

Mcl-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin-resistant ovarian carcinoma cells

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Abstract

Chemoresistance of ovarian carcinoma has been associated previously to the absence of Bcl-x_L expression downregulation in response to cisplatin. Among BH3-mimetic molecules constituting promising anticancer agents able to inhibit the activity of antiapoptotic Bcl-2 family proteins, we evaluated the effect of one of them, HA14-1, on various ovarian carcinoma cell lines. In response to HA14-1, the cisplatin-resistant IGROV1-R10 cell line underwent massive cell death, whereas other cell lines presented a partial response (IGROV1, SKOV3, and A2780) or did not respond to this molecule (OAW42 and OAW42-R). However, the expression of HA14-1 targets (Bcl-2 and Bcl-x_L) did not correlate to these different responses. In contrast, cell death was associated with the disappearance of Mcl-1 after exposure to HA14-1. We showed that, in the HA14-1 nonresponsive cell lines (SKOV3 and OAW42), small interfering RNA-mediated Mcl-1 downregulation allowed HA14-1-induced massive apoptosis in the absence of chemotherapy. Furthermore, cisplatin-induced Mcl-1 downregulation was also able to sensitize highly chemoresistant SKOV3 cells to HA14-1. Taken to-

gether, these results show that Bcl-x_L and Mcl-1 are able to cooperate to protect ovarian carcinoma cells against oncogenic stress or chemotherapy-induced apoptosis and suggest that the development of multitargeted strategies directed against these two antiapoptotic proteins may constitute a major challenge for the therapeutic care of chemoresistant ovarian carcinomas. BH3-mimetic compounds represent promising tools for this purpose either on their own (direct or indirect pan-inhibitors) or in combination with new drugs aiming to inactivate Mcl-1. [Mol Cancer Ther 2009;8(11):3162–70]

Introduction

Ovarian carcinoma is the leading cause of death among patients with gynecologic cancer. Although the majority of tumors initially respond to standard treatments such as debulking surgery and subsequent platinum-based chemotherapy, frequent recurrence and subsequent acquired chemoresistance are responsible for the therapeutic failure, leading to an overall survival rate of ~30% (1).

Because cisplatin exerts its cytotoxic effect on tumor cells by inducing apoptosis as a result of lethal DNA damage, numerous mechanisms can contribute to cisplatin resistance. These mechanisms lead either to a decreased platination of DNA or to a decreased death signal due to alteration of DNA damage detection process and subsequent signal transduction (2). However, a strong cell death signal due to DNA damage generally exists in cells exposed to cisplatin, at least transiently, and it should be hypothesized that cancer cells need strong survival mechanisms to avoid cell death and promote DNA repair. Such a decreased susceptibility to apoptosis has been previously held responsible for chemoresistance (3). The antiapoptotic Bcl-2 family proteins play a major role in the protection against DNA damage-induced apoptosis via the mitochondrial pathway. They constitute a molecular hurdle impeding the chemotherapy-induced apoptosis during the time needed for elimination of cellular damages.

Bcl-2 family members share four Bcl-2 Homology domains with Bcl-2 (BH1-BH4). Antiapoptotic proteins (Bcl-2, Bcl-x_L, etc.) contain the BH1 to BH4 domains, whereas proapoptotic proteins contain either the BH1 to BH3 domains (multidomain members such as Bax or Bak) or only the BH3 domain (BH3-only group such as Bad, Bid, Noxa, Puma, or Hrk).

These proteins are able to physically interact among themselves or with other molecular partners, thus modulating their proapoptotic or antiapoptotic properties (4). These interactions occur via the BH3 domain, which plays a central role in the control of apoptosis. The ratio between

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proapoptotic and antiapoptotic members, their conformation, and their subcellular localization constitute major determinants of cell survival.

Numerous studies have provided evidence for a key function of these proteins in the control of mitochondrial permeability transition and cell death (5). In this context, antiapoptotic members act as sequestrators of multidomain proteins, impeding their active role in apoptosis. In contrast, BH3-only proteins that are considered as stress sensors can dissociate Bax-like proteins from their antiapoptotic sequestrators, thus leading to apoptosis (6).

Expression of Bcl-2 family members is frequently deregulated during carcinogenesis (7), and expression of both Bcl-2 and Bcl-x_L antiapoptotic proteins has been associated with resistance to radiation and antitumor agents (8), notably in ovarian carcinoma (9–11).

Our previous work showed that cisplatin-induced downregulation of Bcl-x_L was strictly associated with apoptosis and absence of recurrence *in vitro*. Conversely, the maintenance of Bcl-x_L expression in response to cisplatin led to chemoresistance (12).

Thus, in ovarian carcinoma, the inhibition of the protective function of Bcl-x_L seems to be an attractive strategy for either restoring normal apoptotic process in cancer cells or circumventing resistance to conventional chemotherapy. In this regard, numerous teams have investigated new potent molecules designed to fit into the hydrophobic pocket of Bcl-2 that binds to the BH3 domain and, by this way, to inhibit the function of the antiapoptotic members (13). These BH3-mimetic compounds are able to dissociate Bax-like proapoptotic multidomain members from their antiapoptotic sequestrators, thus leading to apoptosis.

Among them, HA14-1 was the first Bcl-2 inhibitor to be identified (14). It has been shown to induce apoptosis in HL60 cells through the mitochondrial pathway and to sensitize cells to apoptosis induced by other cytotoxic agents in many cell types (14–19).

Considering all these data, we investigated the effect of HA14-1 on ovarian cancer cell lines either sensitive (OAW42 and A2780) or resistant (IGROV1-R10 and SKOV3) to cisplatin. HA14-1 was used alone or in combination with cisplatin to evaluate its capacity to sensitize to oncogenic stress and to conventional chemotherapy.

Materials and Methods

Cell Lines and Treatment

IGROV1, OAW42, A2780, and SKOV3 cell lines were established from human ovarian adenocarcinomas. Resistant IGROV1-R10 and OAW42-R cells were obtained by reiterating treatments with increasing concentrations of cisplatin (20). These cell lines were grown as described previously (12). Exponentially growing cells were exposed for 2 h to 20 μg/mL CDDP (Merck) in serum-free medium or with HA14-1 dissolved in DMSO (Sigma-Aldrich) in complete medium. For HA14-1 treatment, control cultures received the same dose of DMSO as treated counterparts.

Nuclear Morphology Study

The cells were collected by cyto centrifugation, fixed, and incubated in a 1 μg/mL 4',6-diamidino-2-phenylindole aqueous solution (Boehringer Mannheim).

Cellular Staining with Giemsa

Adherent cells were fixed with 70% ethanol and stained with a Giemsa azur eosin methylene blue solution (Merck) at a 1:5 dilution.

Analysis of Cellular DNA Content by Flow Cytometry

Adherent and detached cells were pooled and fixed in ethanol and the pellets were stained with propidium iodide using the DNA Prep Coulter Reagent kit (Beckman Coulter). Samples were thereafter analyzed using an EPICS XL flow cytometer (Beckman Coulter) with a 488 nm excitation laser.

Electron Microscopy

For transmission electron microscopy, cells were fixed with 2.5% glutaraldehyde in PBS, included in agar, post-fixed in 1% osmium tetroxide in Sorensen's buffer, dehydrated in ethanol, and embedded in EPON resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEOL1011 microscope.

For scanning electron microscopy, cells were fixed with 2.5% glutaraldehyde in PBS, sedimented on L-polylysine-coated thermanox slides, post-fixed in 1% osmium tetroxide in Sorensen's buffer, and dehydrated in ethanol. A critical point drying followed by metalizing cultures with platinum is done before observation using a JEOL6400F microscope.

Western Immunoblotting

Proteins (20 μg) were subjected to immunoblot following procedure described previously (12). The following primary antibodies were used: anti-Bcl-2 (DAKO), anti-Bcl-x_{L/S} and anti-Mcl-1 (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (PARP), anti-cleaved caspase-3, anti-caspase-9 and its cleaved form (Cell Signaling Technology), anti-caspase-3 (clone 19; BD Transduction Laboratories), and anti-α-tubulin (Sigma). Anti-microtubule-associated protein LC3 was kindly given by Dr. Yasuo Uchiyama (Osaka University Graduate School of Medicine).

Small Interfering RNA Transfection

Small interfering RNAs targeting *mcl-1* gene (siMcl-1; 5'-gugccuuuguggcuaaacatt-3') and green fluorescent protein mRNA (used as control of transfection; 5'-gacguaaacggccaagutt-3') were purchased as duplexes from Eurogentec. Oligofectamine (Invitrogen) was used to transfect these siRNA (75 nmol/L) following the manufacturer's instructions. At the optimal time of gene silencing monitored by Western blot (24 h post-transfection), 40 μmol/L HA14-1 was added to the cells for 6 h.

Results

HA14-1 Induces a Massive and Rapid Cell Death in Cisplatin-Resistant IGROV1-R10 Cell Line

IGROV1-R10 cell line was exposed to increasing doses of HA14-1. Whereas no effect was observed with concentrations up to 20 μmol/L, a strong cytotoxic effect was observed in response to higher concentrations (data not

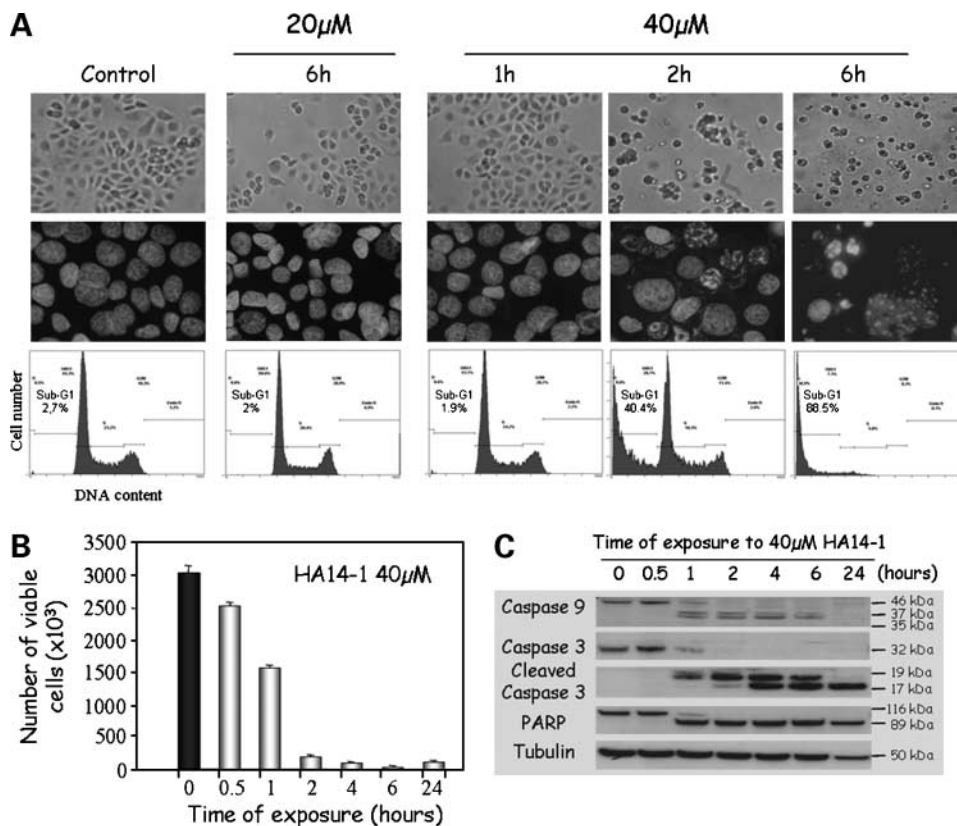


Figure 1. Response of cisplatin-resistant IGROV1-R10 cells to HA14-1. **A**, exponentially growing cells were treated for 1 to 6 h with HA14-1 (20 or 40 $\mu\text{mol/L}$). Response was appreciated by the study of morphologic/nuclear features and DNA content. Cell viability (**B**) and detection of caspase-9, caspase-3, and PARP cleavage (**C**) by Western blot after exposure to 40 $\mu\text{mol/L}$ HA14-1. Representative of three independent experiments.

shown), reaching a maximum when cells were exposed to 40 $\mu\text{mol/L}$. After 2 h of treatment, 40 $\mu\text{mol/L}$ HA14-1 induced a massive cell detachment associated with a high proportion of condensed and fragmented nuclei and with the appearance of a sub-G₁ peak (Fig. 1A and B). Taken together, these features are strongly evocating apoptosis, which is macroscopically nearly completed within 4 to 6 h. We showed that caspase-9, caspase-3, and PARP were totally cleaved as soon as 1 h, showing that caspase activation precedes cell detachment and that mitochondrial apoptotic pathway is involved in this phenomenon (Fig. 1C).

Apoptosis and Autophagy Were Both Involved in Response to HA14-1

Scanning and transmission electron microscopy were used to further characterize cell death in IGROV1-R10 exposed to 40 $\mu\text{mol/L}$ HA14-1 for 6 h. Membrane blebbing (Fig. 2A) and chromatin condensation (Fig. 2B) were observed, confirming the occurrence of apoptosis. Moreover, we observed cells with numerous vesicles filled with organelles debris evocating autophagy features (Fig. 2C, *inset*). The possible involvement of autophagy in the response to 40 $\mu\text{mol/L}$ HA14-1 was also suggested by the appearance of the LC3-II form of the LC3 protein, a specific marker of autophagy (Fig. 2D).

However, it should be noticed that, in the cells containing vesicles filled with organelles debris, we also observed empty vesicles and chromatin condensation, suggesting concomitant or successive autophagic and apoptotic mechanisms.

Intrinsic Resistance to HA14-1

We further investigated the cellular response to HA14-1 in five other cell lines either sensitive (OAW42, IGROV1, and A2780) or resistant (SKOV3 and OAW42-R) to cisplatin (Fig. 3A). In contrast with the massive cell death described with IGROV1-R10, 40 $\mu\text{mol/L}$ HA14-1 for 6 h had little or no effect in these cells. Actually, in IGROV1 cells, only 40% of dead cells were observed after treatment. In SKOV3 cells, HA14-1 induced only a weak apoptotic response, and in OAW42, OAW42-R, and A2780 cells, no sign of cell death was detectable. In these cell lines, nuclear morphology, DNA content, and cell viability of exposed cells were similar to those of the control counterparts. These results were confirmed by Western blot, which showed little or no PARP and caspase cleavage (Fig. 3B). These results suggest that response to HA14-1 depend on cellular context.

Mcl-1 Is a Major Determinant of Response to HA14-1 in Ovarian Carcinoma Cells

To identify the molecular determinants of the differential response to HA14-1 in ovarian carcinoma cells, we studied the expression of the identified targets of this molecule, the antiapoptotic proteins Bcl-2 and Bcl-x_L.

Cells were treated for 6 h with 40 $\mu\text{mol/L}$ HA14-1. Neither the basal level expression nor the expression of these targets after HA14-1 treatment was able to explain the different responses between the cell lines (Fig. 4A). Indeed, neither HA14-1-sensitive IGROV1-R10 cells nor its HA14-1-resistant counterpart IGROV1-R10 cells (data not

shown) express Bcl-2 protein, whereas the two other "HA14-1-resistant" cell lines expressed both Bcl-2 in a similar extent. No modification of this expression was observed after HA14-1 treatment. Thus, Bcl-2 expression could not be correlated to the response to HA14-1.

As far as Bcl-x_L expression was concerned, a similar basal expression level was observed whatever the cell line was, and no modification was observed after HA14-1 treatment.

We then investigated the involvement of another cooperating antiapoptotic protein, Mcl-1. This protein was not described as a HA14-1 target, but its activity is known to be essential for protection against apoptosis (21). Although Mcl-1 protein was detected in all cell lines, its expression level in response to HA14-1 seems to be linked to the cellular response to this compound (Fig. 4B). Indeed, we observed that Mcl-1 expression nearly disappeared in response to HA14-1 in sensitive IGROV1-R10 cells, whereas its expression was increased or at least maintained at similar levels to untreated controls after exposure to HA14-1 in SKOV3 and OAW42 cells (Fig. 4B). Thus, Mcl-1 appeared as an essential determinant to the response to HA14-1 used as a single antitumor agent.

We also examined the expression of Noxa, Puma, and Bim in IGROV1-R10 and SKOV3 cells after a 6-h exposure

to 40 μ mol/L HA14-1. Bim was strongly expressed in IGROV1-R10 but not in SKOV3. However, its expression remained unchanged after treatment. In contrast, Noxa and Puma expression was strongly induced after exposure to HA14-1 in the two cell lines. This upregulation was only observable with sublethal concentrations (20 μ mol/L) and the expression of these proteins sharply decreased in case of massive cell death, suggesting a codegradation of these proteins with Mcl-1 (Fig. 4C).

Mcl-1 Downregulation by RNA Interference Sensitizes SKOV3 and OAW42 cells to HA14-1

Because loss of Mcl-1 seems to be correlated with apoptotic cell death in response to HA14-1, we decided to knock-down this protein with siRNA (siMcl-1) in intrinsically HA14-1-resistant cell lines SKOV3 and OAW42 (Fig. 5). In both cell lines (Fig. 5A), siMcl-1 was able to inhibit Mcl-1 protein expression but did not lead to cell death on its own (Fig. 5). As described previously, a 6-h exposure of 40 μ mol/L HA14-1 alone induced only a weak apoptotic response (SKOV3) or did not have any effect (OAW42). Whereas control siRNA did not modify the response to HA14-1, we observed a strong apoptotic response in both cell lines when 40 μ mol/L HA14-1 was combined with siMcl-1.

These results showed that Mcl-1 knockdown in SKOV3 and OAW42 cells allowed apoptotic cell death in response to HA14-1, thus strongly arguing that Mcl-1 play a pivotal role in the cellular response to HA14-1.

Cisplatin Also Sensitizes SKOV3 Cells to HA14-1

Considering the crucial involvement of Mcl-1 in response to HA14-1 and the decrease of Mcl-1 expression observed in SKOV3 cells in response to cisplatin (Fig. 6A), it could be hypothesized that this chemotherapeutic agent could allow HA14-1-induced apoptosis through the downregulation of Mcl-1.

Indeed, we showed that a 2-h pre-exposure of SKOV3 cells to cisplatin sensitized them to HA14-1 (the exposure to HA14-1 occurring after 48 h, when cisplatin-induced Mcl-1 downregulation appeared optimal). The association of the two compounds led to a strong cytotoxic effect, whereas cisplatin and HA14-1 alone did not induce apoptosis (Fig. 6B). Although cell growth was unaffected by HA14-1 alone, cisplatin-treated cells remained in a latent state for 2 weeks to eventually recover a normal proliferation rate thereafter (Fig. 6C). In contrast, cells exposed to the combination of the two agents did not escape treatment even after 3 weeks as confirmed by Giemsa staining of the cellular layer 22 days after treatment (Fig. 6C). This experiment shows that HA14-1 and cisplatin can be considered as reciprocal sensitizers and suggests that cooperation between Bcl-x_L and Mcl-1 is essential for protection against apoptosis in ovarian cancer cells.

Discussion

Our results show that, in the absence of chemotherapy, HA14-1 is able to induce a massive and rapid cell death in chemoresistant IGROV1-R10 cells. Thus, it could be considered as an antitumoral agent on its own, at least in some

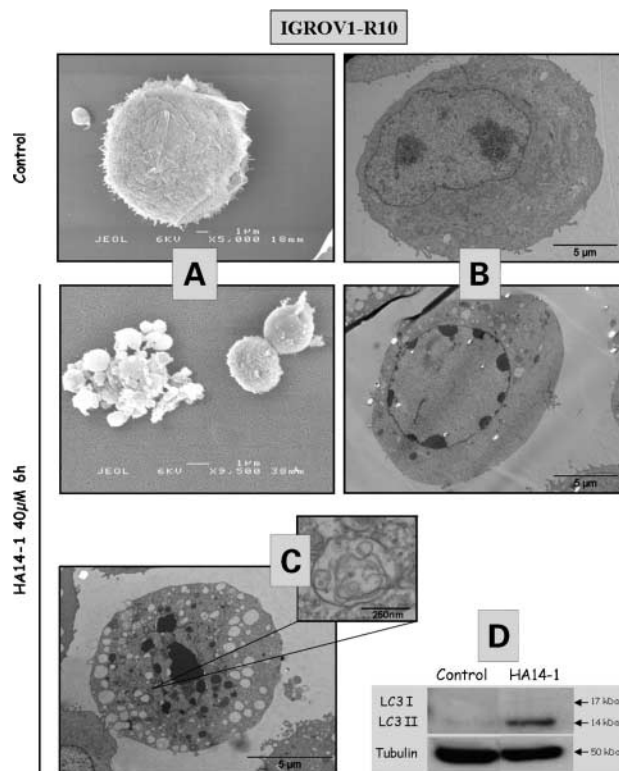


Figure 2. Nature of the cell death. Apoptosis morphologic features were observed by scanning electron microscopy (A) or transmission electron microscopy (B and C) in IGROV1-R10 cells exposed 6 h to 40 μ mol/L HA14-1. C, inset, presence of vesicles filled with organelles debris evocating autophagy features, confirmed by the detection of LC3-II form by Western blot (D).

cases, as described previously in various hematologic malignancies (14, 22, 23) in the 10 to 100 micromolar range. However, its effect on solid tumors remains poorly studied. Our results showed that apoptosis was induced in IGROV1-R10 cells after exposure to HA14-1, at least in part through the mitochondrial pathway, because both caspase-9 and -3 were cleaved within 2 to 6 h. However, both electron microscopy and appearance of the LC3-II form of the LC3 protein suggest that autophagy was also involved in response to HA14-1. The presence of both apoptotic and autophagic features in the same cells suggests that autophagy could precede apoptosis, thus constituting an early response to the HA14-1-generated stress, evocating more a survival response than a cell death mechanism. This compound has also been described previously by others to induce autophagy (24) as well as another BH3-mimetic, ABT-737, which dissociates Beclin-1 from Bcl-x_L and Bcl-2 (25).

Interestingly, the massive cell death induced by HA14-1 in IGROV1-R10 suggests that a strong intrinsic oncogenic stress is probably responsible for the activation of Bax/Bak proteins. However, in cancer cells, the overexpression of antiapoptotic proteins led to sequestration of the activated Bax-like proteins, thus impeding apoptosis induction. These cancer cells are consequently properly "primed to death" (26). In this case, the apoptotic mechanisms induced by HA14-1 should be sufficient on its own to induce cell death.

However, this cell death has not been observed in all ovarian carcinoma cell lines and some of them were only partially or totally unresponsive to HA14-1 treatment. In these cases, the presence of other stresses is needed to allow HA14-1-induced apoptosis (18, 27, 28).

In our models, we did not observe any correlation between the resistance to cisplatin and the absence of response to HA14-1, because the most cisplatin-sensitive cell line

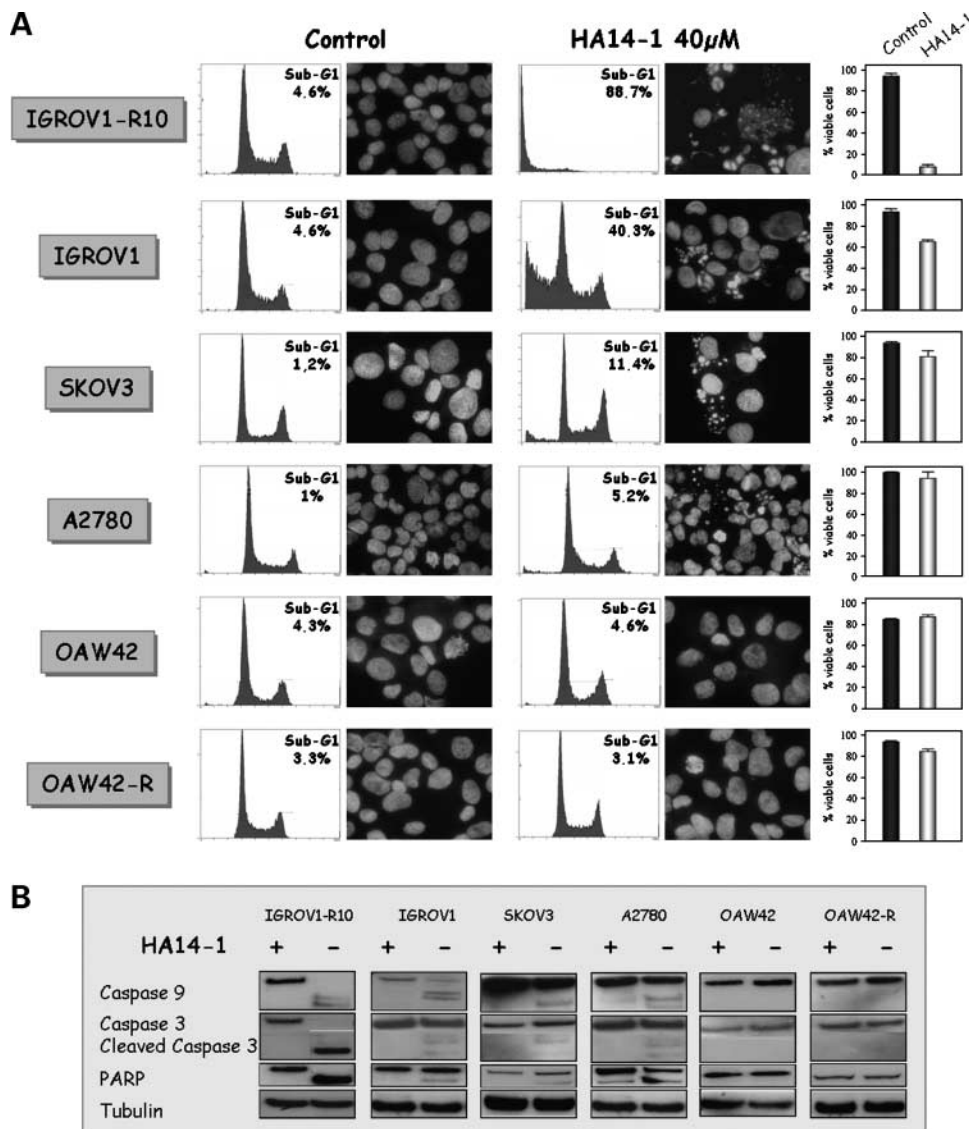


Figure 3. Response of six ovarian carcinoma cell lines to HA14-1. **A**, DNA content, nuclear morphology, and viability were assessed after a 6-h exposure to 40 μmol/L HA14-1 in IGROV1-R10, IGROV1, SKOV3, A2780, OAW42, and OAW42-R. Representative of three independent experiments. **B**, detection of caspase-9, caspase-3, and PARP cleavage by Western blot.

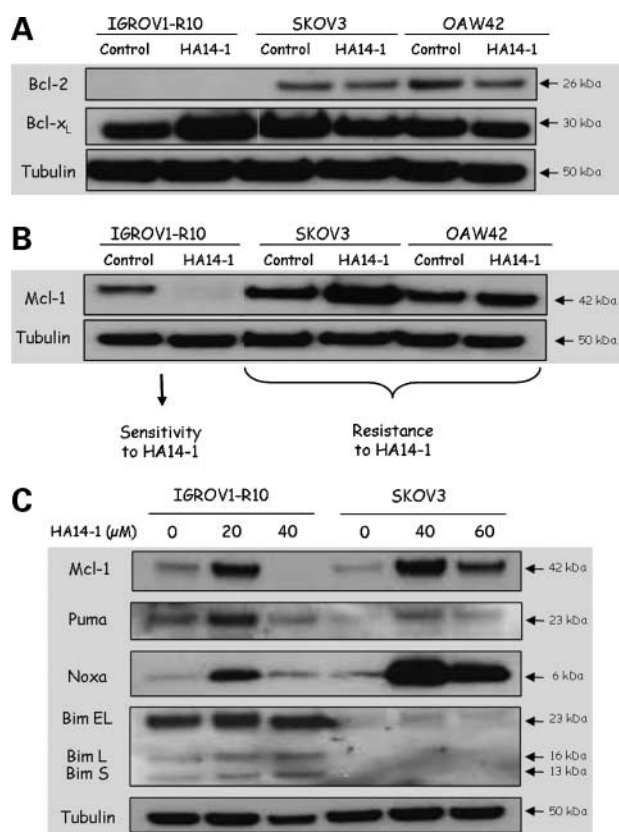


Figure 4. Molecular determinants of response to HA14-1. Exponentially growing IGROV1-R10, SKOV3, and OAW42 cells were exposed to 40 $\mu\text{mol/L}$ HA14-1 for 6 h. **A** to **C**, expression of Bcl-2, Bcl- x_L , Mcl-1, Noxa, Puma, and Bim analyzed by Western blot.

(OAW42) was also the most resistant to HA14-1, thus excluding that the absence of response to HA14-1 could be due to a defective apoptotic machinery (12). An excessive expulsion of the drug out of the cell through MDR-like mechanisms could also be ruled out, because verapamil did not allow HA14-1 to induce cell death (data not shown), consistent with the observation of a massive apoptosis in response to HA14-1 in both MRP and P-glycoprotein-overexpressing cells (17). It could be hypothesized that, in our "HA14-1-resistant" cell lines, the oncogenic stress could be insufficient to allow Bax-like proteins activation. This could be due either to a lower oncogenic stress or, more probably, to the overexpression of multiple antiapoptotic proteins.

The variations observed in response to HA14-1 were neither linked to differences in the basal expression level of the identified targets of HA14-1 (Bcl-2 or Bcl- x_L ; refs. 14, 29) nor to the variation of their expression in response to HA14-1. Although HA14-1 apoptotic effects were described to depend on Bcl-2 expression (18) Bcl- x_L was also described as a potential target of HA14-1 (29) and its well-known overexpression in ovarian carcinoma (9–12) could explain the activity of HA14-1 in the absence of Bcl-2 (IGROV1-R10).

We also investigated the possible involvement of Mcl-1. Indeed, Bcl- x_L (the only HA14-1 target expressed in sensitive

IGROV1-R10 cells) has been shown to cooperate with Mcl-1 to protect cells against apoptosis through the sequestration of Bak (21). Moreover, overexpression of Mcl-1 in ovarian carcinoma led to a poor prognosis (30) and its inactivation has been shown to induce cell death only when Bcl- x_L was also inactivated (31). Although its basal expression was similar in all cell lines, Mcl-1 was strongly downregulated after exposure to HA14-1 in IGROV1-R10 sensitive cells, whereas its expression was maintained or upregulated in resistant cells.

Such a resistance to BH3-mimetic molecules has been described previously. For instance, resistance to YC137 was reported in breast cancer cells and was associated to the disappearance of Bcl-2 expression (32). In lymphoma cells, sensitivity to ABT-737 has also been associated to the expression of Bcl-2 and more precisely to the high level of Bim protein (itself able to inactivate Mcl-1) sequestered

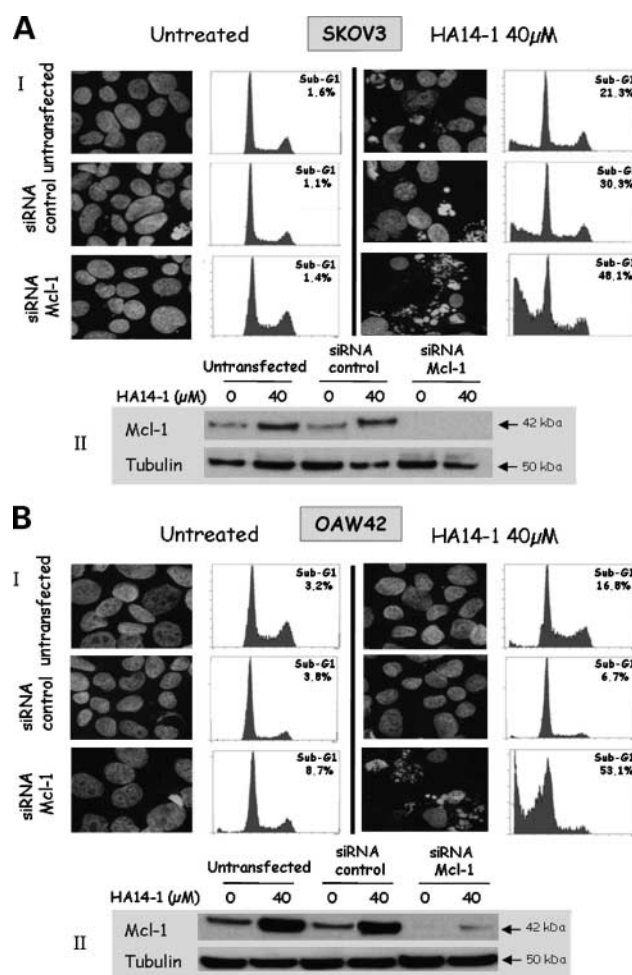


Figure 5. Mcl-1 downregulation by siRNA sensitizes to HA14-1. **A**, effect of siMcl-1 on Mcl-1 protein expression. SKOV3 and OAW42 cells were untransfected (control) or transfected with either an ineffective siRNA (siRNA control) or siMcl-1. Twenty-four hours later, Mcl-1 protein expression level was evaluated by Western blot. **B** and **C**, after siMcl-1 transfection, SKOV3 and OAW42 were exposed to 40 $\mu\text{mol/L}$ HA14-1 for 6 h. Morphologic/nuclear features and DNA content were studied as well as Mcl-1 expression level. Representative of three independent experiments.

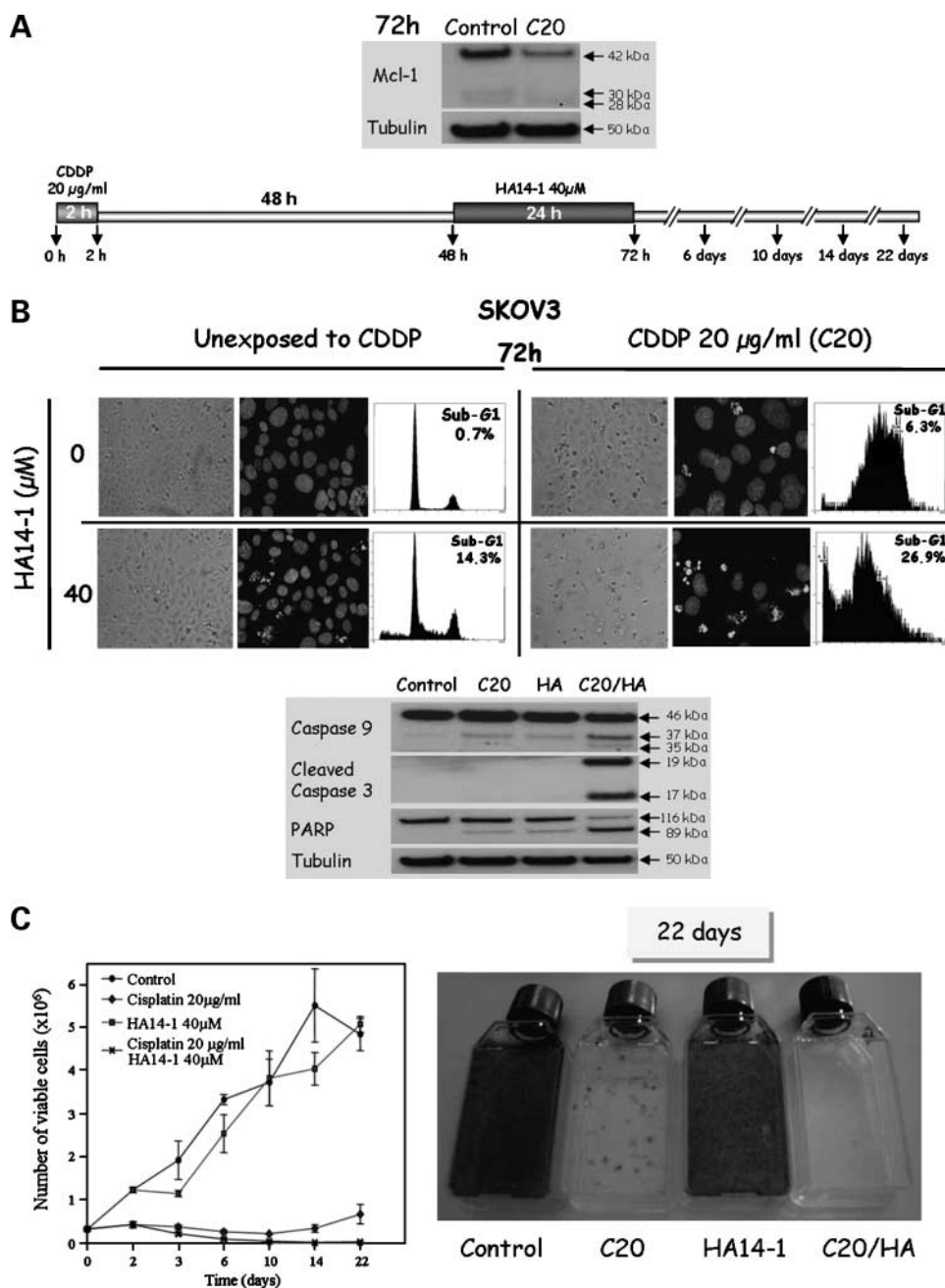


Figure 6. Effect of a treatment combining cisplatin exposure and HA14-1 treatment in SKOV3 cells. **A**, SKOV3 cell line was treated with CDDP (20 µg/mL) and Mcl-1 protein expression level was evaluated after 72 h by Western blot. Representative of two independent experiments. **B**, SKOV3 cells were treated with CDDP 20 µg/mL for 2 h; 48 h later, cells were treated with 40 µmol/L HA14-1 for 24 h. Morphologic/nuclear features and DNA content were studied as well as the cleavage of caspase-9, caspase-3, and PARP. **C**, proliferation resumption quantified by trypan blue and Giemsa coloration.

by Bcl-2 (33). Resistance to ABT-737 has also been correlated with Mcl-1 overexpression (34), and the experimental inhibition of Mcl-1 activity or expression led to sensitization to ABT-737 (35–37).

These results suggest that concomitant inhibition of both Bcl-2/ Bcl-x_L and Mcl-1 could be essential for BH3-mimetics induced cell death. This is also suggested by the observation realized with GX15-070 that binds with moderate affinity to all antiapoptotic Bcl-2 family members (including Mcl-1), because this compound potently induces apoptosis via disruption of both Bcl-x_L/Bak and Mcl-1/Bak complexes (38, 39).

To our knowledge, our study describes for the first time a downregulation of Mcl-1 in response to HA14-1 in sensitive cells. Such a downregulation has also been described in response to ABT-737 in myeloma cells (40), although the mechanisms involved in this Mcl-1 expression modulation remain unknown.

Mcl-1 is submitted to a rapid turnover (41), and the control of its expression could involve both transcriptional and post-translational mechanisms (42). The interaction of Mcl-1 with proapoptotic BH3-only members of Bcl-2 family or with the multidomain proapoptotic protein Bak is determinant for its

function. Its association with some of these partners will lead to stabilization (Bak, Puma, Bim, and Nbk/Bik; refs. 21, 43), whereas other will induce its degradation (Noxa; ref. 44). Mcl-1 degradation can involve the proteasome but can also be a consequence of caspase activity (45). We did not observe caspase-cleaved Mcl-1 fragment, and transcriptional downregulation has been also excluded (data not shown). Thus, we can conclude that Mcl-1 probably disappeared through proteasome activity either due to a modification of its partners after action of HA14-1 on Bcl-x_L or through a c-Jun NH₂-terminal kinase-associated phosphorylation as suggested by Inoshita et al. (46). The upregulation of Noxa and Puma observed in response to HA14-1 could argue in this way. The mechanisms involved in the regulation of these proteins by HA14-1 are currently under investigation.

We showed that Mcl-1 downregulation was essential to induce cell death in response to HA14-1 in SKOV3 and OAW42 cells and that concomitant inhibition of Bcl-x_L (by HA14-1) and Mcl-1 (by siMcl-1) led to apoptosis in the absence of other apoptotic signals, showing that SKOV3 and OAW42 cells were both properly "primed to death." These experiments showed that, in ovarian carcinoma cells, the absence of response to BH3-mimetics could be linked to a reinforced expression of Mcl-1 after exposure rather than to a defect in Bax/Bak activation. We also showed previously that the association of two siRNAs directed against Bcl-x_L and Mcl-1 induced a strong cell death in all cell lines (47), confirming that these two proteins constitute a molecular hurdle to oncogenic stress or chemotherapy-induced apoptosis.

In our models of chemoresistant ovarian carcinoma, the use of HA14-1 was initially conceived to sensitize cells to cisplatin rather than to induce apoptosis on its own. However, because Mcl-1 downregulation has been observed in resistant SKOV3 cells in response to cisplatin, we evaluated the effect of cisplatin on response to HA14-1. We showed that this combination led to massive cell death without recurrence. We did not observe transcriptional variation of Mcl-1 expression in response to cisplatin (12), suggesting a proteasome-dependent Mcl-1 downregulation as observed by others in renal tubular cancer cells exposed to this drug (48). The overexpression of Noxa and Puma also observed in SKOV3 cells in response to cisplatin could thus be involved in this downregulation (data not shown).

Such a platinum sensitization has also been described previously in ovarian carcinoma cells in response to ABT-737 (49). This effect was not associated with Mcl-1 downregulation but rather with cooperation between the apoptotic signal generated by carboplatin and the inhibition of Bcl-x_L activity by ABT-737. Platinum analogues could thus induce the activation of Bax/Bak proteins. We propose that, in complement to these events, the cisplatin-induced Mcl-1 downregulation constitutes an essential determinant of cell death in ovarian carcinoma cells in response to BH3-mimetics.

Bcl-x_L and Mcl-1 appear as key targets in ovarian carcinoma and BH3-mimetics could induce apoptosis in response to oncogenic stress and to chemotherapy, provided that Mcl-1 protein should be either inactivated or downregu-

lated. On this regard, HA14-1 is interesting because it could exert both a conventional BH3-mimetic effect and an indirect effect on Mcl-1. However, we underlined the possible escape to treatment and a subsequent acquired resistance to HA14-1 and associated this resistance to the loss of the mechanisms leading to the downregulation of Mcl-1 in response to treatment (data not shown). It thus appears that the combination of the exposure to a BH3-mimetic with a strategy leading to inactivation or downregulation of Mcl-1 is essential to ensure massive apoptotic cell death and to avoid therapeutic failure.

The development of multitargeted therapies directed against Bcl-x_L and Mcl-1 constitutes a major challenge for the therapeutic care of chemoresistant ovarian cancers. BH3-mimetics represent promising tools for this purpose either on their own in the case of pan-Bcl-2 family inhibitors or in association with new drugs leading directly or not to Mcl-1 inactivation. Such associations represent a real therapeutic hope, which now remains to be materialized.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
- Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007;7:573–84.
- Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov* 2005;4:307–20.
- Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008;9:47–59.
- Schwartz PS, Hockenbery DM. Bcl-2-related survival proteins. *Cell Death Differ* 2006;13:1250–5.
- Borner C. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Mol Immunol* 2003;39:615–47.
- Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007;26:1324–37.
- Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003;22:7265–79.
- Liu JR, Fletcher B, Page C, Hu C, Nunez G, Baker V. Bcl-x_L is expressed in ovarian carcinoma and modulates chemotherapy-induced apoptosis. *Gynecol Oncol* 1998;70:398–403.
- Marone M, Scambia G, Mozzetti S, et al. bcl-2, bax, bcl-x_L, and bcl-x_S expression in normal and neoplastic ovarian tissues. *Clin Cancer Res* 1998;4:517–24.
- Williams J, Lucas PC, Griffith KA, et al. Expression of Bcl-x_L in ovarian carcinoma is associated with chemoresistance and recurrent disease. *Gynecol Oncol* 2005;96:287–95.
- Villedieu M, Louis MH, Dutoit S, et al. Absence of Bcl-x_L downregulation in response to cisplatin is associated with chemoresistance in ovarian carcinoma cells. *Gynecol Oncol* 2007;105:31–44.
- Mohammad R, Giri A, Goustin AS. Small-molecule inhibitors of bcl-2 family proteins as therapeutic agents in cancer. *Recent Patents Anticancer Drug Discov* 2008;3:20–30.
- Wang JL, Liu D, Zhang ZJ, et al. Structure-based discovery of an

- organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci U S A* 2000;97:7124–9.
15. An J, Chen Y, Huang Z. Critical upstream signals of cytochrome *c* release induced by a novel Bcl-2 inhibitor. *J Biol Chem* 2004;279:19133–40.
 16. Chen J, Freeman A, Liu J, Dai Q, Lee RM. The apoptotic effect of HA14-1, a Bcl-2-interacting small molecular compound, requires Bax translocation and is enhanced by PK11195. *Mol Cancer Ther* 2002;1:961–7.
 17. Oliver L, Mahe B, Gree R, Vallette FM, Juin P. HA14-1, a small molecule inhibitor of Bcl-2, bypasses chemoresistance in leukaemia cells. *Leuk Res* 2007;31:859–63.
 18. Manero F, Gautier F, Gallenne T, et al. The small organic compound HA14-1 prevents Bcl-2 interaction with Bax to sensitize malignant glioma cells to induction of cell death. *Cancer Res* 2006;66:2757–64.
 19. Lickliter JD, Wood NJ, Johnson L, et al. HA14-1 selectively induces apoptosis in Bcl-2-overexpressing leukemia/lymphoma cells, and enhances cytarabine-induced cell death. *Leukemia* 2003;17:2074–80.
 20. Poulain L, Lincet H, Duigou F, et al. Acquisition of chemoresistance in a human ovarian carcinoma cell is linked to a defect in cell cycle control. *Int J Cancer* 1998;78:454–63.
 21. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-x_L, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005;19:1294–305.
 22. Reiners JJ, Jr., Kessel D. Susceptibility of myelomonocytic leukemia U937 cells to the induction of apoptosis by the non-peptidic Bcl-2 ligand HA14-1 is cell cycle phase-dependent. *Cancer Lett* 2005;221:153–63.
 23. Skommer J, Wlodkowic D, Matto M, Eray M, Pelkonen J. HA14-1, a small molecule Bcl-2 antagonist, induces apoptosis and modulates action of selected anticancer drugs in follicular lymphoma B cells. *Leuk Res* 2006;30:322–31.
 24. Kessel D, Reiners JJ, Jr. Initiation of apoptosis and autophagy by the Bcl-2 antagonist HA14-1. *Cancer Lett* 2007;249:294–9.
 25. Maiuri MC, Ciriollo A, Tasdemir E, et al. BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L). *Autophagy* 2007;3:374–6.
 26. Certo M, Del GM V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 2006;9:351–65.
 27. Hetschko H, Voss V, Horn S, Seifert V, Prehn JH, Kogel D. Pharmacological inhibition of Bcl-2 family members reactivates TRAIL-induced apoptosis in malignant glioma. *J Neurooncol* 2008;86:265–72.
 28. Pei XY, Dai Y, Grant S. The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. *Leukemia* 2003;17:2036–45.
 29. Doshi JM, Tian D, Xing C. Structure-activity relationship studies of ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA 14-1), an antagonist for antiapoptotic Bcl-2 proteins to overcome drug resistance in cancer. *J Med Chem* 2006;49:7731–9.
 30. Shigemasa K, Katoh O, Shiroyama Y, et al. Increased MCL-1 expression is associated with poor prognosis in ovarian carcinomas. *Jpn J Cancer Res* 2002;93:542–50.
 31. Lee EF, Czabotar PE, van Delft MF, et al. A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. *J Cell Biol* 2008;180:341–55.
 32. Real PJ, Cao Y, Wang R, et al. Breast cancer cells can evade apoptosis-mediated selective killing by a novel small molecule inhibitor of Bcl-2. *Cancer Res* 2004;64:7947–53.
 33. Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, Letai A. BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* 2007;12:171–85.
 34. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* 2006;10:375–88.
 35. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 2006;10:389–99.
 36. Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* 2007;67:782–91.
 37. Tahir SK, Yang X, Anderson MG, et al. Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. *Cancer Res* 2007;67:1176–83.
 38. Perez-Galan P, Roue G, Villamor N, Campo E, Colomer D. The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood* 2007;109:4441–9.
 39. Nguyen M, Marcellus RC, Roulston A, et al. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A* 2007;104:19512–7.
 40. Kline MP, Rajkumar SV, Timm MM, et al. ABT-737, an inhibitor of Bcl-2 family proteins, is a potent inducer of apoptosis in multiple myeloma cells. *Leukemia* 2007;21:1549–60.
 41. Yang-Yen HF. Mcl-1: a highly regulated cell death and survival controller. *J Biomed Sci* 2006;13:201–4.
 42. Warr MR, Shore GC. Unique biology of mcl-1: therapeutic opportunities in cancer. *Curr Mol Med* 2008;8:138–47.
 43. Shimazu T, Degenhardt K, Nur-E-Kamal A, et al. NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition. *Genes Dev* 2007;21:929–41.
 44. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, et al. Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* 2007;67:5418–24.
 45. Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005;37:267–71.
 46. Inoshita S, Takeda K, Hatai T, et al. Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem* 2002;277:43730–4.
 47. Brotin E, Meryet-Figuière M, Simonin K, et al. Bcl-x(L) and MCL-1 constitute pertinent targets in ovarian carcinoma and their concomitant inhibition is sufficient to induce apoptosis. *Int J Cancer*, Epub 2009 Jul 24.
 48. Yang C, Kaushal V, Shah SV, Kaushal GP. Mcl-1 is downregulated in cisplatin-induced apoptosis, and proteasome inhibitors restore Mcl-1 and promote survival in renal tubular epithelial cells. *Am J Physiol Renal Physiol* 2007;292:F1710–7.
 49. Witham J, Valenti MR, Haven-Brandon AK, et al. The Bcl-2/Bcl-x_L family inhibitor ABT-737 sensitizes ovarian cancer cells to carboplatin. *Clin Cancer Res* 2007;13:7191–8.

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