Caspase activation and apoptosis in response to proteasome inhibitors

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Abstract

Several studies have indicated that proteasome inhibitors (PIs) are promising anticancer agents. We have discovered that PIs have the unique ability to activate effector caspases through a mitochondrial Bcl-2 inhibitable but caspase-9 independent pathway. Stabilization of released Smac induced by blockade of the proteasome could explain the apoptosome-independent cell death induced by PIs. In fact, Smac/DIABLO critically supports this PIs-dependent caspase activation. By using a new assay, we confirm that at a single cell level both Smac and PIs can activate caspases in the absence of the apoptosome. Moreover, we have observed two PIs-induced kinetics of caspase activation, with caspase-9 being still required for the rapid caspase activation in response to mitochondrial depolarization, but dispensable for the slow DEVDase activation. In summary, our data indicate that PIs can activate downstream caspases at least in part through Smac/DIABLO stabilization.

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Abbreviations: PIs, proteasome inhibitors; IAPs, Inhibitor of Apoptosis; TSA, trichostatin A; MEFs, murine embryonic fibroblasts; GFP, green fluorescent protein

Introduction

Most chemotherapeutic molecules trigger tumor eradication and patient survival by inducing apoptotic cell death.¹ As a consequence, chemoresistance and tumor progression often arise when cancer cells accumulate mutations in the critical genes regulating the apoptotic program.²

Caspases play important roles in the regulation of the apoptotic program.³ These proteolytic enzymes, synthesized as inactive zymogens, are divided into initiators and effectors depending on the timing of activation. Initiator caspases are activated earlier following adaptor-mediated oligomerization whereas effector caspases are activated after cleavage mediated by initiator caspases.^{4,5} Once activated effector caspases process selected death substrates and other caspases in an amplificatory loop that causes cell fragmentation.⁶

The mitochondrial or intrinsic pathway of caspase activation represents the fundamental switch in the apoptotic response triggered by different cellular stresses.⁷ Anti and proapoptotic Bcl-2 family members are the guardians of mitochondrial integrity. They work in an antagonized manner to control outer mitochondrial membrane permeability and the release of mitochondrial proapoptotic factors into the cytosol.^{8,9} Cytochrome *c* was the first identified proapoptotic factor released from the mitochondria into the cytosol, where it stimulates the formation of the apoptosome, an oligomeric complex containing the adaptor protein Apaf-1 and the initiator caspase-9.⁷

Smac/DIABLO and HtrA2/Omi, similar to cytochrome *c*, are released from mitochondria during apoptosis. Their principal function is to relieve the inhibition imposed by inhibitor of apoptosis (IAPs) on caspase-9, -3 and -7. The synergistic action of cytochrome *c*, Smac/DIABLO and HtrA2/Omi leads to full activation of caspases and cell death.^{7,10}

Signals activated by many chemotherapeutic molecules converge on caspase-9 and the apoptosome after triggering mitochondrial permeabilization.¹¹ The central role of the apoptosome in apoptosis induced by different anti-tumor treatments has been clearly established by a large body of data.^{12–17}

To identify molecules that can trigger cell death bypassing the apoptosome dependence, we screened a number of chemical principles, including many chemotherapeutic molecules, for their ability to trigger apoptosis in human cells engineered to express a dominant negative form of caspase-9. Here, we report the identification of proteasome inhibitors (PIs) as the only molecule able to induce efficient caspase activation and cell death even in the absence of a functional apoptosome. We also provide evidence that Smac/DIABLO plays a role in the caspase-9 independent apoptotic pathway activated by PIs.

Results

PIs trigger caspase activation and cell death independently from caspase-9

To identify compounds able to induce efficient caspase activation and apoptosis in tumor cells independently from the apoptosome, we took advantage of IMR90-E1A human cells, engineered to express a dominant-negative form of caspase-9.¹³ IMR90-E1A (E1A) and IMR90-E1A/C9DN (E1A/

C9DN) cells were treated with different chemical principles provided of anti-cancer activity represented in Figure 1a. The list of anticancer compounds includes antitubulin drugs, nucleoside analogs, topoisomerases inhibitors, anthracycline antibiotics and DNA cross-linking agents. We integrated our list with more recently characterized antineoplastic activities such as PIs, histone deacetylase inhibitors and ether lipids, by using, respectively, ALLnL (*N*-Acetyl-Leu-Leu-*N*-Leu-CHO), trichostatin A (TSA) and ET-18-OCH₃ as prototype molecules.

E1A and E1A/C9DN cells were treated with the different compounds and caspase-3 and -7 activities were measured 24–48 h later using a fluorogenic peptide substrate. The ability to trigger caspase activation independently from caspase-9 was represented as the ratio of caspase-3/-7 activity in E1A/C9DN versus E1A cells (Figure 1a). ALLnL was able to trigger caspase activity at similar levels in both cell lines whereas all the other tested drugs showed a certain degree of dependence from caspase-9. The ability of ALLnL to trigger full DEVDase activity in cells lacking a functional caspase-9 was confirmed in four different experiments (Figure 1b). TSA was used as negative control since it has been demonstrated to be a strictly caspase-9 dependent apoptotic stimulus.¹⁸

To demonstrate that the ability of ALLnL to trigger apoptosis and caspase activation in a caspase-9 independent manner was due to proteasome inhibition and not due to the inhibition of calpains and cathepsins,^{19,20} E1A and E1A/C9DN cells were treated with two more specific PIs such as MG-132 (Z-Leu-Leu-Leu-CHO) and PSI (Z-Ile-Gln(OtBu)-Ala-Leu-CHO). We also used the specific calpain inhibitor ALLM (*N*-acetyl-Leu-Leu-Met-CHO) that does not inhibit the proteasome. Etoposide, a typical caspase-9 dependent drug, was used as a control.

All the PIs tested induced cell death at similar levels in both cell lines. The calpain inhibitor was ineffective in inducing cell death whereas etoposide, as expected, was less efficient in triggering apoptosis in E1A/C9DN cells (Figure 1c). Next, we evaluated the proteolytic processing of caspases and of caspase substrates in E1A/C9DN cells treated with PIs compared to etoposide. E1A and E1A/C9DN cells were incubated with ALLnL, MG-132, or etoposide at doses that had been previously tested to trigger comparable apoptosis in E1A cells (Figure 1c). Accordingly, immunoblots showed similar amounts of processed caspase-2 and -8 after treatment with PIs and etoposide in E1A cells. Comparable processing was also observed for the death substrate PARP. In E1A/C9DN cells apoptosis and processing of caspase-2, -8 and PARP were lower after treatment with etoposide, whereas they were similar after treatment with PIs when compared to E1A cells. ALLM was unable to trigger caspase-2, -8 and PARP processing in neither of the cell lines.

A time-course analysis was next performed to evaluate the kinetics of caspase-2, -7, -8 and PARP processing in response to ALLnL (Figure 1e). Cell death, scored by Trypan-blue staining, was induced with similar kinetics in E1A and E1A/C9DN cells, and also cleavage of caspase-2, -7, -8 and PARP showed a similar schedule.

To confirm the ability of the PIs to overcome the apoptosome, we took advantage of MCF-7/C9DN, which are defective for caspase-3 and express the dominant negative

form of caspase-9. Here again ALLnL and lactacystin but not ALLM induced apoptosis and caspase activation at comparable levels in MCF-7/C9DN and in the control MCF-7/NEO cells (data not shown). To further confirm that PIs trigger caspase activation and apoptosis in the absence of caspase-9, Myc-transformed caspase-9^{+/-} murine embryonic fibroblasts (MEFs) and caspase-9^{-/-} MEFs were treated with MG-132 or etoposide as a control. MG-132 induced cell death at similar levels in both cell lines whereas etoposide, as expected triggered apoptosis less efficiently in Myc-expressing caspase-9^{-/-} MEFs (Figure 1f). In conclusion we have demonstrated that PIs overcome the lack of caspase-9 to trigger caspase activation and apoptosis.

Bcl-2 family members control PIs-mediated cytochrome *c* release and apoptosis

To understand the mechanisms responsible for the apoptosome-independent caspase activation induced by PIs, we took advantage of BJ cells transformed with E1A, Ras and expressing Bcl-2. Parental E1A/Ras cells efficiently entered apoptosis following ALLnL treatment, while the expression of Bcl-2 dramatically suppressed this apoptotic response as revealed by the Trypan blue assay (Figure 2a). We also characterized the pattern and timing of caspase activation in E1A/Ras cells compared to E1A/Ras/Bcl-2 cells. Processing of caspase-2, -3, -7, -8 and that of the death substrate PARP was clearly reduced by the presence of Bcl-2 (Figure 2b).

Increasing evidences support the idea that Bcl-2 can regulate apoptosis also independently of the apoptosome.^{21–23} Our data on the different effects of Bcl-2 and C9DN in counteracting apoptosis induced by PIs sustain this hypothesis. To further corroborate our observation, we sought to analyze the PIs-induced apoptotic response of E1A and E1A/C9DN cells stably expressing Bcl-2. The trypan blue assays (Figure 2c) showed that apoptosis induced by ALLnL was efficiently counteracted by Bcl-2 independently from the presence of C9DN. TSA was used as a control of the apoptosome inhibition by C9DN. Processing of caspase-2, -7, -8 and of the death substrate PARP was dramatically reduced in E1A cells expressing Bcl-2 according to the suppression of the apoptotic response (data not shown).

Recent evidences indicate that the ability of Bcl-2 to prevent cell death independently of Apaf-1 or caspase-9 is consistent with its ability to prevent cytochrome *c* release from mitochondria.²¹ Thus, we evaluated the release of cytochrome *c* from mitochondria in E1A, E1A/Bcl-2, E1A/C9DN and E1A/C9DN/Bcl-2 cells by immunofluorescence after treatment for 12 h with ALLnL. As shown in Figure 2d, cytochrome *c* release from mitochondria was reduced in cells expressing Bcl-2. Similar results in terms of apoptotic suppression and cytochrome *c* release were obtained when Bcl-xL, instead of Bcl-2, was ectopically expressed (data not shown).

Genetic evidences indicate that the multidomain proapoptotic proteins Bax and Bak are required for the response to many apoptotic stimuli.²⁴ To unveil the role of these proteins during PIs-dependent apoptosis, immortalized wild-type MEFs and *bax*^{-/-}*bak*^{-/-} MEFs were treated with ALLnL. We found that the absence of Bax and Bak expression

dramatically reduced the apoptotic response to ALLnL compared to wild-type MEFs (Figure 2e). Accordingly, caspase-3/-7 activities and caspase-2 and β -catenin processing were almost completely suppressed in *bax*^{-/-} *bak*^{-/-}

MEFs challenged with ALLnL (Figure 2f and g). Overall our experiments indicate that the Bcl-2 family members control alternative mitochondrial pathways of caspase activation that overcome caspase-9.

Analysis of caspase activation at the mitochondria surface of a single cell *in vivo*

Recent studies indicate that caspase-9 and the apoptosome have an important role in accelerating rather than in dictating cell death.^{21,22} Therefore, it is possible that, even though PIs can trigger caspase activation in the absence of caspase-9, the kinetic of activation may be affected by the absence of the accelerating function of the apoptosome. Therefore, we decided to study in detail the timing of caspase activation in C9DN cells after treatment with PIs.

Since apoptosis is an asynchronous process, the timing of caspase activation needs to be analyzed in single cells. Our results suggest that mitochondria play a critical role during the apoptotic response triggered by PIs. Mitochondrial membrane potential ($\Delta\psi_m$) is often employed as an indicator of cellular viability and its destruction has been implicated in many apoptotic responses.^{25,26} Single-cell imaging studies have shown that $\Delta\psi_m$ decrease is concomitant to the release of cytochrome-*c*²⁷ and that its rapid loss requires caspase activity.²⁸ Therefore, in order to examine the timing of caspase activation during apoptosis induced by PIs it is important to monitor simultaneously $\Delta\psi_m$ and caspase activation possibly close to mitochondria. For this purpose, we have developed a new assay to monitor, simultaneously, $\Delta\psi_m$ and caspase activity on the outer mitochondria membrane (OMM) of single cells.

We generated a set of hybrid proteins containing a green fluorescent protein (GFP), one (D or AD) two (DD) or no (AA) effector caspases consensus cleavage sites and the transmembrane domain of Bcl-xL (TM), which is needed to target the fusion protein to the OMM (Figure 3a). GFP translocates from the OMM to the cytoplasm only after effector caspases cleave the DD sites allowing us to measure caspase activity in a single cell *in vivo* (Figure 3c and d).

Despite the fact that all the different chimeric proteins localize to the mitochondria (Figure 3b), their cleavage efficiency, following apoptosis induced by etoposide, was different depending on the presence of one or two caspase cleavage sites. As shown in Figure 3c, GF-DD-TM was fully processed in the apoptotic population whereas GFP-AD-TM and GFP-D-TM were only partially processed. As expected GFP-AA-TM was not processed. In all the different experiments apoptosis was similarly induced, as confirmed by PARP processing. These cleavages were inhibited by the addition of the caspase inhibitor Boc-D-fmk (Figure 3e) and the expression of these constructs did not affect cell growth or the apoptotic process (Figure 3f).

The GFP-DD-TM and the control GFP-AA-TM chimeras were chosen for the following studies. E1A cells were

microinjected with the two different constructs and exposed to etoposide. Subcellular localization of the two chimeras and mitochondrial depolarization, measured by the decrease in tetramethyl rhodamine methyl-ester (TMRM) uptake,²⁹ were simultaneously analyzed by time-lapse confocal microscopy. GFP was completely released into the cytosol in a short frame of time only in cells expressing GFP-DD-TM. In cells expressing the uncleavable mutant GFP-AA-TM, the GFP was confined to the OMM even during the late phase of the apoptotic process (Figure 3d).

Our results indicate that the generated GFP chimeras can be used to monitor caspase activity in single living cells by time-lapse analysis.

$\Delta\psi_m$ dissipation and caspase activation during etoposide or PIs-induced apoptosis

To investigate the relationships between caspase activation and mitochondrial depolarization in response to etoposide or MG-132 (used as a prototype of PIs for the succeeding analysis, but similar data were obtained with ALLnL), we analyzed the redistribution of GFP into the cytosol by measuring the decrease in the standard deviation (S.D.) from the average pixel intensity. The S.D. was higher in conditions of mitochondrial GFP localization whereas it was lower during diffuse fluorescence (activated effector caspases). $\Delta\psi_m$ dissipation was analyzed by measuring the changes in total TMRM fluorescence of mitochondria by determining the integrated intensity. Since individual cells depolarize and release GFP from mitochondria at different times from the addition of the proapoptotic insult, the beginning of depolarization (TMRM decrease) was set to min zero. Figure 4a shows that TMRM decrease and GFP translocation were heterogeneous in cells treated with etoposide. In general, cells showing a slower depolarization also exhibited delayed caspase activation (compare the green and violet traces in Figure 4a). In contrast, the kinetic of GFP translocation was rapid (6.76 ± 0.57 min, $n = 17$) and comparable in the different cells indicating that, once caspases are activated, the chimeric protein is efficiently and rapidly cleaved in all the cells analyzed.

Compared to etoposide, MG-132 induced faster and more homogeneous $\Delta\psi_m$ dissipation with many cells reaching the bottom level after 60 min (Figure 4b). As for etoposide the kinetics of GFP translocation induced by MG-132 were rapid (9.57 ± 1.31 min, $n = 14$) and similar in the different cells analyzed (Figure 4b).

Of note, in etoposide treated cells the interval between full caspase activity and the beginning of mitochondrial de-

Figure 1 Proteasome inhibitors induce caspase activation and apoptosis in cells expressing a dominant-negative form of caspase-9. (a) E1A and E1A/C9DN cells were treated with the indicated drugs and caspase-3/-7 activities have been measured with Ac-DEVD-AMC (7-amino-4-methyl-coumarin) fluorogenic substrate. Data are reported as the ratio of DEVDase activity between E1A/C9DN and E1A cells. (b) DEVDase activity in E1A and E1A/C9DN cells treated with ALLnL or TSA as indicated (means $\pm$ S.D.; $n = 4$). Results show the average of four independent experiments. (c) E1A and E1A/C9DN cells were treated with the indicated apoptotic stimuli and the appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$). (d) Processing of caspase-2, -8 and PARP in E1A and E1A/C9DN cells treated with PIs, ALLM or etoposide. Equal amounts of cell lysates were subjected to SDS/PAGE electrophoresis. Immunoblots were performed using the indicated antibodies. Tubulin was used as loading control. (e) Processing of caspase-2, -7 -8 and PARP in E1A and E1A/C9DN cells treated with ALLnL for the indicated time. Equal amounts of cell lysates were subjected to SDS/PAGE electrophoresis. Immunoblots were performed using the indicated antibodies. The appearance of apoptosis was scored by Trypan blue staining. (f) MEFs from *caspase-9*^{-/-} or *-9*^{-/-} mice expressing *c-myc* were either treated or not treated for 24 h with etoposide or MG-132. The appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$)

polarization is shorter (10.24 ± 3.34 min, $n = 17$) than in MG-132 treated cells (27.29 ± 3.75 , $n = 14$), $P < 0.05$. Finally, we verified that GFP translocation into the cytosol was a

consequence of caspase activity and not a secondary event caused by OMM rupture. When the time-lapse analysis was performed with the Asp mutant chimera (GFP-AA-TM) we

were unable to observe GFP translocation in the cytosol even after 2 h from the onset of mitochondrial depolarization (Figure 4c).

$\Delta\psi_m$ dissipation and caspase activation during etoposide or PIs-induced apoptosis in cells defective for caspase-9

As for E1A cells, we investigated the timing of caspase activation in relation to $\Delta\psi_m$ dissipation in 63 E1A/C9DN cells after treatment with MG-132 or etoposide. Etoposide-induced $\Delta\psi_m$ loss was much slower in E1A/C9DN cells than in E1A cells. A new steady-state level is reached and maintained through the time ($\Delta\psi_{m-caspase-9}$) as shown previously in z-VAD-fmk-treated cells.^{30–32} This new steady-state level was about 40–60% of the initial $\Delta\psi_m$ and it was detectable even after 240 min from the onset of depolarization (Figure 4d). In etoposide-treated E1A/C9DN cells, GFP remained on the OMM suggesting that caspase activity was dramatically reduced (Figure 4d). When the same analysis was performed with MG-132, we observed that the dissipation of $\Delta\psi_m$ in E1A/C9DN cells was almost indistinguishable from the one observed in MG-132 treated E1A cells (compare Figure 4b and e). As expected, GFP was efficiently translocated to the cytoplasm confirming the ability of PIs to sustain caspase activation in the absence of a functional apoptosome. However, the time-lapse analysis demonstrates that the time span between the onset of depolarization and full caspase activation was delayed by approximately 120 min in MG-132-treated E1A/C9DN cells (146 ± 16 min, $n = 16$) compared to E1A cells (27.29 ± 3.75 min, $n = 14$) $P < 0.05$.

In summary, PIs can trigger effector caspases activation in the absence of caspase-9, but an increased period of time is required to observe full caspase activation from the onset of $\Delta\psi_m$ decrease.

Biologically active cytosolic Smac/DIABLO can synergize with etoposide to induce efficient apoptosis and caspase activation in cells defective for caspase-9

Some reports suggest that the levels of cytosolic-released Smac/DIABLO, which acts by relieving the inhibitory action of IAPs on caspases,¹⁰ could be controlled by the proteasome.^{33,34} The IAPs can promote Smac/DIABLO ubiquitination and degradation *in vitro*.^{33–38} To shed light on the role of Smac/DIABLO in the apoptosome-independent activation

of the effector caspases, we first examined whether we could restore etoposide sensitivity, in terms of efficient caspase activation and apoptosis, in E1A/C9DN cells by increasing the levels of cytosolic Smac/DIABLO. We took advantage of the ubiquitin-Smac/DIABLO fusion system³⁹ to obtain the mature Smac/DIABLO protein in the cytoplasm beginning with the AVPI sequence ($\Delta 53$). We also used the deletion $\Delta 57$, initiating with the sequence AQKS, which is still able to bind the IAPs and to induce some caspase activation, albeit with lower efficiency compared to $\Delta 53$.^{40–43} Furthermore, we generated a larger deletion ($\Delta 71$), which initiates with the MRRA sequence (Figure 5a) and fails to bind XIAP and to trigger caspase activation.⁴⁰

The different Smac/DIABLO deletion mutants were co-transfected together with GFP in E1A and E1A/C9DN cells; apoptosis was induced by etoposide and it was scored *in vivo* by monitoring the appearance of membrane blebbing in green cells. While overexpression of the different deletion mutants did not significantly modify the apoptotic response in E1A cells (Figure 5b), Smac/DIABLO $\Delta 53$ and $\Delta 57$ significantly lowered the apoptotic threshold of etoposide-treated E1A/C9DN cells, whereas the $\Delta 71$ was ineffective (Figure 5c).

The role of Smac/DIABLO in caspase-9 independent apoptosis was further supported by the increased caspase-3 cleavage/activation observed in E1A/C9DN cells expressing Smac/DIABLO $\Delta 53$ after treatment with etoposide. In this assay, E1A/C9DN cells were transiently cotransfected with caspase-3 containing the GFP at the carboxy terminus and with Smac/DIABLO $\Delta 53$ or $\Delta 71$. β -Gal was used as control for transfection efficiency. A low level of caspase-3 activation was observed in E1A/C9DN cells after overexpression of Smac/DIABLO $\Delta 53$, but it was greatly increased in the presence of etoposide (Figure 5d). Caspase-3 activation can be scored by comparing the rate between the amount of pro-caspase-3-GFP and the amount of the cleaved form (small subunit of caspase-3 fused to GFP). In cells expressing Smac/DIABLO $\Delta 71$ and treated with etoposide only a faint increase in caspase-3 processing can be observed (Figure 5d). MG-132 was able to induce caspase-3 activation even when Smac/DIABLO $\Delta 71$ was expressed and here again the rate of caspase-3 processing was further increased by the presence of Smac/DIABLO $\Delta 53$. Therefore, high levels of biologically active cytosolic Smac/DIABLO can sensitize apoptosome-deficient cells to drug-induced apoptosis bypassing the requirement of active caspase-9.

XIAP can regulate Smac/DIABLO protein levels by acting as E3 ubiquitin ligase.^{33,36,34,38} Ectopic expression of XIAP should efficiently counteract apoptosis induced by PIs. XIAP was able to counteract apoptosis (Figure 5e) and caspase-3

Figure 2 Bcl-2 family members control PIs-mediated cytochrome c release and apoptosis. (a) BJ/E1A/Ras and BJ/E1A/Ras/Bcl-2 cells were treated with ALLnL for the indicated time and the appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$). (b) Processing of caspase-2, -3, -7, 8 and PARP in BJ/E1A/Ras and BJ/E1A/Ras/Bcl-2 cells treated with ALLnL for the indicated time. Equal amounts of cell lysates were subjected to SDS/PAGE electrophoresis. Immunoblots were performed using the indicated antibodies. (c) E1A and E1A/C9DN cells either expressing or not expressing Bcl-2 were treated for the indicated times with ALLnL or TSA as indicated. The appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$). (d) E1A and E1A/C9DN cells either expressing or not expressing Bcl-2 were treated for the indicated times with ALLnL. After fixation, cells were scored for the accumulation of cytochrome c into the cytosol by immunofluorescence. (e) MEFs from *wt* or *bak^{-/-} bax^{-/-}* mice were treated for the indicated time with ALLnL or for 24 h with etoposide. The appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$). (f) DEVDase activity in MEFs from *wt* or *bak^{-/-} bax^{-/-}* mice treated for the indicated time with ALLnL or etoposide (means $\pm$ S.D.; $n = 4$). (g) Processing of caspase-2 and β -catenin in MEFs from *wt* or *bak^{-/-} bax^{-/-}* mice treated with ALLnL or etoposide for the indicated time. Equal amounts of cell lysates were subjected to SDS/PAGE electrophoresis. Immunoblots were performed using the indicated antibodies

Figure 3 Analysis of caspase activity at a single cell level. **(a)** Schematic representation of the different GFP chimeras generated. **(b)** Subcellular localization of the GFP-DD-TM protein in E1A cells. The immunofluorescence assay was performed using the anti-cytochrome *c* antibody 20 h after transfection with the GFP-DD-TM plasmid. **(c)** E1A cells were transfected with the indicated constructs and either treated or not treated with 50 μ M etoposide for 18 h as indicated. NA (nonapoptotic) and A (apoptotic) cells were separated and immunoblots were performed using the indicated antibodies. **(d)** Time-lapse images of E1A cells expressing GFP-DD-TM or GFP-AA-TM incubated with etoposide and TMRM. Frames at selected times after the onset of $\Delta\psi_m$ depolarization of a representative cell expressing GFP-DD-TM or GFP-AA-TM. **(e)** E1A cells were transfected with the indicated constructs and either treated or not treated with 50 μ M etoposide for 18 h, in the presence or not of caspase inhibitor Boc-D-fmk (125 μ M). Cellular lysates were prepared and immunoblots were performed using the indicated antibodies. **(f)** Flow cytometric analysis of the cell cycle profile by propidium iodide staining of U2OS cells stably expressing GFP or GFP-DD-TM treated with 50 μ M etoposide for the indicated time

cleavage (Figure 5f) induced by the proteasome inhibitor MG-132 with high efficiency in E1A/C9DN and in E1A cells, reinforcing the idea that the IAP antagonist Smac/DIABLO could play an important role in mediating caspase activation in the absence of a functional apoptosome.

Time-lapse analysis with the GFP-DD-TM protein confirmed the ability of Smac/DIABLO to sensitize E1A/C9DN cells to etoposide-induced apoptosis. GFP translocation into the cytosol and complete $\Delta\psi_m$ dissipation can be observed in etoposide-treated E1A/C9DN cells when the levels of

cytosolic Smac/DIABLO $\Delta 53$ were ectopically increased. Figure 5g shows eight representative cells out of 30 analyzed cells (compare with Figure 4d). Both the timing of caspase activation and the kinetics of depolarization were heterogeneous. Some cells activated caspases after a prolonged time from the onset of depolarization (192.7 ± 10.4 min)

probably because microinjected cells express different levels of Smac/DIABLO $\Delta 53$.

Overall our data demonstrate that high levels of cytosolic biologically active Smac/DIABLO can sustain caspase activation in the absence of a functional apoptosome.

Figure 4 Cytosolic translocation of GFP-DD-TM and decrease of mitochondrial TMRM uptake in single cells as markers respectively of caspase activation and $\Delta\psi_m$ dissipation. (a) Individual traces for TMRM uptake and GFP localization of eight typical E1A cells expressing GFP-DD-TM after treatment with 50 μ M etoposide. In the TMRM and GFP reported traces the same color marks the same cell. (b) Individual traces for TMRM uptake and GFP localization of eight typical E1A cells expressing GFP-DD-TM after treatment with 2,5 μ M MG-132. In the TMRM and GFP reported traces, the same color marks the same cell. (c) Individual traces for TMRM uptake and GFP localization of seven typical cells expressing GFP-AA-TM after treatment with 50 μ M etoposide. In the TMRM and GFP reported traces the same color marks the same cell. (d) Individual traces for TMRM uptake and GFP localization of eight typical E1A/C9DN cells expressing GFP-DD-TM after treatment with 50 μ M etoposide. In the TMRM and GFP reported traces the same color marks the same cell. (e) Individual traces for TMRM uptake and GFP localization of eight typical E1A/C9DN cells expressing GFP-DD-TM after treatment with 2,5 μ M MG-132. In the TMRM and GFP reported traces the same color marks the same cell

Figure 4 Continued

Endogenous Smac/DIABLO is required for the apoptosome-independent cell death induced by PIs and its level is increased in the cytosol during PIs-induced apoptosis

We explored the role of endogenous Smac in regulating PIs-dependent caspase activation and apoptosis in E1A/C9DN cells. Smac expression was downregulated by specific siRNA (Figure 6b) and the apoptotic response to PIs was measured by trypan blue staining and caspase-2 processing. While no effect of Smac siRNA was observed in control-untreated cells, Smac siRNA significantly reduced the susceptibility to apoptosis of E1A/C9DN cells challenged with

MG-132 (Figure 6a). Reduced apoptosis in Smac-depleted cells was confirmed by the reduced proteolytic activation of caspase-2 (Figure 6b).

To support the hypothesis that released Smac is important to sustain apoptosis induced by PIs in caspase-9-defective cells, we investigated whether PIs could regulate the level of endogenous cytosolic Smac once released from mitochondria. Subcellular fractionation was performed to isolate the mitochondrial and cytosolic fractions from E1A and E1A/C9DN cells after treatment with MG-132 or etoposide at doses that had been previously tested to trigger comparable apoptosis. Trypan blue scoring confirmed that apoptosis was similarly induced in E1A cells by both stimulus (data not

Figure 5 Smac/DIABLO can synergize with etoposide to induce efficient apoptosis and caspase activation in cells containing a caspase-9 dominant negative form. (a) Schematic representation of the different Smac/DIABLO mutants used. (b) The different Smac/DIABLO constructs were transiently expressed in E1A cells together with GFP as reported. Cells were either treated or not treated with etoposide and green cells showing a collapsed morphology and presenting extensive membrane blebbing were scored as apoptotic (means $\pm$ S.D.; $n=6$). (c) The different Smac/DIABLO constructs were transiently expressed in E1A/C9DN cells together with GFP as reported. Cells were either treated or not treated with etoposide and cells showing a collapsed morphology and presenting extensive membrane blebbing were scored as apoptotic (means $\pm$ S.D.; $n=6$). (d) Caspase-3/GFP Smac $\Delta 53$ or Smac $\Delta 71$ together with β -galactosidase were transiently expressed in E1A/C9DN cells. After 20 h of etoposide or MG-132 treatment cell lysates were generated and subjected to Western immunoblotting using the indicated antibodies. (e) XIAP was transiently expressed in E1A or E1A/C9DN cells together with GFP as a reporter. After treatment with MG-132, green cells showing a collapsed morphology and presenting extensive membrane blebbing were scored as apoptotic (means $\pm$ S.D.; $n=6$). (f) Caspase-3/GFP XIAP together with β -galactosidase were transiently expressed in E1A/C9DN cells. After 20 h of etoposide or MG-132 treatment cell lysates were generated and subjected to Western immunoblotting using the indicated antibodies. (g) Individual traces for TMRM uptake and GFP-DD-TM localization of eight typical E1A/C9DN cells treated with 50 μ M etoposide and overexpressing Smac/DIABLO $\Delta 53$. In the TMRM and GFP reported traces the same color marks the same cell

Figure 6 The role of Smac/DIABLO in PIs-dependent apoptosis. **(a)** E1A/C9DN cells transfected with Smac-specific siRNA or with control siRNA were either incubated or not incubated with MG-132. Appearance of apoptosis was scored by Trypan blue staining (means $\pm$ S.D.; $n = 3$). **(b)** Western blot of E1A/C9DN cell lysates either treated or not treated with MG-132 shows the effect of Smac siRNA or control siRNA on caspase-2 processing. **(c)** Subcellular fractionation of E1A and E1A/C9DN cells treated with etoposide or MG-132 by separating soluble cytosolic fractions (SN) from digitonin insoluble fractions including mitochondria (Pellet). Western immunoblots were performed using the indicated antibodies. Molecular weights are in kDa. **(d)** E1A/C9DN cells treated with etoposide or MG-132 were fixed, permeabilized and double stained with the anti-Smac and anti-cytochrome *c* antibodies. **(e)** Images acquired with the confocal microscope after immunofluorescent labeling were subjected to the quantitative analysis using Metamorph software. Randomly chosen fields in each coverslip were acquired for the analysis. The fluorescent intensity for each pixel subtracted from the background was measured using the appropriate function of the analysis program. Values are normalized to the cell area

shown) and the comparable levels of HDAC4, and PARP processing detected in immunoblot (Figure 6c) verified it. The levels of mature Smac were similarly decreased in the

mitochondria of etoposide and PIs treated cells compared to the untreated ones. However, the levels of Smac were higher in the cytosol of cells treated with MG-132 compared to

etoposide (Figure 6c). This increase was not due to alterations in the fractionations procedure as confirmed by F1-ATP synthase and tubulin staining. The fact that in E1A/C9DN cells the level of cytosolic Smac is lower than in E1A cells after treatment with etoposide may be explained by the reduced caspase activity which is important to shutdown the proteasome function during apoptosis.⁴⁴

We confirmed by immunofluorescence analysis that a brighter signal for endogenous Smac can be observed in the cytosol of MG-132-treated E1A/C9DN cells (Figure 6d). To quantify the relative levels of Smac present in the cytosol after MG-132- or etoposide-induced mitochondrial release, confocal images from different fields were selected containing both mitochondrial localized and cytosolic Smac/DIABLO. 400 cells from different fields were individually analyzed and the fluorescence intensity was calculated with respect to the cell area. This analysis shows that the levels of Smac decrease once released into the cytosol, but this decrease is lower in PIs-treated cells. In conclusion, our studies demonstrate that PIs can regulate the amount of cytosolic Smac once released from the mitochondria.

Discussion

The proteasome is a 2.000 kDa multisubunit cylindrical complex that recognizes and proteolyzes proteins that contain multiple ubiquitin molecules. The 19S subunits recognize and subsequently cleave the ubiquitin chain from the protein substrate, whereas the 20S subunit (catalytic core) progressively degrades the targeted protein.¹⁹ Since many proteins that control cell proliferation and apoptosis are degraded after ubiquitination, the proteasome has become an interesting target for anticancer therapy.⁴⁵ Recently, the peptide boronate PS-341/Bortezomib, a reversible inhibitor of the proteasome, has been approved for use in patients with relapse and refractory multiple myeloma.⁴⁶ Interestingly, Bortezomib mechanism of cytotoxicity differs from 60.000 compounds of the National Cancer Institute database.⁴⁷

We have demonstrated that PIs, in contrast to many chemotherapeutic agents, have the unique ability of activating caspases through a mitochondrial pathway that does not need a functional apoptosome. Our studies show that the Bcl-2 family members play a central role in dictating the choice between cell death and survival in response to PIs. It was known that Bcl-2 and Bcl-xL can protect against apoptosis induced by PIs.^{48–50} We have demonstrated that not only Bcl-2 and Bcl-xL can suppress PIs-induced cell death but even the proapoptotic members Bax and Bak are necessary. Overall this scenario indicates that PIs induce the activation of the intrinsic/mitochondrial apoptotic pathway as suggested by other studies.^{49–53} In contrast to other anti-tumor drugs, where Bcl-2/Bcl-xL overexpression and caspase-9 inactivation are equally able to suppress caspase activation and cell death, in the case of PIs, only Bcl-2/Bcl-xL can suppress apoptosis and caspase activation. This strengthened the idea that Bcl-2/Bcl-xL can control the activation of different caspases independently from caspase-9.^{21–23}

The ability of PIs to activate caspases in the absence of a functional caspase-9 was confirmed by analyzing enzymatic

activity, proenzymes and death substrates cleavage in different cellular systems including MEF from mice $-/-$ for caspase-9. Moreover, we have also used a new assay to measure caspase activity in single cells on the OMM, and here again PIs, in contrast to etoposide, were able to trigger caspases activation in the absence of functional apoptosome. This assay allowed us to measure the temporal relationship between caspase activation and mitochondrial depolarization. Previously Rehm *et al*³¹, have used the FRET-based sensors to demonstrate that, in response to staurosporine, activation of DEVDases occurs within 10 min from the onset of depolarization. We have similarly observed that full DEVDase activity in response to etoposide is obtained on the surface of mitochondria in approximately 10 min after depolarization ($10.24 \text{ min} \pm 3.34$), whereas MG-132 induces full caspase activity with a certain delay (27.3 ± 3.75). The reason for this difference is unknown but it could be related to the quicker depolarization induced by MG-132. In caspase-9 defective cells activation of caspases in response to etoposide was impaired up to 4 h from mitochondrial depolarization and cells showed reduced dissipation of $\Delta\psi_m$. This observation confirms similar data obtained by using chemicals inhibitors of caspases or Apaf-1-defective cells.^{30–32} The partial dissipation reflects the absence of a caspase-dependent feedback loop on mitochondria.²⁸

When apoptosis was induced by PIs in caspase-9 defective cells, $\Delta\psi_m$ dissipation was similar to that observed in wt cells; caspases activation was also observed, even though with a delay of approximately 120 min. Therefore, two kinetics of caspase activation can be recognized, with caspase-9 still being required for the rapid caspase activation in response to OMM permeabilization, but dispensable for the slow DEVDases activation.

Our results suggest that, in contrast to etoposide, PIs can induce full mitochondrial depolarization before robust caspase activity. Why can PIs affect mitochondrial function so dramatically? Bortezomib and different types of PIs provoke an elevation of mitochondrial derived reactive oxygen species (ROS),^{54–57} which are critical for the OMM permeabilization, $\Delta\psi_m$ decrease and apoptosis. However, the fact that etoposide also induces ROS production⁵⁸ makes necessary a detailed study on ROS levels generated in single cells after treatment with the different drugs.

We can exclude that the caspase-9 independent apoptosis, triggered by PIs, relies entirely on the compensatory activity of other regulative caspases implicated in the apoptotic process. In fact, caspase-8 inhibition by CrmA only partially affected this apoptotic response (Henderson and Brancolini, unpublished) and downregulation of caspase-2 by siRNA was ineffective (Henderson and Brancolini, unpublished).⁵⁴ Our work indicates that Smac/DIABLO can play a role in regulating caspases activation in the absence of caspase-9. First, we demonstrate that the levels of cytosolic Smac/DIABLO are increased in response to PI treatment. Second, a normal apoptotic response to etoposide can be restored in caspase-9 defective cells by artificially raising the levels of cytosolic active Smac/DIABLO. Third, Smac siRNA reduced the apoptotic response to PIs.

Once released from mitochondria Smac/DIABLO interacts, through a short amino-terminal sequence, with the IAPs

relieving their inhibitory effect on caspases.¹⁰ XIAPs, cIAP1, cIAP2 and Livin are E3 ligases containing a carboxy-terminal RING-finger domain that is involved in auto-ubiquitination and ubiquitination of interacting proteins, mediating their degradation via the proteasome.⁵⁹ Different reports have illustrated that Smac/DIABLO is targeted for degradation by the combined action of the E2/E3 ligase BRUCE and XIAP or of cIAP1 and cIAP2.^{33–37,43}

How could cytosolic Smac activate effector caspases? A possibility is that cancer cells have an intrinsic drive to activate effector caspases, such that functional removal of IAPs triggers apoptosis. This hypothesis has been recently suggested by the identification of XIAP antagonists that specifically target the BIR2 functional region but not the BIR3 and trigger apoptosis in cancer cells.^{60,61} Furthermore, Smac can induce apoptosis in Apollon-deficient cells but not in wild-type cells³³ and zVAD-fmk can increase the levels of Apollon/Bruce in nonapoptotic cells.³⁷

These data together with our results support the hypothesis that in cancer cells caspases are continuously activated at minimal levels and kept in frame by IAPs.^{60,62,63,17} It is clear that further studies are required to confirm this hypothesis. In conclusion, our data indicate that PIs, by sustaining the levels of the IAPs antagonist Smac/DIABLO, can overcome this distal apoptotic checkpoint and they could be powerful drugs to induce cell death of cancer cells that have accumulated mutations in the apoptosome, both as a single agent or in combination with other antitumor treatments.

Materials and Methods

Culture conditions, transfection and retroviral infection

All the used cell lines with the exception of the BJ were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In all Trypan blue assays 160–400 cells, from three independent samples, were counted for each data point. Data were represented as arithmetic means $\pm$ S.D. for at least three independent experiments. Trypan blue assays were verified by analyzing the levels of caspases or caspase substrates processing in immunoblotting. E1A/Bcl-2 and E1A/C9DN/Bcl-2 cells were generated by retroviral infection.¹⁸ Smac siRNA CCACATATGCGTTGATTGAAGCTAT (bp 275–299) and control siRNA CCAGTATTTGCTTAGGAAGCACTAT (scrambled) were purchased from Invitrogen. Cells were transfected 24 h after plating by adding to the medium OptiMEM containing lipofectamine (Invitrogen) plus the siRNAs.

Plasmids generation

The pEGFPC3-HA-TMBcl-xL was generated by subcloning the *Hind*III-*Eco*RI HA tag fragment from pcDNA3HA into pEGFPC3 (Invitrogen). The Bcl-xL transmembrane coding cDNA was amplified by PCR from pGDSV7-Bcl-xL using specific primers and inserted as an *Eco*RI/*Sa*II fragment. Next the DEVD coding sequence was inserted into the pEGFPC3-HA-TMBcl-xL plasmid. The oligonucleotides were annealed and ligated into the plasmid backbone as an *Xho*I/*Hind*III fragment.

In vitro mutagenesis (Invitrogen) was performed to obtain pEGFPC3-DEVA-HA-TMBcl-xL (GFP-AD-TM) and pEGFPC3DEVA-HA(-D)-TMBcl-xL (GFP-AA-TM) plasmids. pUC19-Ub- Δ 71-mSmac/DIABLO was

generated by an overlapping PCR strategy from pUC19-Ub- Δ 53-mSmac/DIABLO. The Ub- Δ 71-mSmac/DIABLO PCR product was digested by *Hind*III/*Xho*I to insert it into the ubiquitin C promoter-containing expression vector pUC19 (courtesy of P Liston). pET32a(+)- Δ 53-mSmac/DIABLO was generated by amplifying the coding region of mouse Smac/DIABLO by PCR. pGEX4T1- Δ 53-mSmac/DIABLO was generated by subcloning the Δ 53-mSmac/DIABLO coding region from pET32a(+)- Δ 53-mSmac/DIABLO into pGEX4T1 (AP Biotech) using the *Bam*HI/*Xho*I sites.

Time-lapse confocal fluorescence microscopy

For time-lapse imaging, cells were grown on 35 mm glass-bottom dishes (Wilco BV) and microinjected using the automated injection system (Zeiss) with 2 ng of plasmid DNA. After 3 h cells were treated with the $\Delta\psi_m$ -sensitive dye-TMRM,²⁹ (20 nM final) and etoposide (50 μ M) or MG-132 (2.5 μ M); the media was then covered with embryo-tested mineral oil to prevent evaporation. After 3–6 h of treatment the culture dishes were subjected to time-lapse analysis. Imaging of GFP and TMRM fluorescence was monitored every 2 min using a confocal microscope equipped with a 488 nm argon laser, a 543 nm HeNe laser and a 63 $\times$ oil fluorescence objective.

Image processing and remodeling of fluorescence kinetics

The image analysis was performed using the MetaMorph 6.04. software. For TMRM analysis in single cells, fluorescent mitochondria (MT) were analyzed by drawing a region around them and by measuring the total brightness (integrated fluorescence intensity or I_{TMRM}) of such a region. The background to be subtracted from the I_{TMRM} (Bk_{TMRM}) was calculated by using a ROI positioned in a nonfluorescent region according to the following equation:

$$Bk_{TMRM} = (I_{ROI}/Area_{ROI}) * Area_{MT}$$

Finally, the background-corrected I_{TMRM} values were presented as the percentage of the average of the 29 values detected before the I_{TMRM} started decreasing. The I_{TMRM} values have been taken for 60 min before and 120/240 min after the beginning of the $\Delta\psi_m$ decrease (set to min, 0).

The release kinetics of GFP have been measured as the S.D. from the average pixel intensity of a ROI positioned on the mitochondria and cytosol of individual cells. Mitochondrial localized TM-DD-GFP (punctuate) contributes to a high S.D. of pixel intensities, whereas homogeneously distributed GFP (diffuse) is represented by a low S.D. To examine the relationship between $\Delta\psi_m$ loss and caspase activation better, the punctuate-diffuse GFP values have been shown as the inverse value of S.D. after background subtraction ($1/S.D. = y$). For direct comparisons and statistical analyses, single cell standard deviation traces were transformed in a 0–100% scale in which 0% equals to the first y punctuate value (y_{MT}) and 100% equals the first complete diffusion value (y_{diff}) according to the following equation:

$$y_{(0-100\%)} = 100 * (y - y_{MT}) / (y_{diff} - y_{MT})$$

For analyzing cells that present $\Delta\psi_m$ decrease but do not release GFP in the cytosol, a relation between I_{GFP} and $SD_{GFP\ diff}$ has been calculated from the linear treadline of the values recovered from 20 cells that do release GFP:

$$SD_{GFP\ diff} = 2706,8 * I_{GFP} - 215,66$$

Such an equation has been used to calculate the hypothetical $SD_{GFP\ diff}$

that a cell, with given I_{GFP} , could reach. After background subtraction it was possible to calculate the y_{diff} necessary to scale the values from 0 to 100.

Antibody production and Western blotting

Rabbits were immunized with recombinant histidin-tagged Smac/DIABLO $\Delta 53$ purified from *Escherichia coli*. For anti-Smac/DIABLO antibody purification from antiserum, Smac/DIABLO was fused to GST and cross-linked to glutathione-Sepharose as previously described.⁶⁴

For Western blotting, proteins were transferred to a 0.2 μ m pore sized nitrocellulose membrane and incubated with anti-caspase-2,¹⁸ anti-Smac/DIABLO anti-GFP,⁶⁴ anti-caspase-8 (Alexis), anti-caspase-7 (Cell Signalling), anti-p85 PARP fragment (Promega), anti-PARP (Cell Signalling), anti-tubulin, anti-Bid (Cell Signalling), anti- β -catenin (Transduction lab), anti-HDAC4 (40), and anti- β -gal (Promega) antibodies.

Immunofluorescence microscopy

For indirect immunofluorescence microscopy, cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed with PBS/0.1 M glycine, pH 7.5, and then permeabilized with 0.1% Triton X 100 in PBS for 5 min. The coverslips were treated with the anti-cytochrome *c* (Promega) or anti-Smac antibodies, diluted in PBS, for 45 min in a moist chamber at 37°C. They were then washed with PBS twice, and incubated with 488 or 543 Alexa-conjugated secondary antibodies (Molecular Probes), for 30 min at 37°C.

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