbekov, 2008). Many different proteins are present in the centrosome and they can be divided into three main group: MT triplet and centriolar matrix proteins, proteins of the PCM and proteins whose associations with centrosome is labile (Alieva and Uzbekov, 2008). Between proteins of centriolar matrix and PCM are centrin, pericentrin, ninein and γ -tubulin (Salisbury, 1995) that are involved in anchoring and nucleation of MT. The centrosome function as a Microtubule Organizing Centre (MTOC), which establishes polarity and orientation of MT during interphase, and direct the assembly of bipolar spindles during mitosis (Fukasawa, 2002). MT nucleated by centrosome are polarized with their rapidly growing (plus) ends in the cytoplasm and their slow-growing (minus) ends anchored at the centrosome (Doxsey, 2001). MT nucleations is due to the presence in the PCM of γ -tubulin Ring Complex (γ -TuRC)(Oakley and Oakley, 1989; Zheng et al, 1991; Moritz et al, 1995). The main subunit of the complex is a tetramer composed by two γ -tubulin molecules and two other proteins GPC2 and GPC3 (Knop and Schiebel, 1997; Oegema et al, 1999). The ring structure, observed by electronic microscopy, reveals the presence of six or seven tetramers together with at least other four proteins. Every daughter cell inherits only one centrosome and so the cell must duplicate the centrosome prior to the next mitosis. Duplication of centrosomes must proceed in coordination with other cell cycle events indeed initiation of centrosome duplication occurs near the G1/S boundary, while elongation of daughter centrioles and maturation of centrosome continue during S and G2 phase (Blagden and Glover, 2003; Doxsey et al, 2005). The presence of more than two centrosomes in a cell result in the formation of defective mitotic spindles

which increase the chromosomes segregation errors and it is responsible of chromosomes instability. This role of centrosomes was initially proposed by Theodor Boveri in 1914 and it was confirmed only many years later with the discovery that centrosome abnormality is a common marker in human cancer (Pihan et al, 1998; Lingle et al, 1998; Brinkley and Goepfert, 1998; Fukasawa, 2002)

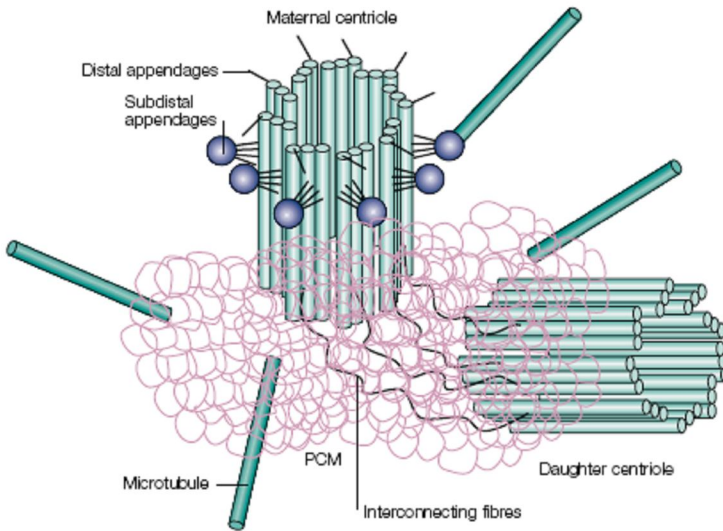


Fig. 3 The centrosome is composed by a pair of centrioles surrounded by pericentriolar material that nucleates microtubules around the ends closest to one another. Only the maternal centriole has two sets of extra appendages, distal and subdistal interconnecting fibres links the closest ends of the two centrioles. (From (Doxsey, 2001)

1.3 MITOTIC SPINDLE

The predominant model of spindle assembly in the presence of centrosomes is based on microtubules dynamic instability and is known as the “search and capture” model (Fig.4)(Kirschner and Mitchison, 1986;Gadde and Heald, 2004). In this scenario microtubules emanating from a centrosome go under cycles of growth and shrinkage, randomly probing the cytoplasm until running into a kinetochore, with which they form a stable attachment (Kirschner and Mitchison, 1986;Gadde and Heald, 2004). This lead to bipolar spindle formation. “Search and capture” mechanism is not relevant for spindle assembly in cell that lack centrosomes like plants and oocyte of animal cells. In this cells, acentrosomal spindle assembly (self organization model) start with microtubules nucleation near chromosomes, in a process that involves Ran/RCC1 activity (Karsenti and Vernos, 2001;Li et al, 2003;Kalab et al, 2006). This two mechanism have been viewed mutually exclusive for many years, however, several lines of evidence have changed this view. When the centrosome is physically removed in vertebrate somatic cells using a laser beam or microsurgery, functional bipolar spindle form nevertheless (Khodjakov et al, 2000;Hinchcliffe et al, 2001;Mahoney et al, 2006). Besides, mutations in *Drosophila* that inactivate centrosomes didn’t inactivate functional spindle formation (Bonaccorsi et al, 1998;Megraw et al, 2001). The effect on mitotic progression in the absence of centrosomes is that spindle are more misoriented. These observations highlight the idea that multiple mechanisms promote bipolar spindle formation. It remains to establish if the acentrosomal mechanism is activated only when cell are not able to use the “search and capture” model. Besides, recent observation suggested that kinetochore could promote microtubules polymerization (K fibres)(Khodjakov et al, 2003).

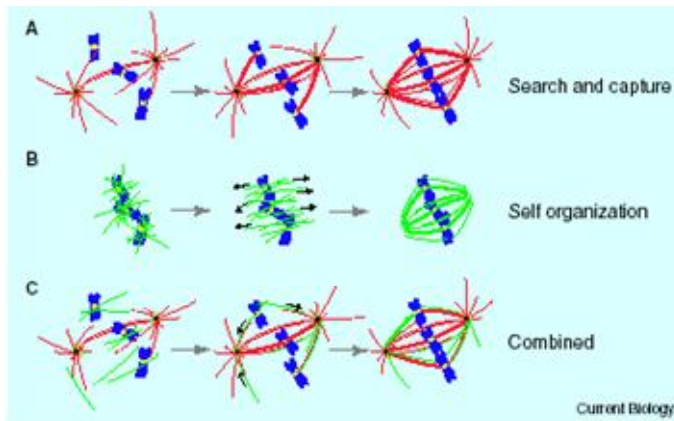


Fig. 4 Models of spindle assembly: search and capture (a); self organization (b); combined (c). (From (Gadde and Heald, 2004))

2. MICROTUBULES AND CANCER THERAPY

Most chemotherapeutic anti-cancer drugs used in the clinic today include agents that target the cell cycle in order to inhibit the hyperproliferation state of tumor cells and, subsequently, to induce apoptosis, which is the desired outcome of chemotherapy (Lee and Schmitt, 2003). Based on their mode of action these chemotherapeutic drugs can be subdivided into distinct groups: (i) drugs that interfere with DNA synthesis, (ii) drugs that introduce DNA damage and (iii) drugs that inhibit the function of the mitotic spindle. The latter have been proven to be exceptionally successful in the clinic and are classically represented by microtubule binding drugs frequently referred to as spindle poisons (Mollinedo and Gajate, 2003; Jordan and Wilson, 2004f; Pasquier et al, 2006). As the microtubule network is highly involved in cell proliferation, it appeared to be a preferential target for cancer therapy.

These drugs bind to and inhibit the function of microtubules of the mitotic spindle apparatus. However, since microtubules fulfill important functions in resting and differentiated cells these drugs exhibit a plethora of unwanted side effects. Therefore, novel drug targets that spare microtubules, but inhibit the progression of mitosis are highly desired and already exploited for the development of novel anti-mitotic drugs (Schmidt and Bastians, 2007).

The interest in MTAs is accompanied with advances in the fundamental understanding of their mechanism of action, that may provide alternative or additional approaches in the treatment of cancers (Esteve et al, 2007a). Their importance in mitosis and cell division makes microtubules an important target for anticancer drugs.

For that matter, great efforts have been devoted to discover drugs that affect microtubules.

2.1 MICROTUBULES DAMAGING AGENTS (MDA)

MDA constitute a class of anti-cancer drugs largely used in the clinic for the treatment of various cancers. MDA have been demonstrated to exert a high level of clinical activity, represented by clinical remissions in advanced ovarian, breast and the upper aerodigestive tract cancers (Rowinsky and Donehower, 1995; Miller and Sledge, 1999; Jordan and Wilson, 2004e).

Microtubule damaging agents are usually classified into two main groups (Fig.5). One group, known as the microtubule-destabilizing agents, inhibits microtubule polymerization at high concentrations and includes several compounds — such as the *Vinca* alkaloids (vinblastine, vincristine, vinorelbine, vindesine and vinflunine), cryptophycins, halichondrins, estramustine, colchicine and combretastatins — that are used clinically or are under clinical investigation for treatment of cancer. The second main group is known as the microtubule-stabilizing agents. These agents stimulate microtubule polymerization and include paclitaxel (the first agent to be identified in this class), docetaxel (taxotere) and the epothilones (Jordan and Wilson, 2004g). The most important action of these drugs is the suppression of spindle-microtubules dynamics, which result in the blocking of mitosis at the metaphase-anaphase transition and induction of apoptotic cell death.

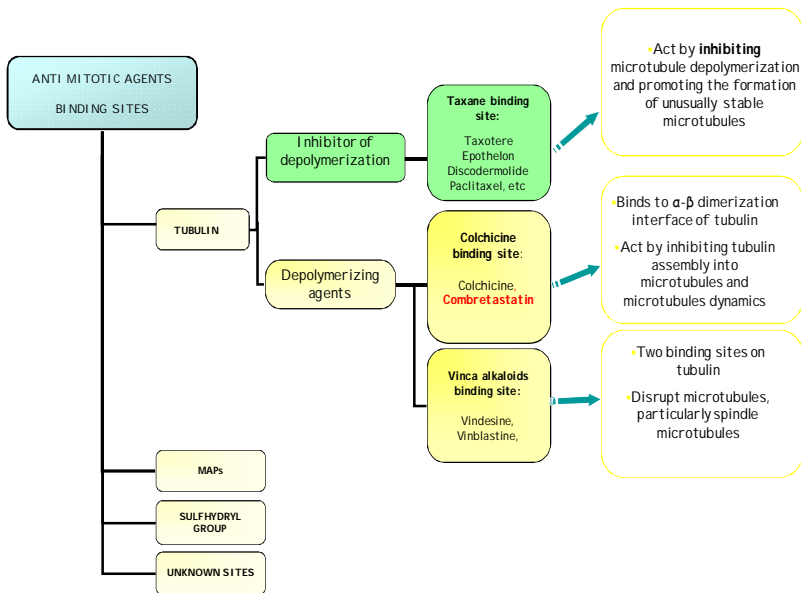


Fig. 5 A representative pattern of MDA and their binding sites.

2.2 COMBRETASTATIN CA-4

Combretastatin A-4 (CA-4) is a natural stilbenoid isolated from the South African bush of *Combretum caffrum* that is structurally related to colchicines but have potent anti-vascular properties at well-tolerated doses (Fig.6)(Tozer et al, 2002). CA-4 is the most active of 17 compounds isolated from the natural source (Uppuluri et al, 1993;Pettit et al, 1995;Jordan et al, 1998). CA-4 is not recognized by the Multi Drug Resistance (MDR) pump which rapidly ejects foreign molecules including many anticancer drugs (Patocka and Strunecka, 1999;Islam and Iskander, 2004).

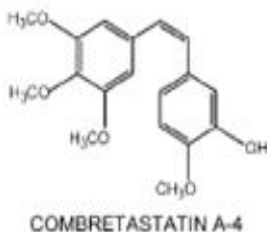


Fig. 6 Chemical structures of combretastatin A-4. (From (Tozer et al, 2002).

3. APOPTOSIS

Cell death is an essential strategy for the control of the dynamic balance in living systems. Briefly there are two different forms of cell death, apoptosis and necrosis. Necrosis is an accidental passive process resulting in an early disruption of the cell membrane and in the breakdown of cell structures in response to violent environmental perturbations. In contrast, apoptosis is tightly regulated and involves the activation of an energy-requiring intracellular machinery (Yuan, 1996; Vermeulen et al, 2005). This form of cell death affects single cells and it is able to eliminate cells exposing the organism to danger (Shibata et al, 1994; Green and Martin, 1995; Liebermann et al, 1995; Vermeulen et al, 2005).

Apoptosis is characterized by many morphological changes like cell shrinkage, nuclear pyknosis (chromatin condensation), karyorrhexis (nuclear fragmentation), blebbing and phosphatidylserine exposure on the surface of the plasma membrane (Kerr et al, 1972; Wyllie et al, 1984; Martin et al, 1995; Zamzami et al, 1996; Kroemer et al, 2007). Apoptosis is a genetically predetermined mechanism characterized by two major biochemical events, namely the activation of caspases and the permeabilization of the outer mitochondrial membrane, with the consequent release of many death effectors from the mitochondrial intermembrane space (Kerr et al, 1972; Brenner and Kroemer, 2000; Ferri and Kroemer, 2000; Ravagnan et al, 2002). Apoptosis may be elicited by different pathways of which the best characterized are called the extrinsic and intrinsic (Fig.7).

In the extrinsic pathway apoptosis is triggered by activation of death receptors at the cell surface. This causes the recruitment and oligomerization of the adapter molecule FADD (Fas-Associating Death Domain-containing protein) within the Death-Inducing Signaling Complex (DISC). Finally this oligomerization led to activation of caspase-8 and 10 (Debatin and Krammer, 2004; Kroemer et al, 2007). The intrinsic or mitochondrial pathway is the predominant form of cell death in vertebrates. The critical event in this pathway is the mitochondrial membrane permeabilization (MMP) that is responsible for caspases activation. Normally cytochrome C (cytC) resides in the inner mitochondrial space where it functions as an electron shuttle in the respiratory chain (Bernardi and Azzzone, 1981). During the intrinsic

pathway of apoptosis cytC is released into the cytosol where it can bind to APAF-1 (Apoptosis Protease Activating Factor-1). APAF-1 exist in the cytosol as a monomer and its activation depend on the presence of cytochrome C and ATP/ADP (Cain et al, 2002). The multimerization of APAF-1 led to activation of caspase-9 and to initiation of mitochondrial apoptosis pathway through activation of effector caspases. Effectors caspases act on a variety of substrates resulting in proteolysis of cellular proteins and death by apoptosis. One of the best characterized and the earliest substrate of caspase is Poly-(ADP-Ribose) Polymerase (PARP), a nuclear protein implicated in DNA repair (Duriez and Shah, 1997). Caspase cleavage and inactivation of ICAD (Inhibitor of Caspase-Activated DNase) allow CAD (DNA fragmentation factor) to translocate the nucleus where it is responsible for internucleosomal DNA cleavage, generating DNA fragments (Liu et al, 1997; Sakahira et al, 1998; Vermeulen et al, 2005). Besides caspase cleavage of lamins and cytoskeletal proteins leads to nuclear shrinkage and cytosolic reorganization (Martin et al, 1995; Mashima et al, 1995; Orth et al, 1996; Liu et al, 1997; Vermeulen et al, 2005).

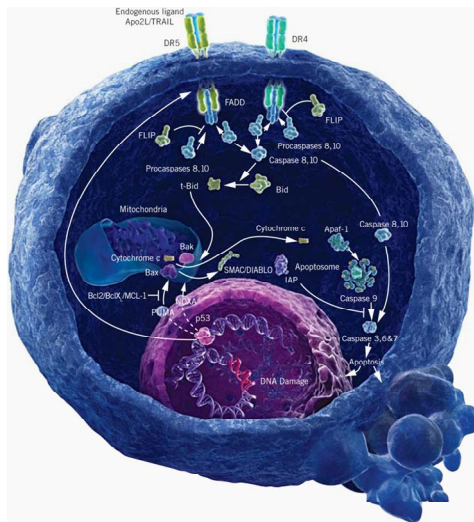


Fig. 7. Extrinsic and intrinsic pathways of apoptosis. A representative image from [http:// www.biooncology.com/bioonc/research/apoptosis/index.m](http://www.biooncology.com/bioonc/research/apoptosis/index.m)

3.1 CASPASE-INDEPENDENT CELL DEATH (CICD)

Many studies have shown that apoptosis is linked to caspases activity and that caspases are required to induce cell death (Taylor et al, 2008;Tait and Green, 2008). Recently it has been described a different type of cell death that occurs when a signal that normally induced apoptosis fails to activate caspases (Taylor et al, 2008;Tait and Green, 2008). This type of cell death often shares common characteristic with apoptosis (Table 1).

	Apoptosis	CICD
Nucleus	Cleavage of caspase targets DNA laddering Nuclear fragmentation Chromatin condensation	Partial chromatin condensation Chromatin marginalization around the nuclear membrane No DNA laddering Nuclear shrinkage but no fragmentation
Mitochondrion	Outer-membrane permeabilization Cleavage of caspase targets Rapid loss of membrane potential	Outer-membrane permeabilization Swollen cristae Gradual loss of membrane potential
Cytoplasm	Cleavage of caspase targets Increased [Ca ²⁺] Protein crosslinking Condensation	Abundant autophagosomes Ribosome aggregation Vacuolated
Plasma Membrane	Cleavage of caspase targets Loss of membrane-phospholipid asymmetry Annexin V-positive Membrane blebbing	Annexin V-negative
Cell	Loss of proliferation Detachment from matrix Phagocytosis of dying cell	Loss of proliferation Often remain attached to matrix Removal through unknown means

Table 1. Characteristics of apoptotic and caspase-independent cell death. (From (Tait and Green, 2008) modified).

Many studies in both cell culture models and utilizing mice deficient in apoptotic signaling supported the existence of CICD typically by inducing apoptosis. In the presence of chemical caspase inhibitors such as zVAD-fmk (Hirsch et al, 1997;Ohta et al, 1997;Sarin et al, 1997;Tait and Green, 2008). CICD has also been observed in cell expressing genetically encoded caspase inhibitors such as XIAP, CrmA or p35 following induction of apoptosis (Okuno et al, 1998;Denmeade et al, 1999;Wilkinson et al,

2004;Tait and Green, 2008). Besides cells that lack components of the intrinsic apoptotic pathway such as Apaf-1, cytochrome c or caspase-9 demonstrated the existence of an alternative type of cell death (Hakem et al, 1998;Kuida et al, 1998;Li et al, 2000;Tait and Green, 2008).

3.2 MITOTIC CATASTROPHE

Mitotic catastrophe has been first described in *Schizosaccharomyces pombe* as a temperature-sensitive lethal phenotype, linked to gross abnormalities of chromosome segregation, that was observed in some mutant strains (Ayscough et al, 1992;Castedo et al, 2004). Some authors view mitotic catastrophe of mammalian cells as the failure to undergo complete mitosis which usually ends in the formation of large cells with multiple micronuclei and decondensed chromatin (Swanson et al, 1995;Ianzini and Mackey, 1997;Roninson et al, 2001;Castedo et al, 2004). However, reports describing mitotic catastrophe frequently show cells with some phenotypic characteristic of apoptosis (Castedo et al, 2004). Mitotic catastrophe would be a type of cell death occurring during mitosis, as a result of DNA damage or deranged spindle formation coupled to the debilitation of different checkpoint mechanism that would normally arrest progression into mitosis and hence suppress catastrophic events until repair has been achieved. (Castedo et al, 2004;Tait and Green, 2008). Damage leading to mitotic catastrophe can be induced, in particular, by microtubules damaging agents. So, mitotic catastrophe can be considered as a type of cell death occurring during mitosis or resulting from mitotic failure.

4. BCL-2 PROTEINS

The major regulators of apoptosis belong to Bcl-2 family that is composed of up to 30 proteins (Strasser et al, 2000a;Esteve et al, 2007b). Bcl-2 was the first human oncoprotein described to promote cell survival (Vaux et al, 1988). Classification of other members has then been based on their Bcl-2 homology (BH) domains (Fig. 8)(Yin et al, 1994;Chittenden et al, 1995a;Zha et al, 1996;Gibson et al, 1996;O'Connor et al, 1998). Bcl-2 and

its closest homologs (Bcl-xL, Bcl-w, Mcl-1) (Boise et al, 1993;Gibson et al, 1996) share three or four regions of homology (BH1-BH4 regions) and are able to inhibit apoptosis (Vaux et al, 1988;Strasser et al, 2000a). Other members of this family have the capability to promote apoptosis and for this reason they are called pro-apoptotic proteins. These proteins can be divided into the Bax subfamily (Bax, Bak, and Bok multidomain proteins) (Oltvai et al, 1993;Farrow et al, 1995;Kiefer et al, 1995;Chittenden et al, 1995b;O'Connor et al, 1998) or the BH3-only subfamily (Bid, Bim and PUMA). The N-terminal BH4 domain is present only in some anti-apoptotic proteins, while BH1, BH2 and BH3 can be found in both sub-families. Some members of the Bcl-2 family have a hydrophobic C-terminal region that allows their localization on intracellular membranes (Schinzel et al, 2004;Lucken-Ardjomande and Martinou, 2005). Homotypic and heterotypic interaction between individual members of the family are important mechanism that controls their activity (Cheng et al, 2001;Cory et al, 2003;Lucken-Ardjomande and Martinou, 2005;Chen et al, 2005).

The relative level of anti- and pro-apoptotic Bcl-2 proteins arbitrate cell life or death decision (Oltvai et al, 1993;Oltvai and Korsmeyer, 1994). Bcl-2 like members are mainly localized to mitochondrial membranes and prevent permeabilization of the mitochondrial outer membrane. There are two models that explain how Bcl-2 proteins can induce mitochondrial membrane permeabilization: indirect and direct model. The indirect model proposes that anti-apoptotic proteins must continually inhibit the function of Bax and Bak to ensure mitochondrial integrity and survival. So in this scenario, mitochondrial membrane permeabilization arise when all anti-apoptotic proteins are functionally neutralized by activated BH3-only proteins. At the contrary, in the direct model BH3-only proteins (Bid and Bim) directly interact with Bax or Bak leading to mitochondrial membrane permeabilization (Chipuk et al, 2004). Data present today in literature do not allow to clarify which is the correct model.

Association of an anti-apoptotic with a pro-apoptotic protein, such as Bcl-2 or Bcl-xL) with Bax (or Bak), requires the BH1 and BH2 domains of the former (Yin et al, 1994;Hanada et al, 1995;Sedlak et al, 1995) and the BH3 domain (Chittenden et al, 1995a;Zha et al, 1996;Simonian et al, 1996). In the tertiary structure of Bcl-xL,the BH1, BH2 and BH3 domains form an elongated hydrophobic cleft (Muchmore et al, 1996) along which the amphipathic helix formed by BH3 domains of the pro-apoptotic proteins

can bind (Sattler et al, 1997). The importance of the BH3 region for facilitating apoptosis has been underscored by the discovery of several BH3-containing proteins: Bik/Nbk (Boyd et al, 1995; Han et al, 1996) Bid (Wang et al, 1996) and Hrk/DP5 (Imaizumi et al, 1997; Inohara et al, 1997), which are otherwise potent activators of apoptosis when overexpressed.

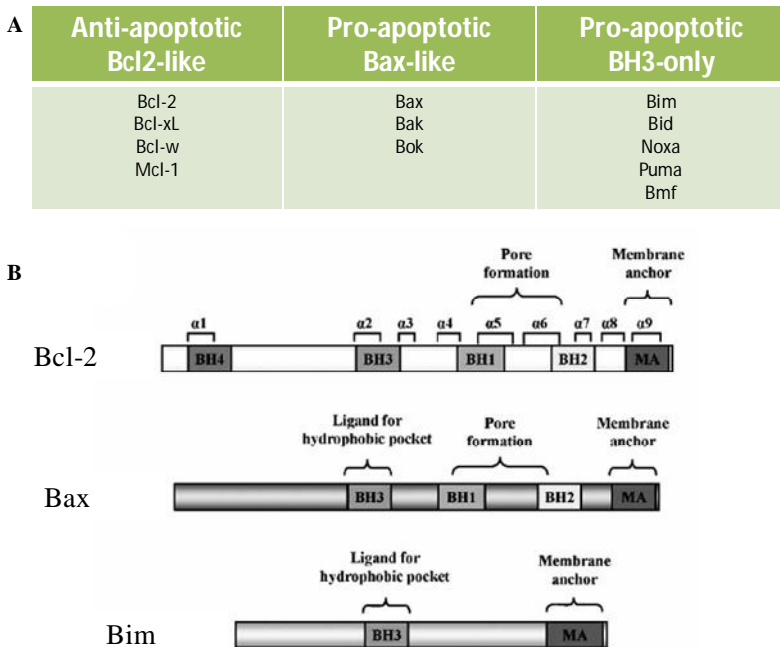


Fig. 8 The Bcl-2 family members classified in three groups (A). Linear structure of Bcl-2, Bax and Bim. (From (Esteve et al, 2007a)modified)

4.1 BH3-ONLY protein: BIM

The BH3-only proteins require only their BH3 domain for binding to and neutralizing the activity of antiapoptotic Bcl-2 family members, and it appears that this is the only mechanism by which they induce cell death. (Strasser et al, 2000b).

At least nine mammalian BH3-only family members (Bad, Bik, Blk, Hrk, Bid, Bim, Noxa, PUMA, Bmf) have been identified. Bim was initially isolated by screening a cDNA expression library with a Bcl-2 protein probe (O'Connor et al, 1998;Miyashita et al, 2001). Three Bim isoforms have been reported and designated as BimEL, BimL and BimS, BimS being the most potent. These isoforms all have a BH3 domain, but vary in a region toward the N-terminus (O'Connor et al, 1998;Strasser et al, 2000b). Whether the Bim isoforms are expressed differentially in different tissues or developmental stages or in response to particular apoptotic stimuli is currently under study. Bim can interact with Bcl-2, Bcl-xL, Bcl-w and also with two more distantly related family members that promote cell survival, Mcl-1 (Kozopas et al, 1993;O'Connor et al, 1998) and A1 (Lin et al, 1993;O'Connor et al, 1998).

The pro-apoptotic activity of Bim is controlled by several mechanisms (Puthalakath and Strasser, 2002;Willis and Adams, 2005;Ley et al, 2005;Czernick et al, 2009):

transcriptional regulation: the Bim promoter contains two putative FOXO3a binding sites and FOXO3a has been implicated in regulating Bim expression in several cell types (Gilley et al, 2003;Essafi et al, 2005;Urbich et al, 2005;Cai and Xia, 2008). FOXO3a directly binds to the Bim promoter and activate Bim transcription. Furthermore, members of the MAP kinase family, such as JNK and ERK5 have been reported to directly or indirectly regulate FOXO3a expression. Also ERK1/2 phosphorylates FOXO3a leading to the degradation and subsequent inhibition of FOXO3a (Yang et al, 2008). JNK regulates FOXO3a nuclear translocation in breast cancer cells after paclitaxel treatment (Sunters et al, 2006). Recently it has been demonstrated that inhibition of p38 increases FOXO3a phosphorylation at T32: an inhibitory phosphorylation site that is phosphorylated by the pro-survival kinase Akt (Brunet et al, 1999;Cai and Xia, 2008).

post translational modifications: Bim's pro-apoptotic activity is stimulated by phosphorylation through pro-apoptotic JNK and p38 MAP kinase and inhibited by phosphorylation through pro-survival ERK. (phosphorylation of Bim-EL by Erk1/2 on S69 is necessary and sufficient to promote its rapid degradation by the proteasome and to protect cells from apoptosis (Luciano et al, 2003).

intracellular localization: BimL and BimEL, but not BimS, bound to LC8 dynein light chain both in yeast and mammalian cells (Puthalakath et al, 1999). Single amino acid substitutions in BimL that disrupt its interaction with LC8 increased its killing potency to that of BimS, proving that interaction with LC8 is a physiological regulator of Bim proapoptotic activity (Puthalakath et al, 1999).

In healthy cells, most endogenous BimL and BimEL molecules were bound via LC8 to the microtubule-associated dynein motor complex, and thereby sequestered from Bcl-2 and its homologs. Certain apoptotic stimuli, disrupted the interaction between LC8 and the dynein motor complex. This freed Bim to translocate together with LC8 to Bcl-2 and neutralize its antiapoptotic activity. This process did not require caspase activity, and therefore constitutes an initiating event in apoptosis signaling (Puthalakath et al, 1999;Strasser et al, 2000b)

Recently it has been demonstrated that in cells exposed to UV radiation, activated JNK can phosphorylates Bim on T56 leading to its release from dynen (Lei and Davis, 2003).

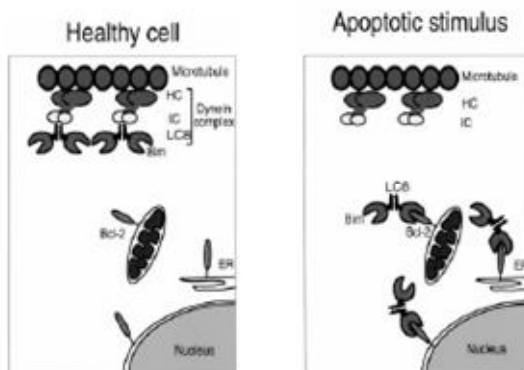


Fig. 9. Model for the regulation of Bim. (From (Puthalakath and Strasser, 2002).

5. MECHANISMS OF CELL DEATH INDUCED BY MDA

MDAs act on microtubule network integrity in different ways and they induce diverse signals responsible for cell death initiation and execution. MDA effectiveness is largely accepted as a consequence of caspase activation through the intrinsic apoptotic pathway (Andre et al, 2002; Pourroy et al, 2004; Wu et al, 2005) that is mainly regulated by members of the Bcl-2 family (Walensky, 2006). Up-regulation of the proapoptotic proteins Bad, PUMA, Bax and/or Bak has been observed after treatment with paclitaxel, epothilone B as well as *Vinca* alkaloids (Jones et al, 1998; Tudor et al, 2000; Yamaguchi et al, 2004; Pourroy et al, 2004). Likewise, Bcl-XS, Bax or Bad overexpression sensitizes cancer cells to paclitaxel and vincristine (Sumantran et al, 1995; Sawa et al, 2000). Paclitaxel, epothilones and vinblastine trigger Bax activation through its conformational change (Makin et al, 2001; Yamaguchi et al, 2004). It allows N-terminal domain exhibition and Bax stable insertion into the outer mitochondrial membrane (Antonsson et al, 2001; Cartron et al, 2003).

MDAs may also activate Bim translocation from microtubules to mitochondria, as recently proposed for paclitaxel (Li et al, 2005). Specifically, the BH3-only protein Bim, has been shown to play a major role in paclitaxel-induced cell death (Sunters et al, 2003;Li et al, 2005;Li et al, 2007). Direct depletion of Bim or indirect depletion of Bim through siRNA repression of Bim transcriptional activators, delays paclitaxel-mediated apoptosis in cell based models (Sunters et al, 2003;Li et al, 2005;Li et al, 2007). As well, in vivo mouse models of bim^{-/-} cells confirmed the importance of Bim expression for paclitaxel cytotoxicity (Tan et al, 2005).

Modulating the activity and the expression levels of Bcl-2 family proteins, MDAs increase the amounts of active proapoptotic members at mitochondrial membranes while they decrease those of active anti-apoptotic members. The ratio among Bcl-2 family members thus turns in favor of mitochondrial permeability and caspases activation.

6. p53 and its INDEPENDENT TRANSCRIPTION ACTIVITY

The p53 tumor suppressor protein is stabilized and activated by different genotoxic and non-genotoxic stress, playing a central role in the chemosensitivity and radiosensitivity of cancer cells by inducing cell cycle arrest and apoptosis (Vogelstein et al, 2000;Fei and El Deiry, 2003;Yamaguchi et al, 2004). p53 triggers apoptosis by inducing mitochondrial membrane permeabilization through transcription dependent mechanism: many pro-apoptotic proteins of the Bcl-2 family such as Bax, PUMA, Noxa and Bid are transcriptional target of p53 (Miyashita and Reed, 1995;Oda et al, 2000;Nakano and Vousden, 2001;Yu et al, 2001;Sax et al, 2002). Interestingly it has been recently demonstrated that upon DNA damage p53 translocate to mitochondria where it directly binds to Bcl-XL/Bcl-2 or Bak and induces Bax/Bak activation and cytochrome C release (Marchenko et al, 2000;Mihara et al, 2003;Dumont et al, 2003;Chipuk et al, 2004;Deng et al, 2006) and in the recent years have been accumulating evidences for transcription-independent p53-mediated apoptosis. Moreover the transcriptionally inactive p53 mutants (1-214) and p53 22/23QS act as potent death inducers in tumor cells (Haupt et al, 1995;Chen et al,

1996;Mihara et al, 2003). Also in cell-free cytoplasts, activation of cytosolic p53 can induce mitochondrial cytochrome C release (Schuler and Green, 2001). Targeting p53 to mitochondria by fusing it with mitochondrial address peptides, thereby bypassing the nucleus, is sufficient to launch apoptosis (Marchenko et al, 2000;Sansome et al, 2001;Moll et al, 2005). Pro-apoptotic protein and p53 rapidly translocate to mitochondria after a death stimulus, bind and inhibit the protective Bcl-XL and Bcl-2 proteins. The complex formed by p53 and BH3-only proteins with Bcl-XL/Bcl-2 is critical for their apoptogenicity: interference with their binding, blocks the mitochondrial killing activity (Oda et al, 2000;Cheng et al, 2001;Nakano and Vousden, 2001;Mihara et al, 2003). thus, tumor-derived p53 mutations may represent “double hits”, eliminating the trascriptional as well as the direct mitochondrial functions of p53 (Moll et al, 2005). it was determined that p53 DNA interface and the BH4 domain of Bcl-XL are involved in the formation of the complex : this result was confirmed by NMR studies (Petros et al, 2004;Moll et al, 2005). Besides p53-Bcl-2 binding appears to disrupt Bcl-2-Bax binding and thereby promotes free Bax to be potentially activated by a BH3-only proapoptotic protein (Deng et al, 2006) infact p53 can directly disrupt the Bcl-2/Bax complex by binding to a novel regulatory region of Bcl-2's FLD (Flexible Loop Regulatory Domain)(aa 32 to 68) that has been identified as regulatory domain in Bcl-2 (Deng et al, 2006). Phosphorylation in the FLD can positively regulate Bcl-2's antiapoptotic function, inhibiting p53 binding and its apoptogenic role at mitochondria (Deng et al, 2006) but the mechanism is not clear. It has been proposed that localization of p53 on mitochondria could represent a rapid and direct way to induce cell death, amplifying its transcription-based apoptotic action that requires more time to take effect (Mihara et al, 2003;Moll et al, 2005). However the role of p53 in apoptosis induction varies between cell types and death signals, the role of p53 in MDA-induced apoptosis of human cancer cells remain controversial. Besides it has been recently demonstrated that p53 is associated with cellular microtubules through its interaction with dynein and that only in cells with a functional microtubules network it accumulated in the nucleus after DNA damage (Moll et al, 1996;Giannakakou et al, 2000). Treatment with MDA, disrupting microtubules network and dynamics can impair nuclear accumulation of p53 after DNA damage. Result obtained after microinjection of antibody

against the dynein intermediate chain, further supporting the idea that dynein functions in p53 transport (Giannakakou et al, 2000).

RATIONALE AND AIM OF THE PROJECT

Microtubules, together with actin and intermediate filaments are the major components of the cytoskeleton of eukaryotic cells and play a critical role in many cellular processes, including cell division, cell motility, intracellular trafficking, and cell shape maintenance. The principal Microtubule Organizing Center (MTOC) in mammalian cells is the centrosome that is composed of a pair of centrioles surrounded by an osmiophilic matrix termed pericentriolar material (PCM). As the microtubule network is highly involved in cell proliferation, it appeared to be a preferential target for cancer therapy. Microtubule-Damaging Agents represent an important class of anticancer drugs. Although some of these agents promote microtubule stabilization and others induce microtubules disruption, all MDA share the common property of suppressing microtubules dynamics, and thereby microtubules function, leading to the disruption of the mitotic spindle and the blockade of cell cycle progression at the transition from metaphase to anaphase that leads to apoptosis or mitotic catastrophe commitment. Mitotic catastrophe has been described as cell death that occurs from metaphase of mitosis in response to agents that cause mitotic-spindle damage. Recently we focused our attention on the antineoplastic spindle poison agent CA-4. CA-4 is an MDA isolated from the South African tree *Combretum Caffrum* that is not recognized by the multi drug resistance pump, a cellular pump which rapidly ejects foreign molecules including many anticancer drugs. MDAs also promote several effects on the centrosome including abnormal centrioles structure, centrosome fragmentation and inappropriate centrosome duplication. Aim of this project was to clarify the relationship between microtubular dynamics, dismantling of pericentriolar components and induction of apoptosis after exposure of H460 non-small lung cancer cells to this antimitotic drug.

CA-4 is able to arrest cells at mitosis in a dose depending manner accompanied by an increasing disorganization of chromosomal arrangement, led to formation of particular structure named “star-like”. These structures contain pericentrosomal matrix components like γ -tubulin, pericentrin and ninein. Confocal and immunofluorescence microscopy revealed that “star like” structures are spherical structures formed by residual fragments of microtubules connected to kinetochores. At the centre of this

structure are present some component of the pericentriolar matrix like γ -tubulin, pericentrin and ninein.

After the analysis of structural alteration induced by Combretastatin we have focused our attention on the capability of this drug to induce cell death. Treatment with CA-4, which produced high frequency of “star-like” mitosis, was accompanied by mitotic catastrophe commitment characterized by activation of caspases-3/-9 and DNA fragmentation. The mechanisms that lead from microtubules depolymerization to cell death are still not completely clear. Recently it has been demonstrated that Bim, a proapoptotic Bcl-2 protein, is associated to the dynein motor complex and sequestered by microtubules. MDA, through the alteration of microtubules structure, might lead to the release of Bim from cytoskeleton and to its translocation to the mitochondria. Moreover, recent data show that further mechanisms might be at the base of the Bim-induced apoptosis such as regulation of Bim transcriptional levels. In a similar way, p53 another well known pro-apoptotic molecule, is associated with cellular microtubules and is transported to the nucleus by dynein. Based on these data, we have investigated whether upon tubulin depolymerization, Bim and p53 could be involved, and eventually in which manner, in the first steps of apoptosis. CA-4 treatment induce the release of Bim from microtubules and its increase at transcriptional level. In addition, Bim silencing strongly reduced apoptotic commitment. It has recently reported that p53 can also induce apoptosis independently of new protein synthesis however the functional importance of p53 in MDA-induced apoptosis of human cancer cells remain poorly understood. Concerning the role of p53 in CA-4-induced apoptosis, we found that treatment determined its release from cytoskeleton and re-localization to mitochondria. It remains to evaluate which are the Bcl-2 proteins involved in independent-transcription p53-induced apoptosis. For this purpose we are investigating the interaction of p53 with some members of the Bcl-2 family protein like Bcl-2, Bcl-XL, Bak and Bax.

RESULT

1. Interfering with spindle microtubule-dynamics arrests cells at metaphase stage with misaligned chromosomes

H460 (non-small-cell lung cancer) cells were incubated for 8 h with the “tubulin depolymerising agent” CA-4 in a dose-range of 1 nM to 100 nM. Treatments caused a dose-dependent block of the cell cycle in mitosis (Fig. 1a). Cells were arrested mainly at metaphase stage as evaluated by Giemsa staining (Fig. 1b), with mitotic index values of 35% and 47% and with ana-telophase ratio close to 0 at concentrations ≥ 5 nM for combretastatin (Fig. 1a). After low concentrations of Ca-4 a high degree of metaphases showed misaligned chromosomes (approximately 20% at 2.5 nM) (Fig. 1c). With increasing concentrations the vast majority of combretastatin-treated cells presented collapsed chromatin (98% at 10 nM).

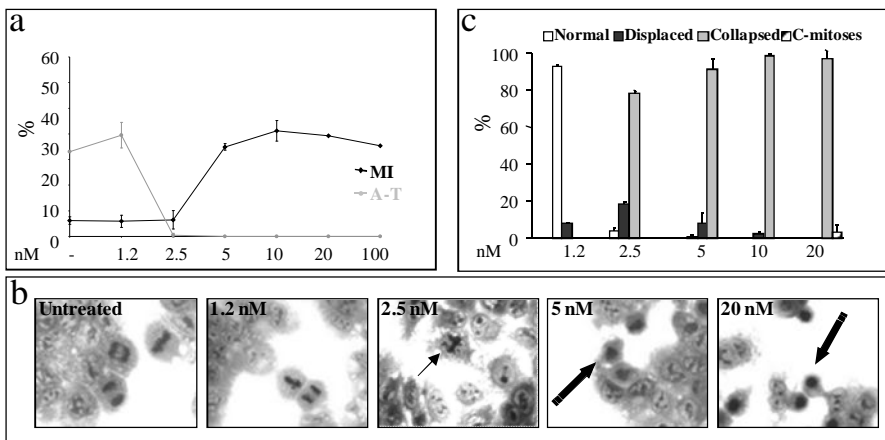


Figure 1. CA-4 caused mitotic arrest and generation of abnormal metaphases in H460 cells. (a, b). Mitotic index (M.I.) and ana-telophase ratio (A-T) of H460 cells left untreated or treated for 8 h with different concentrations of CA-4 (a). Data are mean of three independent experiments \pm SE. Metaphase organization in cells treated with CA-4 or (c). Mitoses were classified into four categories based on the GIEMSA staining profiles: normal metaphases (normal), metaphases with displaced chromosomes (displaced, thin black arrow) or collapsed chromatin (thick black arrows), C-mitoses. Images are representative of three independent experiments. Panel c reports quantitative data recorded from three independent experiments (mean \pm SE).

2. Combretastatin A-4 affects spindle organization giving rise to “star-like” mitosis.

In both control and treated cells, the mitotic spindle arrangements were analyzed by immunostaining with antibodies directed against microtubules (α -, α -acetylated-tubulin) and centrosomes (γ -tubulin). Mitotic spindles of cells blocked in metaphase by low concentrations of combretastatin (0.1 nM) were bipolar with few chromosomes displaced from the metaphase plate and positioned in the vicinity of the spindle poles (spindle type stage I) (Fig. 2a). With increasing concentrations of combretastatin, spindle morphology became more abnormal and increasing numbers of chromosomes were located near the poles of a shorter bipolar spindle rather than in the metaphase plate (spindle type stage II) (not shown). After 7.5 nM CA-4 treatment many spindles (95%) had a multipolar organization with ball-shaped aggregations of condensed chromosomes containing multiple asters of microtubules (spindle types stage III and IV); this structure is called “star-like” (Fig. 2a). These small spindles contained residual kinetochore microtubules that were acetylated, as shown in immunostaining using an antibody that recognizes the acetylated form of α -tubulin (Fig. 2b). Acetylated microtubules are more stable and commonly resist to drug-induced disassembly (32). The “star-like” structures were characterized by the presence of multiple signals of γ -tubulin (Fig. 2c), and occurred only in mitosis as observed after co-staining with phospho-histone H3 (not shown). Following prolonged exposure or high concentrations of drug (≥ 20 nM) causing complete microtubules depolymerisation, non-centrosomal gamma-tubulin “star-like” structures did not formed; whereas centrosomes retained γ -tubulin signals (spindle type stage V) (Figs. 2a, b, c). Immunoblot analysis aimed at quantitating the amount of γ -tubulin after combretastatin-treatment showed no variation due to drug treatment (not shown)

3. Pericentriolar matrix components are present in CA-4-induced “star-like” structures

To better understand the spatial organization and the reciprocal localization of the components of the “star-like” structure, 7.5 nM combretastatin-treated cells were analyzed by transmission electron microscopy and by confocal microscopy, after immunolabeling with antibodies against α -tubulin, γ -tubulin and CREST. After combretastatin treatment, γ -tubulin was located in close proximity to the chromosomes, at the center of a reduced monopolar spindle of α -tubulin that was connected to kinetochores and chromosomes (Fig. 3). The localization of centrioles and of other centrosome or PCM components after CA-4 treatments was evaluated by fluorescence microscopy using specific antibodies against centrin, pericentrin and ninein (Fig. 3c, d, e). In control cells, two distinct dots of centrin that, represent a pair of centrioles, colocalized with γ -tubulin, pericentrin and ninein on each spindle pole (Figs. 3c, d, e). On the other hand, in combretastatin-treated cells, the two dots of centrin colocalized only with one γ -tubulin pole of the multiple “star-like structure” (Fig. 3c); this means that the structural integrity of centrioles was not affected by treatment. In some cases four spots of centrin were visible in close proximity to each other, indicating that combretastatin also interfered with the centriole-pairs separation that occurred during early mitosis. Drug-induced “star-like” arrangements were also observed for pericentrin (Fig. 3d) and ninein (Fig. 3e).

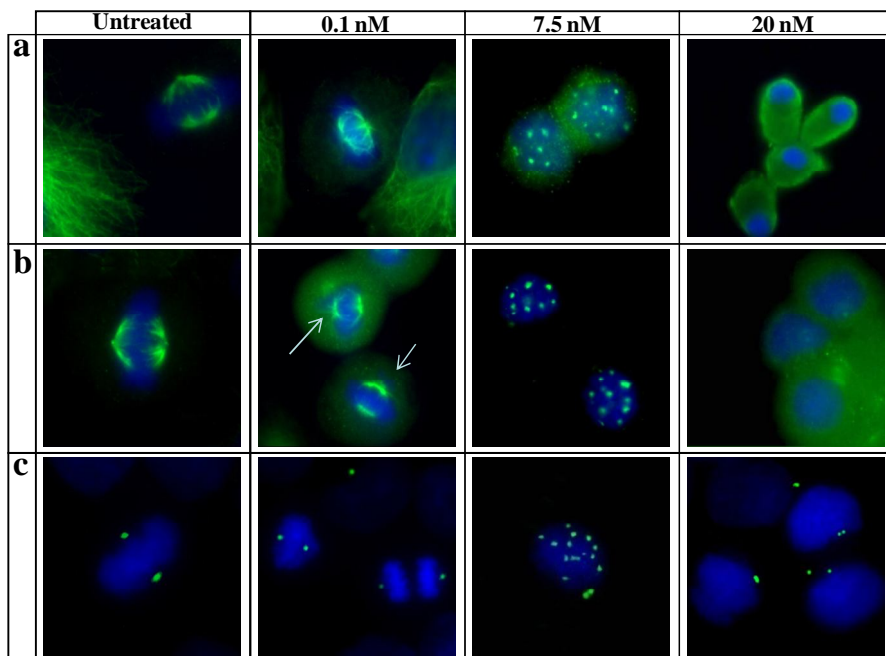


Figure 2. Interferences in microtubular dynamics induce spindle abnormalities. (a-c). Organization of mitotic spindles following treatment for 8 h with the indicated concentrations of CA-4 (a-c). Cells were stained with antibodies that specifically recognize α -tubulin (a), α -acetylated tubulin (b) or γ -tubulin (c) (all emitting in green). DNA was counterstained with DAPI. Representative examples of three independent experiments are shown. White arrows show chromosomes displaced from the metaphase plate. See the text for a detailed description of abnormal spindles.

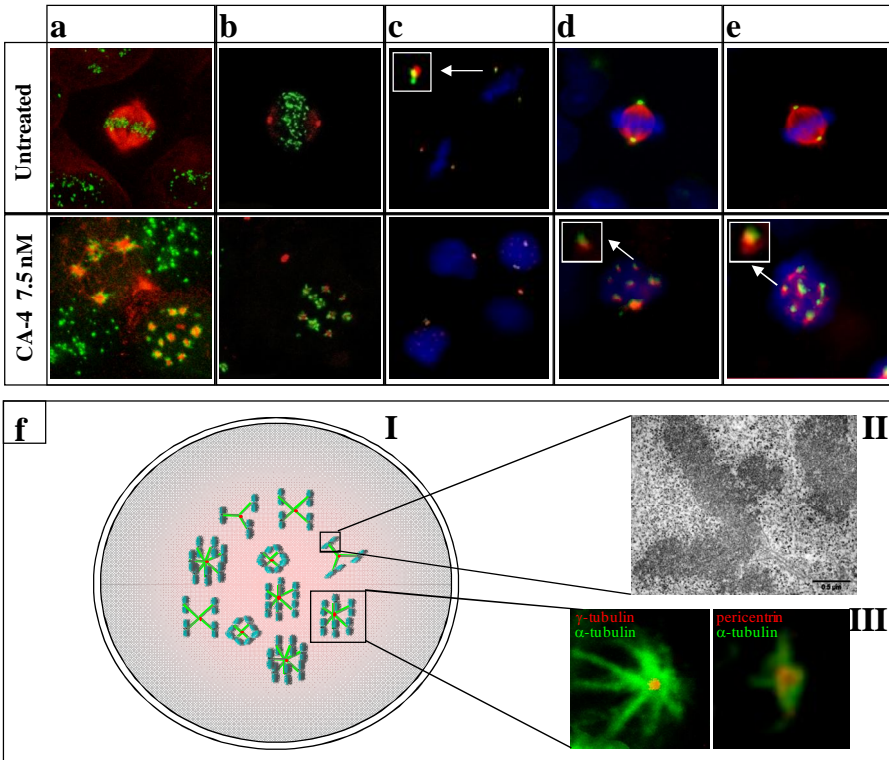


Figure 3. Spatial arrangement of mitotic spindle and centrosome components upon CA-4 treatment. H460 cells were left untreated or treated for 8 h with 7.5 nM Ca-4 prior to immunostaining with different combinations of antibodies and observation by fluorescence microscopy. The following coimmunostaining were performed: (a). CREST serum (green signal) and α -tubulin (in red). (b) CREST serum (in green) and γ -tubulin (in red). (c). γ -tubulin (in red) and centrin (in green) (d). α -tubulin (in red) and pericentrin (green). (e). α -tubulin (in red) and ninein (in green). In all cases DAPI was employed to counterstain DNA (in blue). Please note that the structural integrity of centrioles is not affected by the treatments (centrin, c), whereas the microtubular interference induced by CA-4 led to the generation of the “star-like” structures accompanied by the dismantling of the PCM components (γ -tubulin, b; pericentrin, d; and ninein, e). (f). Proposed model of the arrangement of the “star-like” structure induced by CA-4 treatment (I). As observed by ultra structural assessment by TEM and by confocal microscopy co-immunostaining with antibodies specific for α -tubulin (in green) and γ -tubulin or pericentrin (red) (II and III, respectively), γ -tubulin and pericentrin are located at the centre of each monopolar spindle, in turn composed of small and stable α -tubulin microtubules connected to kinetochores. Representative examples of three independent experiments are shown. See text for a detailed description.

4. Combretastatin induces pericentriolar matrix dismantling and caspase-dependent mitotic catastrophe

To evaluate the relationship between redistribution of the pericentriolar matrix in the form of “star-like” structures and the apoptotic process, biochemical markers like caspase activity and DNA fragmentation, were analyzed. Caspase-involvement in CA-4-induced cell death, was analyzed at whole cellular level, in fluorogenic assays. In fact, a 2-3 fold increase of caspase-9 and -3 expression was detected in cells exposed for 8 h to 7.5 nM CA-4. Such increase was even higher at 24 h with values ranging between 3- and 4- fold, and very close to values of caspase activation obtained after treatment with 2 μ M staurosporine for 6 h (Fig. 4a). After treatment with 7.5 nM CA-4 for 8 h, Caspase 3-7 activation was also monitored at single cell level in time-lapse experiments by using the Magic Red substrate. Positive staining was observed in mitotic arrested cells as a result of a prolonged arrest which was not followed by resumption of cell cycle or attempt to divide, giving rise to a multinucleated cell (mitotic catastrophe) (Fig. 4b). Apoptosis was further confirmed by DNA fragmentation, evaluated by cytofluorimetric analysis after Fluorescent-FragEL™ staining, showing about 40 and 90% apoptotic cells, at 8 and 24 h, respectively (Fig. 4c).

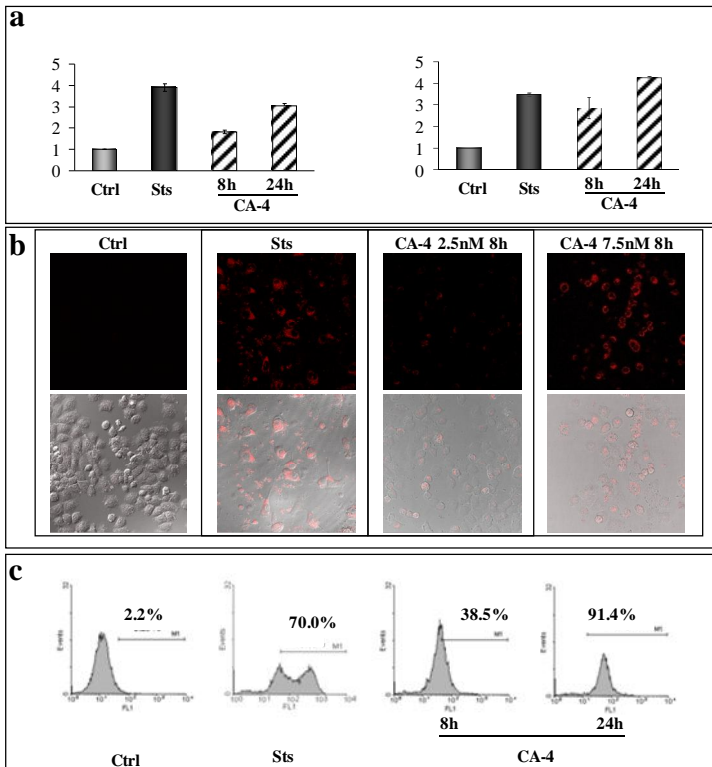


Fig. 4 PCM dismantling induced by CA-4 is followed by apoptosis induction. (a) Caspase-9 and -3 activities in protein extracts from cells left untreated or treated for 8h or 24h with CA-4, as assessed by fluorogenic assays after incubation with caspase-9 (Ac-LEHD-AMC) or caspase-3 (Ac-DEVD-AMC) substrate. As a positive control, cells were treated for 4h with 2 μ M staurosporine, a known activator of the caspases. Columns represent the fold increase in the activity of caspases as compared to untreated cells. Results are expressed as the mean of 3 independent experiments \pm SE. (b) Activation of caspase3-7 has been analyzed in untreated and treated cells using the magic red fluorescent substrate. Magic Red/BF or DIC images were collected at regular intervals throughout experiments; selected images are shown in the panel. (c) Cells were treated or not for the indicated time with CA-4, then analyzed for apoptosis-associated DNA fragmentation using Fluorescent-FragELTM DNA fragmentation kit by flow-cytometry. The percentage of cells with cleaved DNA is reported. The results reported are representative of 3 independent experiments.

5. Inhibition of Bim strongly reduced caspase-3 cleavage and apoptosis

To investigate whether Bim protein mediates CA-4-induced cell death by apoptosis, siRNA were used to inhibit endogenous Bim expression and then we evaluated the cleavage and hence the activation of caspase-3. Infact Real Time analysis clearly showed that BimEL mRNA is induced by CA-4 treatment in a time-dependent manner in H460 cells (Fig. 5a), as also confirmed at protein level (Fig. 5b). As a positive control, taxol, known to up-regulate Bim was included in the experiment (not shown). In parallel experiments, cells were exposed to Bim siRNA or non specific siRNA for 24h then, treated with CA-4 for 0, 8, 24 and 48h. All cells have been collected after 72h trasfection for preparation of whole cell lysates and for isolation of RNA. The level of cellular mRNA and Bim proteins were substantially suppressed after 72h treatment with Bim siRNA (Fig. 5c) in cells treated with CA-4. In contrast, non specific scrambled siRNA had no significant effect on expression of endogenous Bim. Based on our previous results, showing the capability of CA-4 to induce apoptosis in H460 cells in a caspase-3 dependent manner (Cenciarelli et al, 2008) we evaluated whether the inhibition of endogenous Bim following CA-4 induction may suppress cell death. Such analysis was carried out using the activation of pro-caspase-3 to caspase-3 as readout in both immunoblot assay (Fig. 5d) and in time-lapse analysis using the Magic red substrate (Fig. 5e). In both assays, the activation of caspase-3 was strongly reduced when CA-4 treatment was performed in the presence of Bim siRNA, thus indicating the active role played by Bim in CA-4-induced apoptosis.

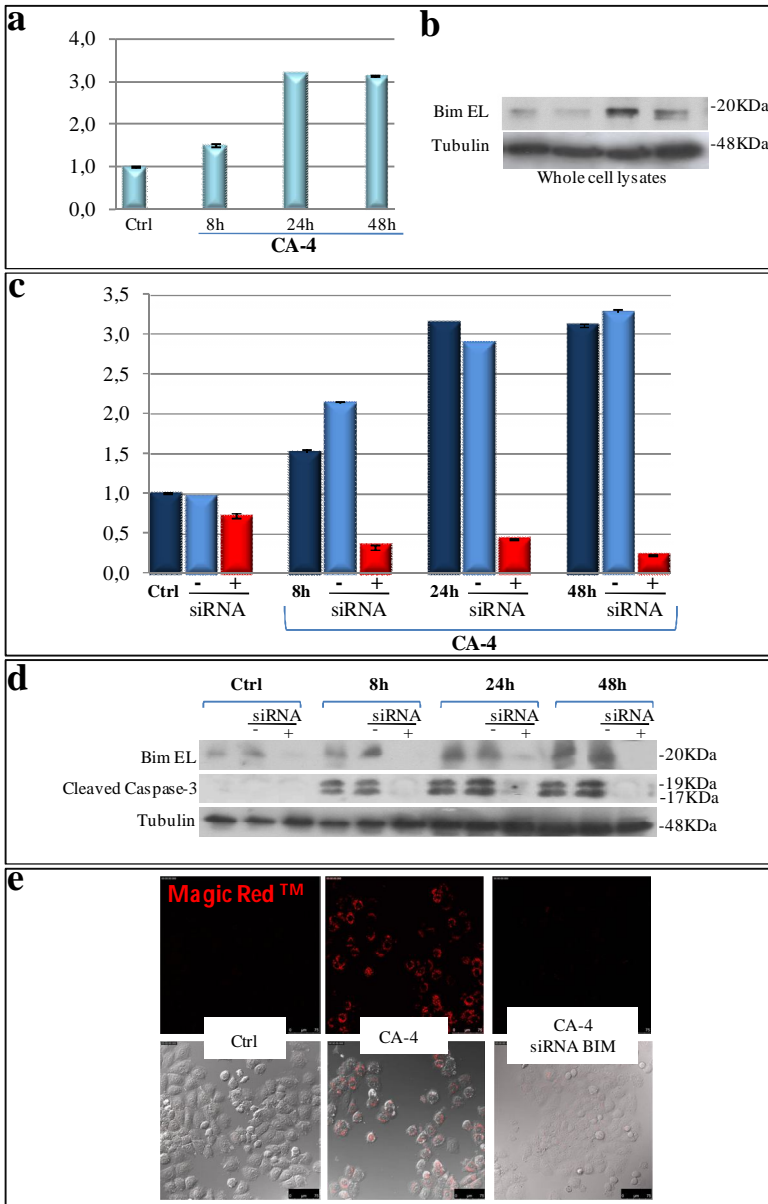


Fig. 5 Real Time and Western Blot analysis showed clearly that Bim mRNA and protein were induced by CA-4 treatment (a, b). H460 cells treated with Bim siRNA or non specific siRNA were also treated with CA-4 for 0, 8, 24 and 48h. All cells have been collected after trasfection for preparation of whole cell lysates and for isolation of RNA. The level of cellular BimEL proteins and mRNA were substantially suppressed after 48h treatment with Bim siRNA (c, d) in cells treated with CA-4. In contrast, non specific siRNA had no significant effect on expression of endogenous Bim. In the presence of Bim siRNA and so during the inhibition of endogenous Bim there's no cleavage of caspase-3 as evaluated by Western Blot and Time Lapse analysis using Magic RedTM(d, e).

6. Bim bound physically to microtubules by interaction with dynein, is released after CA-4 treatment and relocalizes to mitochondria

By means of co-immunoprecipitation assay with a dynein antibody, it was analysed whether CA-4 could induce Bim release from microtubules as a response to tubulin depolymerization. For this purpose, whole cells lysate were prepared after treatment with CA-4 for up to 48h. As reported in Fig. 6a, we found that Bim interacts in a physical manner with dynein. Such interaction was partly retained for the 8h treatment, whereas at longer incubation times of 24 and 48h, the physical interaction between Bim and dynein was markedly impaired, indicating that with increasing in the rate of microtubules depolymerization Bim was released from the microtubular network. Once assessed that CA-4 caused Bim release from microtubule, we analyzed its relocalization on mitochondria. Western Blot analysis of both cytosolic and mitochondrial fraction proteins of cells treated with 7.5nM CA-4 for 0-48h shows a detectable enrichment of Bim in mitochondria (Fig. 6b). In untreated cells the amount of Bim at mitochondria was very low, contrastingly to that observed in T cells (Zhu et al. 2004). Interestingly an increase of Bim was detectable even in the cytosolic extract indicating that beside relocalization, CA-4 promoted also a transcriptional up-regulation of Bim as also suggested by RT-PCR experiments. The occurrence of Bim localization at mitochondria was also confirmed by means of confocal analysis in immunofluorescence experiments using the specific mitotracker staining (Fig. 6c). Taxol was used as internal control (not shown).

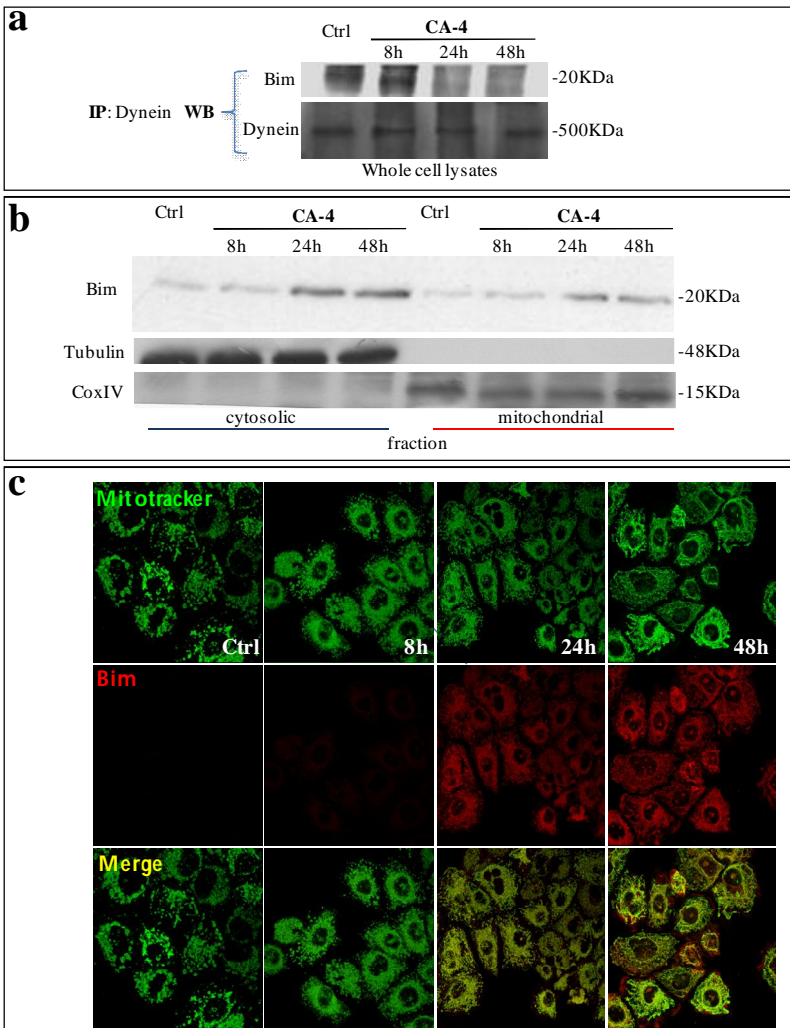


Fig. 6 Mitochondrial relocation of Bim after CA-4 treatment. Bim is sequestered on the microtubule-array and after CA-4 treatment and tubulin depolymerization Bim is released from dynein as shown by coimmunoprecipitations experiments (a). Western Blot analysis of cells treated with CA-4 for 0-48h shows a detectable enrichment of Bim in mitochondrial proteins fraction (b). The confocal analysis of Bim together with mitotracker staining shows that, after 24h and 48h CA-4 treatment, Bim localizes to mitochondria (c).

7. p53 is linked to dynein and localized on mitochondria after Ca-4 treatment

To clarify the role of p53 in Ca-4 induced apoptosis and the relation between microtubule network integrity and p53 localization we performed coimmunoprecipitation assay. Coimmunoprecipitation experiments revealed that p53 is linked to dynein and that the increase in microtubules depolymerization lead to a different accumulation of p53 on dynein and hence on microtubules (Fig. 7a). Besides it has been recently demonstrated that p53 has a transcription independent mediated apoptosis activity. To test this ipotesis we have performed WB analysis on mitochondrial protein fraction. This has revealed the presence of p53 on mitochondria. Infact with the increase in treatment time there is an enrichment of such protein on mitochondria (Fig. 7b).

8. Bim coimmunoprecipitated with p53 in the mitochondrial protein fraction

To determine whether p53 associates with Bim on mitochondria, we carried out co-immunoprecipitation experiments. First of all we have incubated mitochondrial cell lysate from H460 treated cells (0-48h) with anti-Bim antibody for immunoprecipitation followed by immunoblotting with an anti-p53 antibody (Fig. 8). Coimmunoprecipitation experiments showed that CA-4 treatment lead to an enrichment in the interaction between Bim and p53 on mitochondria.

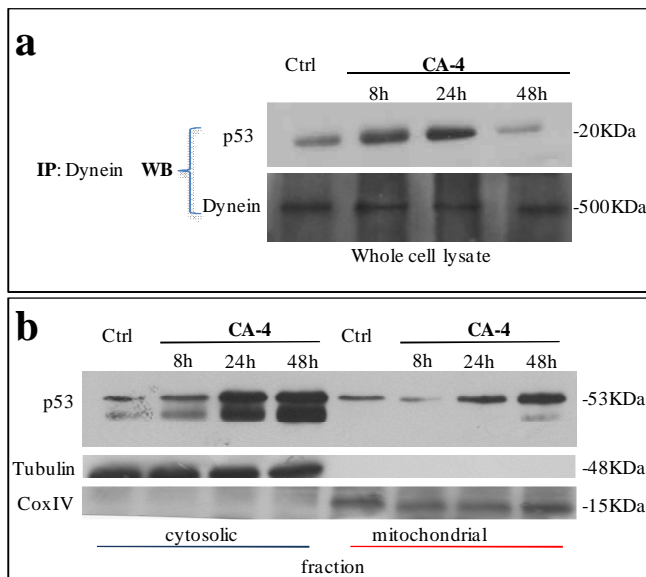


Fig. 7 Co-immunoprecipitation assay revealed a physical interaction between p53 and dynein (a). WB analysis of p53 on cytosolic and mitochondrial protein fraction showed that with the increase in treatment time there is an enrichment of p53 protein on mitochondria; Tubulin and CoxIV have been used as loading controls (b).

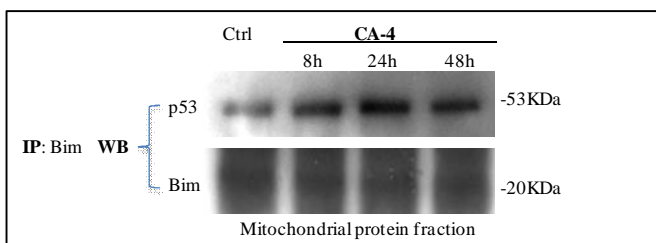


Fig.8 Co-immunoprecipitation assay showed that Bim and p53 are linked together in the mitochondrial protein fraction.

DISCUSSION

By treating NSCLC H460 cells with CA-4, we found a dose-dependent mitotic arrest accompanied by an increasing disorganization of chromosomal arrangement. In this respect, CA-4 behaved similarly to other drugs leading to chromosomal disposition according to stages I-V as described in cells treated with nocodazole or vinca-alkaloids (Jordan et al, 1992). Immunostaining of drug-induced stage III metaphases indicated the presence of multiple signals of α -tubulin and drug-disassembly resistant acetylated- α -tubulin (Takemura et al, 1992), arranged in the shape of “star-like” structures. Such aggregates of α -tubulin could represent residual fragments of microtubules following the depolymerisation of mitotic spindle, ectopic centrosome-independent centres of microtubule nucleation, or splitting of pre-existing centrosomal material. The γ -tubulin immunostaining allowed us to display differences in the effects obtained after treatment with either drug. The co-localization analysis carried out by confocal microscopy by using pairs of antibodies, together with TEM analysis and 3D image reconstruction, revealed that each “star-like” structure presents γ -tubulin at the centre of a spherical structure characterized by short drug-resistant microtubules connected to kinetochores. A “star-like” pattern of staining was also detected in stage III metaphases obtained after treatment with colchicine, vinblastine or combretastatin-derivatives (Jordan et al, 1992; Vitale et al, 2007). Though, agents like ionizing radiation (Sato et al, 1983; Sato et al, 2000) or arsenic and its metabolites (Ochi et al, 1999; Ochi, 2000; Yih et al, 2006) have been shown to produce multiple centrosomal signals, the single detection of γ -tubulin for the centrosome did not allow us to unambiguously distinguish between centrosome overduplication/fragmentation, dismantling of the centrosomal matrix or activation of ectopic assembly of nucleating centres. We found that in the majority of CA-4-treated cells the structural integrity of centrioles was not affected, as shown by the presence of two dots of centrine, a recognized marker of centrioles (Paoletti et al, 1996; Bornens, 2002), in both control and treated-cells. In a few cells, however, four spots of centrine were visible in close proximity to each other, indicating that combretastatin might also affect centriole-pair separation, which occurs during late in the G2-phase, as observed in arsenite-treated cells (Yih et al, 2006). Overall these findings led us to

exclude true centriolar fragmentation or centrosome overduplication and favour a model based on disassembly and relocalization of pericentriolar matrix after combretastatin-treatment. Moreover, we investigated the effect of microtubules perturbation on the release of centrosomal material which localizes either at the matrix, as the scaffold and anchoring protein pericentrin (Dictenberg et al, 1998; Bornens, 2002; Yih et al, 2006), or to the subdistal appendages of mother centriole, like the anchoring protein ninein (Mogensen et al, 2000), which also promotes microtubules nucleation by docking the γ -TuRC at the centrosome (Delgehyr et al, 2005). CA-4 was effective in displacing pericentrin as well as ninein from the centrosome, and in agreement with their role in γ -TuRC docking, cytoplasmic aggregates of pericentrin and ninein recruited γ -tubulin. Furthermore, our findings show that the “star-like” structures arise as a result of a critical level of drug-mediated microtubular disruption as evaluated in combined experiment with CA-4 and either a microtubule depolymerising agent (nocodazole) or a stabilising one (paclitaxel)(not shown). The observation that such structures occurred only at restricted concentrations or length of treatment, suggests that the maintenance of microtubule integrity in mitosis plays a relevant role in stabilising the interaction between pericentriolar matrix components and centrioles. In centrosome perturbed by centrosomin depletion (Mahoney et al, 2006) or in monastrol-treated cells it has been shown that kinetochores are able to enucleate microtubules (Khodjakov et al, 2003), suggesting that γ -tubulin functions as a microtubule nucleator in the chromosome-mediated assembly pathway. Hence, the “star-like” structures might arise as result of the rescue of kinetochore microtubules that undergo drug-mediated catastrophe. As recently observed in yeast, plant and *Drosophila* cells (Janson et al, 2005; Murata et al, 2005; Mahoney et al, 2006), γ -tubulin can interact with pre-existing microtubules, so it is conceivable that, after partial depolymerisation, the centrosome-unassociated γ -tubulin cytoplasmic pool could bind to drug-resistant microtubules at kinetochores. Then, through the presence of cytoplasmic complexes of γ -tubulin-pericentrin or γ -tubulin-ninein interacting domains these proteins are concentrated to kinetochores giving rise to “star-like” structures. On the contrary, in the case of complete microtubule depolymerisation, as those occurring during mitosis at stage IV or by co-incubating cells with combretastatin and nocodazole, γ -tubulin would not have preformed microtubules to interact with, and therefore would be

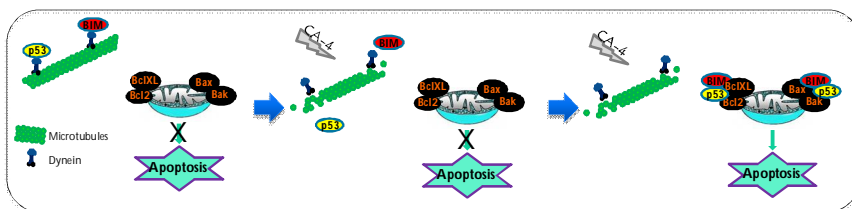
unable to recruit pericentrin and ninein complexes. In this respect, recent studies have demonstrated that silencing of pericentrin A/B leads to uncoupling of the pericentrin- γ -TuRC interaction, disruption of astral microtubules and induction of apoptosis at the G2/antephase in U2OS cells (Zimmerman et al, 2004). In agreement with data obtained in HUVEC primary endothelial cells (Kanthou et al, 2004), we found that combretastatin-treated H460 cells accumulated in mitosis rather than in a G2/antephase stage, as shown by the level of chromosome condensation. These observations led us to rule out that combretastatin activates a G₂-phase checkpoint that senses spindle pole assembly/centrosome maturation because of disruption of the α -tubulin- γ -tubulin/pericentrin/ninein interaction. On the other hand, at these concentrations of drug, though the depolymerisation of α -tubulin is not complete, the mitotic spindle assembly checkpoint is activated as we previously showed in H460 cells (Vitale et al, 2007). We found that the appearance of mitosis with “star-like” structures occurred few hours after treatment with CA-4 and, at 8h, they represented about 40% of the total cell population encompassing virtually all mitotic arrested cells. The use of drug concentrations which failed to modify the mitotic index support the notion that the activation of the apoptotic process is strictly dependent on the mitotic-block and level of tubulin polymerisation. Activation of spindle checkpoint, mitotic block and failure of H460-CA-4 treated cells to correctly exit mitosis, lead to cell death through a caspase-3 dependent mitotic catastrophe as shown by several results as fluorogenic assay for activation of caspase-3 and-9, activation of caspase-3/7 in time-lapse confocal microscopy and DNA fragmentation assay (Vitale et al, 2007; Cenciarelli et al, 2008); present data). However the link between microtubules depolymerization, mitotic arrest and subsequent cell death is largely unknown, particularly for the initiating steps. Recent studies have identified an involvement of Bim in the apoptotic pathway activated by different physical and chemical agents in normal as well as in tumor cells (O'Connor et al, 1998; Li et al, 2005). On the other end, the role of Bim in apoptosis engaged by agents known to affect the microtubular integrity, as the spindle poisons has been only poorly investigated so far, except for microtubular-stabilizing agents as taxanes (Li et al, 2005; Tan et al, 2005; Czernick et al, 2009) and persins (Butt et al, 2006). Both Bim-dependent apoptosis, as in lymphocytes (Erlacher et al, 2005), non-small-cell lung cancer lines (Li et al, 2005), kidney epithelial cells (Tan et al,

2005) and mouse models (Sunters et al, 2003;Li et al, 2005;Li et al, 2007) and Bim-independent apoptosis as in HeLa and breast cancer cells (Czernick et al, 2009) have been reported, suggesting cell specificity in paclitaxel. Then, based on the observation that Bim is sequestered on the microtubular array by interaction with dynein (Puthalakath et al, 1999), we analyzed the involvement of this protein in apoptosis induced cell death. We first realized that the microtubular array disruption and cell death, were accompanied by an up-regulation of the Bim gene and consequent accumulation of its proteic product, particularly after 24 and 48h of treatment. The BimEL form is predominant over BimL in H460 as well as in many investigated normal and tumor cell lines established from lung, breast and prostate (O'Connor et al, 1998;Li et al, 2005), and for this reason data here presented are restricted to this isoform. Transcriptional up-regulation of Bim occurred after exposure to CA-4, as for other stress stimuli. Targeting Bim with siRNA, clearly showed that drug-induced apoptosis in this cell line is strongly dependent on such pro-apoptotic protein as confirmed by inhibition of caspase-3 activation. However, since it is known that Bim activity is controlled by several mechanism including post-translational one, that are responsible for variations in intracellular localization, we investigated the effect of treatment on Bim location. Bim may be either sequestered to microtubular dynein motor complex as reported in different cell lines (human embryonic kidney, human breast carcinoma, mouse promyelocytic and mouse fibroblastoid lines (Puthalakath et al, 1999) or bound to Bcl-2 and Bcl-XL at mitochondria as shown for in normal, resting T cells (Zhu et al, 2004). We found that in H460 cells drug treatment promoted the release of Bim from dynein and its translocation to mitochondria as a function of time. The dissociation from dynein motor complex and hence by microtubules could be one of the explanation for the presence of Bim at mitochondria for tubulin depolymerizing agents, though for γ -irradiated or IL-3-starved FDC-P1 cells the migration of Bim was not dependent on the disruption of microtubular array (Puthalakath et al, 1999). Besides, it has been recently demonstrated a transcription-independent p53-mediated apoptosis (Caelles et al, 1994;Marchenko et al, 2000;Giannakakou et al, 2000;Moll et al, 2005). Our results showed a direct involvement of such protein in CA-4 induced apoptosis. Infact, accordingly with precedent data (Giannakakou et al, 2000;Moll et al, 2005) our experiments showed that p53 is linked to dynein and relocalized to mitochondria after CA-4

treatment. On mitochondria p53 is able to physically interact with Bim. This interaction could be responsible for apoptosis activation and could represent the link between microtubules depolymerization and apoptosis induction. Further experiments will be required to explain which Bcl-2 proteins are involved and how they interact on mitochondria after CA-4 treatment.

CONCLUSION

In conclusion, we show here that chemicals characterized by depolymerising activity like combretastatin A-4 release subclasses of proteins involved either in microtubule-nucleating or -scaffolding and -anchoring components of the centrosome, which organize in mitotic cells in the form of “star-like” structures. Combretastatin-treated H460 cells are killed via a mitotic catastrophe mechanism strictly dependent on the achievement of mitotic block, Bim and p53 translocation to mitochondria and caspases activation.



Model for the regulation on Bim and p53. In healthy cells, Bim and p53 are sequestered on microtubules. CA-4 treatment cause release of such proteins from microtubules. Free Bim and p53 relocate to mitochondria probably interacting with anti-apoptotic proteins neutralizing their action and promoting apoptosis.

References

- Alieva,I.B. and Uzbekov,R.E. 2008. The centrosome is a polyfunctional multiprotein cell complex. *Biochemistry-Moscow*, 73:626-643.
- Andre,N., Carre,M., Brasseur,G., Pourroy,B., Kovacic,H., Briand,C., and Braguer,D. 2002. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *Febs Letters*, 532:256-260.
- Antonsson,B., Montessuit,S., Sanchez,B., and Martinou,J.C. 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *Journal of Biological Chemistry*, 276:11615-11623.
- Ayscough,K., Hayles,J., MacNeill,S.A., and Nurse,P. 1992. Cold-sensitive mutants of p34cdc2 that suppress a mitotic catastrophe phenotype in fission yeast. *Mol.Gen.Genet.*, 232:344-350.
- Bernardi,P. and Azzone,G.F. 1981. Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. *J.Biol.Chem.*, 256:7187-7192.
- Bhat,K.M.R. and Setaluri,V. 2007. Microtubule-associated proteins as targets in cancer chemotherapy. *Clinical Cancer Research*, 13:2849-2854.
- Blagden,S.P. and Glover,D.M. 2003. Polar expeditions - provisioning the centrosome for mitosis. *Nature Cell Biology*, 5:505-511.

Boise,L.H., Gonzalezgarcia,M., Postema,C.E., Ding,L.Y., Lindsten,T., Turka,L.A., Mao,X.H., Nunez,G., and Thompson,C.B. 1993. Bcl-X, A Bcl-2-Related Gene That Functions As A Dominant Regulator of Apoptotic Cell-Death. *Cell*, 74:597-608.

Bonaccorsi,S., Giansanti,M.G., and Gatti,M. 1998. Spindle self-organization and cytokinesis during male meiosis in asterless mutants of *Drosophila melanogaster*. *Journal of Cell Biology*, 142:751-761.

Bornens,M. 2002. Centrosome composition and microtubule anchoring mechanisms. *Current Opinion in Cell Biology*, 14:25-34.

Boyd,J.M., Gallo,G.J., Elangovan,B., Houghton,A.B., Malstrom,S., Avery,B.J., Ebb,R.G., Subramanian,T., Chittenden,T., Lutz,R.J., and Chinnadurai,G. 1995. Bik, A Novel Death-Inducing Protein Shares A Distinct Sequence Motif with Bcl-2 Family Proteins and Interacts with Viral and Cellular Survival-Promoting Proteins. *Oncogene*, 11:1921-1928.

Brenner,C. and Kroemer,G. 2000. Apoptosis - Mitochondria - the death signal integrators. *Science*, 289:1150-1151.

Brinkley,B.R. and Goepfert,T.M. 1998. Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. *Cell Motility and the Cytoskeleton*, 41:281-288.

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell*, 96:857-868.

Butt,A.J., Roberts,C.G., Seawright,A.A., Oelrichs,P.B., Macleod,J.K., Liaw,T.Y., Kavallaris,M., Somers-Edgar,T.J.,

Lehrbach,G.M., Watts,C.K., and Sutherland,R.L. 2006. A novel plant toxin, persin, with in vivo activity in the mammary gland, induces Bim-dependent apoptosis in human breast cancer cells. *Mol.Cancer Ther.*, 5:2300-2309.

Caelles,C., Helmborg,A., and Karin,M. 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*, 370:220-223.

Cai,B.B. and Xia,Z.G. 2008. p38 MAP kinase mediates arsenite-induced apoptosis through FOXO3a activation and induction of Bim transcription. *Apoptosis*, 13:803-810.

Cain,K., Bratton,S.B., and Cohen,G.M. 2002. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie*, 84:203-214.

Cartron,P.F., Priault,M., Oliver,L., Meflah,K., Manon,S., and Vallette,F.M. 2003. The N-terminal end of Bax contains a mitochondrial-targeting signal. *Journal of Biological Chemistry*, 278:11633-11641.

Cassimeris,L. 1993. Regulation of Microtubule Dynamic Instability. *Cell Motility and the Cytoskeleton*, 26:275-281.

Cassimeris,L. and Spittle,C. 2001. Regulation of microtubule-associated proteins. *International Review of Cytology - A Survey of Cell Biology*, Vol 210, 210:163-226.

Castedo,M., Perfettini,J.L., Roumie,T., Andreau,K., Medema,R., and Kroemer,G. 2004. Cell death by mitotic catastrophe: a molecular definition. *Oncogene*, 23:2825-2837.

Cenciarelli,C., Tanzarella,C., Vitale,I., Pisano,C., Crateri,P., Meschini,S., Arancia,G., and Antoccia,A. 2008. The tubulin-depolymerising agent combretastatin-4 induces ectopic aster assembly and mitotic catastrophe in lung cancer cells H460. *Apoptosis*, 13:659-669.

Chen,L., Willis,S.N., Wei,A., Smith,B.J., Fletcher,J.I., Hinds,M.G., Colman,P.M., Day,C.L., Adams,J.M., and Huang,D.C.S. 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular Cell*, 17:393-403.

Chen,X.B., Ko,L.J., Jayaraman,L., and Prives,C. 1996. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes & Development*, 10:2438-2451.

Cheng,E.H.Y.A., Wei,M.C., Weiler,S., Flavell,R.A., Mak,T.W., Lindsten,T., and Korsmeyer,S.J. 2001. BCL-2, BCL-X-L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell*, 8:705-711.

Chipuk,J.E., Kuwana,T., Bouchier-Hayes,L., Droin,N.M., Newmeyer,D.D., Schuler,M., and Green,D.R. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, 303:1010-1014.

Chittenden,T., Flemington,C., Houghton,A.B., Ebb,R.G., Gallo,G.J., Elangovan,B., Chinnadurai,G., and Lutz,R.J. 1995a. A Conserved Domain in Bak, Distinct from Bh1 and Bh2, Mediates Cell-Death and Protein-Binding Functions. *Embo Journal*, 14:5589-5596.

Chittenden,T., Harrington,E.A., Oconnor,R., Flemington,C., Lutz,R.J., Evan,G.I., and Guild,B.C. 1995b. Induction of Apoptosis by the Bcl-2 Homolog Bak. *Nature*, 374:733-736.

Cory,S., Huang,D.C.S., and Adams,J.M. 2003. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene*, 22:8590-8607.

Czernick,M., Rieger,A., and Goping,I.S. 2009. Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. *Biochemical and Biophysical Research Communications*, 379:145-150.

Debatin,K.M. and Krammer,P.H. 2004. Death receptors in chemotherapy and cancer. *Oncogene*, 23:2950-2966.

Delgehyr,N., Sillibourne,J., and Bornens,M. 2005. Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. *Journal of Cell Science*, 118:1565-1575.

Deng,X.M., Gao,F.Q., Flagg,T., Anderson,J., and May,W.S. 2006. Bcl2's flexible loop domain regulates p53 binding and survival. *Molecular and Cellular Biology*, 26:4421-4434.

Denmeade,S.R., Lin,X.H.S., Tombal,B., and Isaacs,J.T. 1999. Inhibition of caspase activity does not prevent the signaling phase of apoptosis in prostate cancer cells. *Prostate*, 39:269-279.

Dictenberg,J.B., Zimmerman,W., Sparks,C.A., Young,A., Vidair,C., Zheng,Y.X., Carrington,W., Fay,F.S., and Doxsey,S.J. 1998. Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. *Journal of Cell Biology*, 141:163-174.

Doxsey,S. 2001. Re-evaluating centrosome function. *Nature Reviews Molecular Cell Biology*, 2:688-698.

Doxsey,S., Zimmerman,W., and Mikule,K. 2005. Chromosome segregation and aneuploidy series: Centrosome control of the cell. *Trends in Cell Biology*, 15:303-311.

Dumont,P., Leu,J.I.J., Della Pietra,A.C., George,D.L., and Murphy,M. 2003. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nature Genetics*, 33:357-365.

Duriez,P.J. and Shah,G.M. 1997. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem.Cell Biol.*, 75:337-349.

Dutertre,S., Descamps,S., and Prigent,C. 2002. On the role of aurora-A in centrosome function. *Oncogene*, 21:6175-6183.

Erlacher,M., Michalak,E.M., Kelly,P.N., Labi,V., Niederegger,H., Coultas,L., Adams,J.M., Strasser,A., and Villunger,A. 2005. BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. *Blood*, 106:4131-4138.

Essafi,A., de Mattos,S.F., Hassen,Y.A.M., Soeiro,I., Mufti,G.J., Thomas,N.S.B., Medema,R.H., and Lam,E.W.F. 2005. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene*, 24:2317-2329.

Esteve,M.A., Carre,M., and Braguer,D. 2007a. Microtubules in apoptosis induction: Are they necessary? *Current Cancer Drug Targets*, 7:713-729.

Esteve,M.A., Carre,M., and Braguer,D. 2007b. Microtubules in apoptosis induction: are they necessary? *Curr.Cancer Drug Targets*, 7:713-729.

Farrow,S.N., White,J.H.M., Martinou,I., Raven,T., Pun,K.T., Grinham,C.J., Martinou,J.C., and Brown,R. 1995. Cloning of A Bcl-2 Homolog by Interaction with Adenovirus E1B 19K (Vol 374, Pg 731, 1995). *Nature*, 375:431.

Fei,P.W. and El Deiry,W.S. 2003. P53 and radiation responses. *Oncogene*, 22:5774-5783.

Ferri,K.F. and Kroemer,G. 2000. Control of apoptotic DNA degradation. *Nature Cell Biology*, 2:E63-E64.

Fukasawa,K. 2002. Centrosome - Introduction. *Oncogene*, 21:6140-6145.

Gadde,S. and Heald,R. 2004. Mechanisms and molecules of the mitotic spindle. *Current Biology*, 14:R797-R805.

Giannakakou,P., Sackett,D.L., Ward,Y., Webster,K.R., Blagosklonny,M.V., and Fojo,T. 2000. p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nature Cell Biology*, 2:709-717.

Gibson,L., Holmgreen,S.P., Huang,D.C.S., Bernand,O., Copeland,N.G., Jenkins,N.A., Sutherland,G.R., Baker,E., Adams,J.M., and Cory,S. 1996. bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene*, 13:665-675.

Gilley,J., Coffey,P.J., and Ham,J. 2003. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *Journal of Cell Biology*, 162:613-622.

Green,D.R. and Martin,S.J. 1995. The Killer and the Executioner - How Apoptosis Controls Malignancy. *Current Opinion in Immunology*, 7:694-703.

Gregoret,I.V., Margolin,G., Alber,M.S., and Goodson,H.V. 2006. Insights into cytoskeletal behavior from computational modeling of dynamic microtubules in a cell-like environment. *Journal of Cell Science*, 119:4781-4788.

Hakem,R., Hakem,A., Duncan,G.S., Henderson,J.T., Woo,M., Soengas,M.S., Elia,A., de la Pompa,J.L., Kagi,D., Khoo,W., Potter,J., Yoshida,R., Kaufman,S.A., Lowe,S.W., Penninger,J.M., and Mak,T.W. 1998. Differential requirement for Caspase 9 in apoptotic pathways in vivo. *Cell*, 94:339-352.

Han,J., Sabbatini,P., and White,E. 1996. Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. *Molecular and Cellular Biology*, 16:5857-5864.

Hanada,M., AimeSempe,C., Sato,T., and Reed,J.C. 1995. Structure-Function Analysis of Bcl-2 Protein Identification of Conserved Domains Important for Homodimerization with Bcl-2 and Heterodimerization with Bax. *Journal of Biological Chemistry*, 270:11962-11969.

Haupt,Y., Rowan,S., Shaulian,E., Vousden,K.H., and Oren,M. 1995. Induction of Apoptosis in Hela-Cells by Trans-Activation-Deficient P53. *Genes & Development*, 9:2170-2183.

Hinchcliffe,E.H., Miller,F.J., Cham,M., Khodjakov,A., and Sluder,G. 2001. Requirement of a centrosomal activity for cell cycle progression through G(1) into S phase. *Science*, 291:1547-1550.

Hirsch,T., Marchetti,P., Susin,S.A., Dallaporta,B., Zamzami,N., Marzo,I., Geuskens,M., and Kroemer,G. 1997. The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene*, 15:1573-1581.

Ianzini,F. and Mackey,M.A. 1997. Spontaneous premature chromosome condensation and mitotic catastrophe following irradiation of HeLa S3 cells. *International Journal of Radiation Biology*, 72:409-421.

Imaizumi,K., Tsuda,M., Imai,Y., Wanaka,A., Takagi,T., and Tohyama,M. 1997. Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death. *Journal of Biological Chemistry*, 272:18842-18848.

Inohara,N., Ding,L.Y., Chen,S., and Nunez,G. 1997. harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X-L. *Embo Journal*, 16:1686-1694.

Islam,M.N. and Iskander,M.N. 2004. Microtubulin binding sites as target for developing anticancer agents. *Mini.Rev.Med.Chem.*, 4:1077-1104.

Janson,M.E., Setty,T.G., Paoletti,A., and Tran,P.T. 2005. Efficient formation of bipolar microtubule bundles requires microtubule-bound gamma-tubulin complexes. *Journal of Cell Biology*, 169:297-308.

Jones,N.A., Turner,J., McIlwrath,A.J., Brown,R., and Dive,C. 1998. Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between Bax and Bak up-regulation and the functional status of p53. *Molecular Pharmacology*, 53:819-826.

Jordan,A., Hadfield,J.A., Lawrence,N.J., and McGown,A.T. 1998. Tubulin as a target for anticancer drugs: Agents which interact with the mitotic spindle. *Medicinal Research Reviews*, 18:259-296.

Jordan,M.A., Thrower,D., and Wilson,L. 1992. Effects of Vinblastine, Podophyllotoxin and Nocodazole on Mitotic Spindles - Implications for the Role of Microtubule Dynamics in Mitosis. *Journal of Cell Science*, 102:401-416.

Jordan,M.A. and Wilson,L. 2004f. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004e. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004g. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004b. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004a. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004d. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Jordan,M.A. and Wilson,L. 2004c. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4:253-265.

Kalab,P., Pralle,A., Isacoff,E.Y., Heald,R., and Weis,K. 2006. Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature*, 440:697-701.

Kanthou,C., Greco,O., Stratford,A., Cook,I., Knight,R., Benzakour,O., and Tozer,G. 2004. The tubulin-binding agent combretastatin A-4-phosphate arrests endothelial cells in mitosis and induces mitotic cell death. *American Journal of Pathology*, 165:1401-1411.

Karsenti,E. and Vernos,I. 2001. The mitotic spindle: a self-made machine. *Science*, 294:543-547.

Kerr,J.F., Wyllie,A.H., and Currie,A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br.J.Cancer*, 26:239-257.

Khodjakov,A., Cole,R.W., Oakley,B.R., and Rieder,C.L. 2000. Centrosome-independent mitotic spindle formation in vertebrates. *Current Biology*, 10:59-67.

Khodjakov,A., Copenagle,L., Gordon,M.B., Compton,D.A., and Kapoor,T.M. 2003. Minus-end capture of preformed kinetochore fibers contributes to spindle morphogenesis. *Journal of Cell Biology*, 160:671-683.

Kiefer,M.C., Brauer,M.J., Powers,V.C., Wu,J.J., Umansky,S.R., Tomei,L.D., and Barr,P.J. 1995. Modulation of Apoptosis by the Widely Distributed Bcl-2 Homolog Bak. *Nature*, 374:736-739.

Kirschner,M. and Mitchison,T. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell*, 45:329-342.

Kline-Smith,S.L. and Walczak,C.E. 2004. Mitotic spindle assembly and chromosome segregation: Refocusing on microtubule dynamics. *Molecular Cell*, 15:317-327.

Knop,M. and Schiebel,E. 1997. Spc98p and Spc97p of the yeast gamma-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. *Embo Journal*, 16:6985-6995.

Kozopas,K.M., Yang,T., Buchan,H.L., Zhou,P., and Craig,R.W. 1993. Mcl1, A Gene Expressed in Programmed Myeloid Cell-Differentiation, Has Sequence Similarity to Bcl2. *Proceedings of the National Academy of Sciences of the United States of America*, 90:3516-3520.

Kroemer,G., Galluzzi,L., and Brenner,C. 2007. Mitochondrial membrane permeabilization in cell death. *Physiological Reviews*, 87:99-163.

Kuida,K., Haydar,T.F., Kuan,C.Y., Gu,Y., Taya,C., Karasuyama,H., Su,M.S.S., Rakic,P., and Flavell,R.A. 1998. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking Caspase 9. *Cell*, 94:325-337.

Lee,S. and Schmitt,C.A. 2003. Chemotherapy response and resistance. *Current Opinion in Genetics & Development*, 13:90-96.

Lei,K. and Davis,R.J. 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, 100:2432-2437.

Ley,R., Ewings,K.E., Hadfield,K., and Cook,S.J. 2005. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death and Differentiation*, 12:1008-1014.

Li,H.Y., Cao,K., and Zheng,Y. 2003. Ran in the spindle checkpoint: a new function for a versatile GTPase. *Trends Cell Biol.*, 13:553-557.

Li,K., Li,Y.C., Shelton,J.M., Richardson,J.A., Spencer,E., Chen,Z.J., Wang,X.D., and Williams,R.S. 2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell*, 101:389-399.

Li,R., Moudgil,T., Ross,H.J., and Hu,H.M. 2005. Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim. *Cell Death and Differentiation*, 12:292-303.

Li,Z., Zhang,J., Liu,Z., Woo,C.W., and Thiele,C.J. 2007. Downregulation of Bim by brain-derived neurotrophic factor activation of TrkB protects neuroblastoma cells from paclitaxel but not etoposide or cisplatin-induced cell death. *Cell Death and Differentiation*, 14:318-326.

Liebermann,D.A., Hoffman,B., and Steinman,R.A. 1995. Molecular Controls of Growth Arrest and Apoptosis - P53-Dependent and Independent Pathways. *Oncogene*, 11:199-210.

Lin,E.Y., Orlofsky,A., Berger,M.S., and Prystowsky,M.B. 1993. Characterization of A1, A Novel Hematopoietic-Specific Early-Response Gene with Sequence Similarity to Bcl-2. *Journal of Immunology*, 151:1979-1988.

Lingle,W.L., Lutz,W.H., Ingle,J.N., Maihle,N.J., and Salisbury,J.L. 1998. Centrosome hypertrophy in human breast tumors: Implications for genomic stability and cell polarity. *Proceedings of the National Academy of Sciences of the United States of America*, 95:2950-2955.

Liu,X.S., Zou,H., Slaughter,C., and Wang,X.D. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, 89:175-184.

Luciano,F., Jacquel,A., Colosetti,P., Herrant,M., Cagnol,S., Pages,G., and Auberger,P. 2003. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene*, 22:6785-6793.

Lucken-Ardjomande,S. and Martinou,J.C. 2005. Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane. *Comptes Rendus Biologies*, 328:616-631.

Mahoney,N.M., Goshima,G., Douglass,A.D., and Vale,R.D. 2006. Making microtubules and mitotic spindles in cells without functional centrosomes. *Current Biology*, 16:564-569.

Makin,G.W.J., Corfe,B.M., Griffiths,G.J., Thistlethwaite,A., Hickman,J.A., and Dive,C. 2001. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. *Embo Journal*, 20:6306-6315.

Marchenko,N.D., Zaika,A., and Moll,U.M. 2000. Death signal-induced localization of p53 protein to mitochondria - A potential role in apoptotic signaling. *Journal of Biological Chemistry*, 275:16202-16212.

Martin,S.J., O'Brien,G.A., Nishioka,W.K., McGahon,A.J., Mahboubi,A., Saido,T.C., and Green,D.R. 1995. Proteolysis of Fodrin (Nonerythroid Spectrin) During Apoptosis. *Journal of Biological Chemistry*, 270:6425-6428.

Mashima,T., Naito,M., Fujita,N., Noguchi,K., and Tsuruo,T. 1995. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-

induced U937 apoptosis. *Biochem.Biophys.Res.Commun.*, 217:1185-1192.

Megraw,T.L., Kao,L.R., and Kaufman,T.C. 2001. Zygotic development without functional mitotic centrosomes. *Current Biology*, 11:116-120.

Mihara,M., Erster,S., Zaika,A., Petrenko,O., Chittenden,T., Pancoska,P., and Moll,U.M. 2003. p53 has a direct apoptogenic role at the mitochondria. *Molecular Cell*, 11:577-590.

Miller,K.D. and Sledge,G.W. 1999. Taxanes in the treatment of breast cancer: A prodigy comes of age. *Cancer Investigation*, 17:121-136.

Miyashita,M.U.T., Shikama,Y., Tadokoro,K., and Yamada,M. 2001. Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. *Febs Letters*, 509:135-141.

Miyashita,T. and Reed,J.C. 1995. Tumor-Suppressor P53 Is A Direct Transcriptional Activator of the Human Bax Gene. *Cell*, 80:293-299.

Mogensen,M.M., Malik,A., Piel,M., Bouckson-Castaing,V., and Bornens,M. 2000. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. *Journal of Cell Science*, 113:3013-3023.

Moll,U.M., Ostermeyer,A.G., Haladay,R., Winkfield,B., Frazier,M., and Zambetti,G. 1996. Cytoplasmic sequestration of wild-type p53 protein impairs the G(1) checkpoint after DNA damage. *Molecular and Cellular Biology*, 16:1126-1137.

- Moll,U.M., Wolff,S., Speidel,D., and Deppert,W. 2005. Transcription-independent pro-apoptotic functions of p53. *Curr.Opin.Cell Biol.*, 17:631-636.
- Mollinedo,F. and Gajate,C. 2003. Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis*, 8:413-450.
- Moritz,M., Braunfeld,M.B., Sedat,J.W., Alberts,B., and Agard,D.A. 1995. Microtubule Nucleation by Gamma-Tubulin-Containing Rings in the Centrosome. *Nature*, 378:638-640.
- Muchmore,S.W., Sattler,M., Liang,H., Meadows,R.P., Harlan,J.E., Yoon,H.S., Nettesheim,D., Chang,B.S., Thompson,C.B., Wong,S.L., Ng,S.C., and Fesik,S.W. 1996. X-ray and NMR structure of human Bcl-x(L), an inhibitor of programmed cell death. *Nature*, 381:335-341.
- Murata,T., Sonobe,S., Baskin,T.I., Hyodo,S., Hasezawa,S., Nagata,T., Horio,T., and Hasebe,M. 2005. Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. *Nature Cell Biology*, 7:961-U52.
- Nakano,K. and Vousden,K.H. 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Molecular Cell*, 7:683-694.
- O'Connor,L., Strasser,A., O'Reilly,L.A., Hausmann,G., Adams,J.M., Cory,S., and Huang,D.C.S. 1998. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *Embo Journal*, 17:384-395.
- Oakley,C.E. and Oakley,B.R. 1989. Identification of gamma-tubulin, a new member of the tubulin superfamily encoded by mipA gene of *Aspergillus nidulans*. *Nature*, 338:662-664.

Ochi,T. 2000. Induction of centrosome injury, multipolar spindles and multipolar division in cultured V79 cells exposed to dimethylarsinic acid: role for microtubules in centrosome dynamics. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, 454:21-33.

Ochi,T., Nakajima,F., Shimizu,A., and Harada,M. 1999. Induction of multinucleated cells in V79 Chinese hamster cells exposed to dimethylarsinic acid, a methylated derivative of inorganic arsenics: Mechanism associated with the formation of aberrant mitotic spindles. *Toxicology in Vitro*, 13:11-25.

Oda,E., Ohki,R., Murasawa,H., Nemoto,J., Shibue,T., Yamashita,T., Tokino,T., Taniguchi,T., and Tanaka,N. 2000. Nora, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, 288:1053-1058.

Oegema,K., Wiese,C., Martin,O.C., Milligan,R.A., Iwamatsu,A., Mitchison,T.J., and Zheng,Y.X. 1999. Characterization of two related *Drosophila* gamma-tubulin complexes that differ in their ability to nucleate microtubdes. *Journal of Cell Biology*, 144:721-733.

Ohta,T., Kinoshita,T., Naito,M., Nozaki,T., Masutani,M., Tsuruo,T., and Miyajima,A. 1997. Requirement of the caspase-3/CPP32 protease cascade for apoptotic death following cytokine deprivation in hematopoietic cells. *Journal of Biological Chemistry*, 272:23111-23116.

Okuno,S., Shimizu,S., Ito,T., Nomura,M., Hamada,E., Tsujimoto,Y., and Matsuda,H. 1998. Bcl-2 prevents caspase-independent cell death. *Journal of Biological Chemistry*, 273:34272-34277.

Oltvai,Z.N. and Korsmeyer,S.J. 1994. Checkpoints of Dueling Dimers Foil Death Wishes. *Cell*, 79:189-192.

Oltvai,Z.N., Milliman,C.L., and Korsmeyer,S.J. 1993. Bcl-2 Heterodimerizes In-Vivo with A Conserved Homolog, Bax, That Accelerates Programmed Cell-Death. *Cell*, 74:609-619.

Orth,K., Chinnaiyan,A.M., Garg,M., Froelich,C.J., and Dixit,V.M. 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *Journal of Biological Chemistry*, 271:16443-16446.

Osborn,M. and Weber,K. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc.Natl.Acad.Sci.U.S.A*, 73:867-871.

Paoletti,A., Moudjou,M., Paintrand,M., Salisbury,J.L., and Bornens,M. 1996. Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J.Cell Sci.*, 109 (Pt 13):3089-3102.

Pasquier,E., Honore,S., and Braguer,D. 2006. Microtubule-targeting agents in angiogenesis: Where do we stand? *Drug Resistance Updates*, 9:74-86.

Patocka,J. and Strunecka,A. 1999. The most important microtubule natural inhibitors. *Acta Medica (Hradec.Kralove)*, 42:3-8.

Petros,A.M., Gunasekera,A., Xu,N., Olejniczak,E.T., and Fesik,S.W. 2004. Defining the p53 DNA-binding domain BcL-(XL)-binding interface using NMR. *Febs Letters*, 559:171-174.

Pettit,G.R., Singh,S.B., Boyd,M.R., Hamel,E., Pettit,R.K., Schmidt,J.M., and Hogan,F. 1995. Antineoplastic Agents .291.

Isolation and Synthesis of Combretastatins A-4, A-5, and A-6 (Vol 38, Pg 1666, 1995). *Journal of Medicinal Chemistry*, 38:2994.

Pihan,G.A., Purohit,A., Wallace,J., Knecht,H., Woda,B., Quesenberry,P., and Doxsey,S.J. 1998. Centrosome defects and genetic instability in malignant tumors. *Cancer Research*, 58:3974-3985.

Pourroy,B., Carre,M., Honore,S., Bourgarel-Rey,V., Kruczynski,A., Briand,C., and Braguer,D. 2004. Low concentrations of vinflunine induce apoptosis in human SK-N-SH neuroblastoma cells through a postmitotic G(1) arrest and a mitochondrial pathway. *Molecular Pharmacology*, 66:580-591.

Puthalakath,H., Huang,D.C., O'Reilly,L.A., King,S.M., and Strasser,A. 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol.Cell*, 3:287-296.

Puthalakath,H. and Strasser,A. 2002. Keeping killers on a tight leash: transcriptional and posttranslational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death and Differentiation*, 9:505-512.

Ravagnan,L., Roumier,T., and Kroemer,G. 2002. Mitochondria, the killer organelles and their weapons. *Journal of Cellular Physiology*, 192:131-137.

Rogers,S.L. and Gelfand,V.I. 2000. Membrane trafficking, organelle transport, and the cytoskeleton. *Current Opinion in Cell Biology*, 12:57-62.

Roninson,I.B., Broude,E.V., and Chang,B.D. 2001. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist.Updat.*, 4:303-313.

Rowinsky,E.K. and Donehower,R.C. 1995. Drug-Therapy - Paclitaxel (Taxol). *New England Journal of Medicine*, 332:1004-1014.

Sakahira,H., Enari,M., and Nagata,S. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, 391:96-99.

Salisbury,J.L. 1995. Centrin, Centrosomes, and Mitotic Spindle Poles. *Current Opinion in Cell Biology*, 7:39-45.

Sansome,C., Zaika,A., Marchenko,N.D., and Moll,U.M. 2001. Hypoxia death stimulus induces translocation of p53 protein to mitochondria - Detection by immunofluorescence on whole cells. *Febs Letters*, 488:110-115.

Sarin,A., Williams,M.S., AlexanderMiller,M.A., Berzofsky,J.A., Zacharchuk,C.M., and Henkart,P.A. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity*, 6:209-215.

Sato,C., Kuriyama,R., and Nishizawa,K. 1983. Microtubule-organizing centers abnormal in number, structure, and nucleating activity in x-irradiated mammalian cells. *J.Cell Biol.*, 96:776-782.

Sato,N., Mizumoto,K., Nakamura,M., Ueno,H., Minamishima,Y.A., Farber,J.L., and Tanaka,M. 2000. A possible role for centrosome overduplication in radiation-induced cell death. *Oncogene*, 19:5281-5290.

Sattler,M., Liang,H., Nettesheim,D., Meadows,R.P., Harlan,J.E., Eberstadt,M., Yoon,H.S., Shuker,S.B., Chang,B.S., Minn,A.J., Thompson,C.B., and Fesik,S.W. 1997. Structure of Bcl-x(L)-Bak peptide complex: Recognition between regulators of apoptosis. *Science*, 275:983-986.

Sawa,H., Kobayashi,T., Mukai,K., Zhang,W., and Shiku,H. 2000. Bax overexpression enhances cytochrome c release from mitochondria and sensitizes KATOIII gastric cancer cells to chemotherapeutic agent-induced apoptosis. *International Journal of Oncology*, 16:745-749.

Sax,J.K., Fei,P.W., Murphy,M.E., Bernhard,E., Korsmeyer,S.J., and El Deiry,W.S. 2002. BID regulation by p53 contributes to chemosensitivity. *Nature Cell Biology*, 4:842-849.

Schinzel,A., Kaufmann,T., and Borner,C. 2004. Bcl-2 family members: intracellular targeting, membrane-insertion, and changes in subcellular localization. *Biochimica et Biophysica Acta-Molecular Cell Research*, 1644:95-105.

Schmidt,M. and Bastians,H. 2007. Mitotic drug targets and the development of novel anti-mitotic anticancer drugs. *Drug Resistance Updates*, 10:162-181.

Schuler,M. and Green,D.R. 2001. Mechanisms of p53-dependent apoptosis. *Biochem.Soc.Trans.*, 29:684-688.

Sedlak,T.W., Oltvai,Z.N., Yang,E., Wang,K., Boise,L.H., Thompson,C.B., and Korsmeyer,S.J. 1995. Multiple Bcl-2 Family Members Demonstrate Selective Dimerizations with Bax. *Proceedings of the National Academy of Sciences of the United States of America*, 92:7834-7838.

Shibata,S., Kyuwa,S., Lee,S.K., Toyoda,Y., and Goto,N. 1994. Apoptosis Induced in Mouse Hepatitis Virus-Infected Cells by A Virus-Specific Cd8(+) Cytotoxic T-Lymphocyte Clone. *Journal of Virology*, 68:7540-7545.

Simonian,P.L., Grillot,D.A.M., Merino,R., and Nunez,G. 1996. Pax can antagonize Bcl-X(L) during etoposide and cisplatin-induced cell death independently of its heterodimerization with Bcl-X(L). *Journal of Biological Chemistry*, 271:22764-22772.

Strasser,A., O'Connor,L., and Dixit,V.M. 2000a. Apoptosis signaling. *Annual Review of Biochemistry*, 69:217-245.

Strasser,A., Puthalakath,H., Bouillet,P., Huang,D.C., O'Connor,L., O'Reilly,L.A., Cullen,L., Cory,S., and Adams,J.M. 2000b. The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. *Ann.N.Y.Acad.Sci.*, 917:541-548.

Sumantran,V.N., Ealovega,M.W., Nunez,G., Clarke,M.F., and Wicha,M.S. 1995. Overexpression of Bcl-X(S), Sensitizes Mcf-7 Cells to Chemotherapy-Induced Apoptosis. *Cancer Research*, 55:2507-2510.

Sunters,A., de Mattos,S.F., Stahl,M., Brosens,J.J., Zoumpoulidou,G., Saunders,C.A., Coffey,P.J., Medema,R.H., Coombes,R.C., and Lam,E.W.F. 2003. FoxO3a transcriptional regulation of bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *Journal of Biological Chemistry*, 278:49795-49805.

Sunters,A., Madureira,P.A., Pomeranz,K.M., Aubert,M., Brosens,J.J., Cook,S.J., Burgering,B.M.T., Coombes,R.C., and Lam,E.W.F. 2006. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-jun NH2-Terminal kinase and Akt. *Cancer Research*, 66:212-220.

Swanson,P.E., Carrol,S.B., Zhang,X.F., and Mackey,M.A. 1995. Spontaneous Premature Chromosome Condensation, Micronucleus Formation, and Non-Apoptotic Cell-Death in Heated Hela S3 Cells - Ultrastructural Observations. *American Journal of Pathology*, 146:963-971.

Tait,S.W.G. and Green,D.R. 2008. Caspase-independent cell death: leaving the set without the final cut. *Oncogene*, 27:6452-6461.

Takemura,R., Okabe,S., Umeyama,T., Kanai,Y., Cowan,N.J., and Hirokawa,N. 1992. Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. *J.Cell Sci.*, 103 (Pt 4):953-964.

Tan,T.T., Degenhardt,K., Nelson,D.A., Beaudoin,B., Nieves-Neira,W., Bouillet,P., Villunger,A., Adams,J.M., and White,E. 2005. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell*, 7:227-238.

Taylor,R.C., Cullen,S.P., and Martin,S.J. 2008. Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology*, 9:231-241.

Tozer,G.M., Kanthou,C., Parkins,C.S., and Hill,S.A. 2002. The biology of the combretastatins as tumour vascular targeting agents. *International Journal of Experimental Pathology*, 83:21-38.

Tudor,G., Aguilera,A., Halverson,D.O., Laing,N.D., and Sausville,E.A. 2000. Susceptibility to drug-induced apoptosis correlates with differential modulation of Bad, Bcl-2 and Bcl-x(L) protein levels. *Cell Death and Differentiation*, 7:574-586.

Uppuluri,S., Knippling,L., Sackett,D.L., and Wolff,J. 1993. Localization of the Colchicine-Binding Site of Tubulin. *Proceedings*

of the National Academy of Sciences of the United States of America, 90:11598-11602.

Urbich,C., Knau,A., Fichtlscherer,S., Walter,D.H., Bruhl,T., Potente,M., Hofmann,W.K., de Vos,S., Zeiher,A.M., and Dimmeler,S. 2005. FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells. *Faseb Journal*, 19:974-+.

Vaux,D.L., Cory,S., and Adams,J.M. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, 335:440-442.

Vermeulen,K., Van Bockstaele,D.R., and Berneman,Z.N. 2005. Apoptosis: mechanisms and relevance in cancer. *Annals of Hematology*, 84:627-639.

Vitale,I., Antoccia,A., Cenciarelli,C., Crateri,P., Meschini,S., Arancia,G., Pisano,C., and Tanzarella,C. 2007. Combretastatin CA-4 and combretastatin derivative induce mitotic catastrophe dependent on spindle checkpoint and caspase-3 activation in non-small cell lung cancer cells. *Apoptosis*, 12:155-166.

Vogelstein,B., Lane,D., and Levine,A.J. 2000. Surfing the p53 network. *Nature*, 408:307-310.

Walensky,L.D. 2006. BCL-2 in the crosshairs: tipping the balance of life and death. *Cell Death and Differentiation*, 13:1339-1350.

Wang,K., Yin,X.M., Chao,D.T., Milliman,C.L., and Korsmeyer,S.J. 1996. BID: A novel BH3 domain-only death agonist. *Genes & Development*, 10:2859-2869.

WatermanStorer,C.M. and Salmon,E.D. 1997. Microtubule dynamics: Treadmilling comes around again. *Current Biology*, 7:R369-R372.

Wilkinson,J.C., Cepero,E., Boise,L.H., and Duckett,C.S. 2004. Upstream regulatory role for XIAP in receptor-mediated apoptosis. *Molecular and Cellular Biology*, 24:7003-7014.

Willis,S.N. and Adams,J.M. 2005. Life in the balance: how BH3-only proteins induce apoptosis. *Current Opinion in Cell Biology*, 17:617-625.

Wu,K.D., Cho,Y.S., Katz,J., Ponomarev,V., Chen-Kiang,S., Danishefsky,S.J., and Moore,M.A.S. 2005. Investigation of antitumor effects of synthetic epothilone analogs in human myeloma models in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 102:10640-10645.

Wyllie,A.H., Morris,R.G., Smith,A.L., and Dunlop,D. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J.Pathol.*, 142:67-77.

Yamaguchi,H., Chen,J., Bhalla,K., and Wang,H.G. 2004. Regulation of Bax activation and apoptotic response to microtubule-damaging agents by p53 transcription-dependent and -independent pathways. *J.Biol.Chem.*, 279:39431-39437.

Yang,J.Y., Zong,C.S., Xia,W.Y., Yamaguchi,H., Ding,Q.Q., Xie,X.M., Lang,J.Y., Lai,C.C., Chang,C.J., Huang,W.C., Huang,H., Kuo,H.P., Lee,D.F., Li,L.Y., Lien,H.C., Cheng,X.Y., Chang,K.J., Hsiao,C.D., Tsai,F.J., Tsai,C.H., Sahin,A.A., Muller,W.J., Mills,G.B., Yu,D.H., Hortobagyi,G.N., and Hung,M.C. 2008. ERK

promotes tumorigenesis by inhibiting FOXO3a via MCM2-mediated degradation (vol 10, pg 138, 2008). *Nature Cell Biology*, 10:370.

Yih,L.H., Tseng,Y.Y., Wu,Y.C., and Lee,T.C. 2006. Induction of centrosome amplification during arsenite-induced mitotic arrest in CGL-2 cells. *Cancer Research*, 66:2098-2106.

Yin,X.M., Oltvai,Z.N., and Korsmeyer,S.J. 1994. Bh1 and Bh2 Domains of Bcl-2 Are Required for Inhibition of Apoptosis and Heterodimerization with Bax. *Nature*, 369:321-323.

Yu,J., Zhang,L., Hwang,P.M., Kinzler,K.W., and Vogelstein,B. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular Cell*, 7:673-682.

Yuan,J. 1996. Evolutionary conservation of a genetic pathway of programmed cell death. *J.Cell Biochem.*, 60:4-11.

Zamzami,N., Susin,S.A., Marchetti,P., Hirsch,T., GomezMonterrey,I., Castedo,M., and Kroemer,G. 1996. Mitochondrial control of nuclear apoptosis. *Journal of Experimental Medicine*, 183:1533-1544.

Zha,H.B., AimeSempe,C., Sato,T., and Reed,J.C. 1996. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BN3) distinct from BH1 and BH2. *Journal of Biological Chemistry*, 271:7440-7444.

Zheng,Y.X., Jung,M.K., and Oakley,B.R. 1991. Gamma-Tubulin Is Present in *Drosophila-Melanogaster* and *Homo-Sapiens* and Is Associated with the Centrosome. *Cell*, 65:817-823.

Zhu,Y.N., Swanson,B.J., Wang,M., Hildeman,D.A., Schaefer,B.C., Liu,X.Q., Suzuki,H., Mihara,K., Kappler,J., and Marrack,P. 2004.

Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101:7681-7686.

Zimmerman,W.C., Sillibourne,J., Rosa,J., and Doxsey,S.J. 2004. Mitosis-specific anchoring of gamma tubulin complexes by pericentrin controls spindle organization and mitotic entry. *Molecular Biology of the Cell*, 15:3642-3657.

Materials and methods

Cell cultures and treatments

The human non-small-cell lung cancer (NSCLC) cell line H460 was maintained in RPMI or DMEM with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 5,000 UI/ml penicillin and 5 mg/ml streptomycin (all from Gibco, Paisly, UK). Cells were grown in a 5% CO₂ atmosphere at 37°C. Forty-eight hours before treatment 200,000 cells were seeded in 35 mm Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) containing sterile glass coverslips, whereas for time-lapse experiments cells were seeded in appropriate μ Slide I chamber slides (Ibidi, Germany). Combretastatin A-4 (CA-4) (Sigma–Aldrich, St. Louis, MO, USA), taxol and staurosporine (Sigma–Aldrich, St. Louis, MO, USA), were freshly dissolved in DMSO (Dimethyl Sulfoxide) prior to each experiment at 100-fold concentration. Cells were treated for 8, 24 and 48 h with drugs, whereas control cultures received 1% (v/v) DMSO.

Mitotic index

Cells were fixed in 3:1 (v/v) absolute methanol:acetic acid for 30 min and stained with 5% (v/v) GIEMSA. The mitotic index was calculated by scoring mitotic figures in a total of 1,000 cells per experimental point in repeated experiments.

Immunofluorescence microscopy

Cells were fixed in absolute methanol for 10 min and in acetone for 20 s at -20°C. After washing with PBS, cells were incubated for 1 h at 37°C with 1:50 mouse anti- α -tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA),

1:10 mouse anti- α -acetylated tubulin antibody, non-diluted mouse anti- γ -tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA), 1:250 rabbit anti-ninein antibody (Abcam, Cambridge, UK) or 1:500 rabbit anti-pericentrine antibody (Novus Biologicals, Littleton, CO) in 2% (w/v) BSA/PBS. As an alternative, cells were permeabilized in 0.1% (v/v) Triton-X-100 in PBS, and incubated for 1 h at 37°C with 1:1000 anti-centrine rabbit antibody (kindly provided by Dr. Bornens) and 1:1 mouse anti- γ -tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA) in 3% (w/v) BSA/ PBS. After primary antibody incubation, slides were rinsed in 1% (w/v) BSA/PBS and incubated for 1 h at 37°C with 1:30 FITC-conjugated secondary anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) FITC-conjugated secondary anti-rabbit antibody (Molecular Probes, Oregon, USA) or with 1:100 Texas Red-conjugated secondary anti-mouse antibody (Amersham Biosciences, UK) in 2% (w/v) BSA/PBS. DNA was counterstained for 10 min with 0.2 μ g/ml 40,6-diamidine-2-phenylindole (DAPI, Sigma–Aldrich, St. Louis, MO, USA) and slides mounted with an anti-fade solution (Vectashield; Vector Laboratories, CA, USA). Slides were analyzed by means of an Axiophot fluorescent microscope (Zeiss, Germany) equipped with a charged cooled device (CCD) camera. Image re-elaborated with Adobe Photoshop 7.0 software (Adobe Systems Inc. Mountain View, CA, USA). Experiments were repeated at least three times.

Transmission electron microscopy and confocal microscopy analyses

Methanol-fixed cells were permeabilized in 0.01% (v/v) Tween in PBS, and incubated for 1 h at 37°C with 1:50 mouse anti- α -tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA); 1:50 rabbit anti- α -tubulin antibody (Immunological sciences); 1:1 mouse anti- γ -tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA); 1:1 CREST anti kinetochore antibody (Sigma–Aldrich, St. Louis, MO, USA) in 0.1% Tween/PBS. In some experiments, cells were first incubated for 1 h at 37°C with 1 mM

Mitotracker green Fm (Molecular Probes, Oregon, USA) in culture medium. Then slides were rinsed in 1% (w/v) BSA/PBS and incubated for 1 h at RT with 1:30– 1:100 FITC or Texas Red-conjugated secondary antibodies. Confocal microscopy analysis was performed as previously described (Vitale et al, 2007;Cenciarelli et al, 2008). Briefly, the excitation wavelengths used were 488 and 543 nm; emission lines were collected after passage through a DD488/543 filter in a spectral window ranging from 515 to 700 nm. Signals from different fluorescent probes were taken in sequential scan mode, allowing elimination of channel crosstalk. Co-localization was detected in an overlay model. Images were processed by using LCS SP5 (Leica Microsystems, Germany). For transmission electron microscopy assessment, treated and untreated cells, grown to near confluence, were fixed and stained as previously described (Vitale et al, 2007;Cenciarelli et al, 2008). Ultrathin sections were examined with a Philips 208S electron microscope.

Protein extraction, co-immunoprecipitation and immunoblotting

Mitochondrial cell lysates have been prepared by using the Mitochondria Isolation Kit (Pierce, USA), according to manufacturer's instructions and whole cell lysates extract and Western Blot analyses were performed as described previously (Vitale et al. 2007). Briefly, forty µg of proteins were subjected electrophoresis, and blotted to PVDF; then the filters were incubated with 1 µg/ml of specific antibodies recognizing Bim (Cell Signaling), α-tubulin (Santa Cruz Biotechnology), CoxIV (Abcam), Dynein (Santa Cruz Biotechnology). Primary antibodies were detected using secondary anti-mouse or anti-rabbit antibodies HRP conjugates (1:2000)(Amersham Biosciences) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences). For immunoprecipitation, cellular lysates were incubated with Dynein or Bim polyclonal antibodies or Bcl2 monoclonal antibody (Cell Signaling) and

15µl of Protein A agarose Beads (Invitrogen) at 4°C for 4h. Immunocomplexes were washed three times with lysis buffer and examined by immunoblotting analysis.

Apoptosis assays

Several assays to assess apoptosis were performed at both single cell level and on whole cellular population. Timelapse experiments were performed by using the Magic Red_ (Immunochemistry Technologies, MN, USA) substrate according to the producer's protocol. This substrate easily penetrates the cell membrane, entering in a nonfluorescent state. If caspases 3–7 are present in an activated form, the substrate is cleaved off yielding a red fluorescent product. Cells were maintained in a 5% CO₂ atmosphere at 37°C by means of a microscope cage incubator (Pecon, Germany). Live cell imaging were carried out with a Leica TCS SP5 confocal microscopy. The fluorogenic assay for Caspases-3 and -9 were performed as described (Vitale et al. 2006) using Ac-Asp-Glu- Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and Ac-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac- LEHD-AMC) as substrates (all from Alexis Biochemicals, San Diego, CA, USA). Cleavage of the substrates was assessed by using an LS-50B fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, USA). Apoptosis induction was also analyzed by means of the in situ Fluorescent-FragELTM DNA fragmentation kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions by using a Galaxy flow cytometer (Dako, Glostrup, Denmark). For each sample, 10,000 events were collected. All experiments were repeated twice.

Time Lapse analysis using Magic Red

Timelapse experiments were performed both on siRNA Bim trasfected cells and control cells, by using the MagicRed™ (Immunochemistry Technologies) substrate according to the producer's protocol adding 1X MagicRed™ to cell cultures before CA-4 treatment. This substrate easily penetrates the cell membrane, entering in a nonfluorescent state. If caspases 3–7 are present in an activated form, the substrate is cleaved off yielding a red fluorescent product emitting at 610nm. During the experiments, cells were maintained in a 5% CO₂ atmosphere at 37°C by means of a microscope cage incubator (Pecan). Live cell imaging were carried out with a Leica TCS SP5 confocal microscopy. Images were collected every 15 minutes and the experiments repeated two times.

siRNA transfection

The Bim small interfering RNA (siRNA) and the non-targeting negative controls were purchased from Dharmacon Research Inc.. For cell trasfection with siRNA, H460 cells were placed on six-well plates 24h prior to trasfection and grown in RPMI 1640 medium with 10% fetal bovine serum. Then 1µl of 20µM siRNA was added to a tube containing 189µl of RPMI serum-free medium and 10µl of Interferin (Polyplus-transfection Inc.). They were incubated at room temperature for 10 min, and added to each well that contained 2ml of RPMI medium. After 4h, 2 ml of fresh complete medium was added. 24h after trasfection cells were treated with CA-4 7,5nM for 48, 24, and 8h. Both the suspension and the adherent cells were collected for Western Blot and gene knockdown analysis. Experiments were repeated twice.

Real-time Quantitative RT-PCR

Total RNA was isolated by using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. Equal amounts of total RNA (1µg) was reverse transcribed by using the MMLV reverse transcriptase (Invitrogen), and the resulting first strand cDNA was diluted and used as template in the real-time quantitative-PCR analysis. All measurements were performed in triplicate. The mRNAs analyzed were Bim and GAPDH, which served as internal control and was used to normalize for variances in input CDNA. The following gene-specific primer pairs were designed using Autoprime software (<http://www.autoprime.de/AutoPrimeWeb>, © Gunnar Wrobel & Felix Kokocinski, DKFZ, 2003 - 2007): Bim EL-sense (5'-TCTCAGTGCAATGGCTTC-3') and Bim EL-antisense (5'-GCGTTAACTCGTCTCCAATAC-3'); GAPDH-sense (5'-CCATGGAGAAGGCTGGGG-3') and GAPDH-antisense (5'-CAAAGTTGTCATGGATGACC-3'). Real-Time PCR was performed using a Rotor-Gene 6000 thermal cycling system (Corbett Research, Sidney, Australia) with Quantimix Easy Syg kit (Biotools, Madrid) as the detection system. Detection of Bim expression was calculated using the $2^{-\Delta\Delta CT}$ method. Experiments were repeated three times.

