A Small-Molecule Inhibitor of Bcl-X<sub>L</sub> Potentiates the Activity of Cytotoxic Drugs In vitro and In vivo

Alex R. Shoemaker,1 Anatol Oleksijew,1 Joy Bauch,1 Barbara A. Belli,2 Tony Borre,1 Milan Bruncko,1 Thomas Deckwirth,2 David J. Frost,1 Ken Jarvis,1 Mary K. Joseph,1 Kennan Marsh,1 William McClellan,1 Hugh Nellen,1 ShiChung Ng,2 Paul Nimmer,1 Jacqueline M. O’Connor,1 Tilman Oltersdorf,2 Weiguo Qing,1 Wang Shen,1 Jason Stavropoulos,1 Stephen K. Tahir,1 Baole Wang,1 Robert Warner,1 Haichao Zhang,1 Stephen W. Fesik,1 Saul H. Rosenberg,1 and Steven W. Elmore2

1Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, Illinois and 2Idun Pharmaceuticals, San Diego, California

Abstract

Inhibition of the prosurvival members of the Bcl-2 family of proteins represents an attractive strategy for the treatment of cancer. We have previously reported the activity of ABT-737, a potent inhibitor of Bcl-2, Bcl-X<sub>L</sub>, and Bcl-w, which exhibits monotherapy efficacy in xenograft models of small-cell lung cancer and lymphoma and potentiates the activity of numerous cytotoxic agents. Here we describe the biological activity of A-385358, a small molecule with relative selectivity for binding to Bcl-X<sub>L</sub> versus Bcl-2 (K<i>i</i>'s of 0.80 and 67 nmol/L for Bcl-X<sub>L</sub> and Bcl-2, respectively). This compound efficiently enters cells and co-localizes with the mitochondrial membrane. Although A-385358 shows relatively modest single-agent cytotoxic activity against most tumor cell lines, it has an EC<sub>50</sub> of <500 nmol/L in cells dependent on Bcl-X<sub>L</sub> for survival. In addition, A-385358 enhances the in vitro cytotoxic activity of numerous chemotherapeutic agents (paclitaxel, etoposide, cisplatin, and doxorubicin) in several tumor cell lines. In A549 non-small-cell lung cancer cells, A-385358 potentiates the activity of paclitaxel by as much as 25-fold. Importantly, A-385358 also potentiates the activity of paclitaxel in vivo. Significant inhibition of tumor growth was observed when A-385358 was added to maximally tolerated or half maximally tolerated doses of paclitaxel in the A549 xenograft model. In tumors, the combination therapy also resulted in a significant increase in mitotic arrest followed by apoptosis relative to paclitaxel monotherapy. (Cancer Res 2006; 66(17): 8731-9)

Introduction

Defects in the ability to appropriately regulate apoptotic processes are one of the fundamental occurrences that underlie cancer (1). Tumor initiation, progression to metastatic disease, and resistance to chemotherapeutic intervention all have been linked to apoptotic dysregulation (2–7). The central mediators of apoptosis are the Bcl-2 family of proteins that are composed of multiple prosurvival (Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Mcl-1, and A1) as well as proapoptotic (Bax, Bak, Bad, Bid, Bim, Noxa, etc.) members (8). These proteins control apoptosis through a complex panoply of protein-protein interactions between the prosurvival members and a subset of the BH3-only proapoptotic class (e.g., Bim and Noxa; refs. 8–11). This, in turn, facilitates the function of the second class of proapoptotic proteins, Bax and Bak, resulting in the induction of a network of proteins (e.g., caspases) that execute cellular destruction (9, 10).

The elucidation of the three-dimensional structures of several Bcl-2 family members, as well as an improved understanding of the roles of the protein-protein interactions in regulating apoptosis, has suggested opportunities for developing chemotherapeutic agents that interfere with the prosurvival components of this process (12–19). Strategies currently under investigation include inhibition of protein expression as well as disruption of protein-protein interactions by the use of modified peptides, natural product analogues, and small organic molecules (20–27). An important unresolved question is whether inhibition of one or multiple prosurvival proteins will provide the optimal therapeutic advantage. The importance of Bcl-2 in neoplastic diseases is well established owing to the overexpression of this protein in multiple tumor types as well as the identification of the t(14;18) translocation as an initiating event in non-Hodgkin’s lymphoma (4, 28). The importance of Bcl-X<sub>L</sub> as a target is suggested by the fact that this protein is overexpressed in numerous tumor types and is associated with the development of disease progression (5, 29). In addition, Bcl-X<sub>L</sub> expression is strongly correlated with resistance to a large variety of chemotherapeutic agents, suggesting that inhibition of this protein could be especially important as a potentiator of chemotherapeutic activity (30–32).

Previously, we have reported the biological activity of ABT-737, a potent inhibitor of Bcl-2, Bcl-X<sub>L</sub>, and Bcl-w (27). This broad-spectrum Bcl-2 family inhibitor elicits robust single-agent activity in xenograft models, in addition to potentiating the activity of numerous cytotoxic agents. Here, we describe the activity of A-385358, a compound with relative selectivity for inhibition Bcl-X<sub>L</sub>. Although this compound does not show single-agent activity in vivo, it was found to effectively potentiate the activity of numerous cytotoxic agents in a variety of cancer cells. In addition, the combination of A-385358 plus paclitaxel resulted in improved in vivo efficacy relative to paclitaxel monotherapy.
Materials and Methods

A-385358
A-385358 [(R)-4-(3-dimethylamino-1-phenylsulfonylmethyl-propylamino)-N-[4-(4,4-dimethyl-piperidin-1-yl)-benzoyl]-3-nitro-benzensulfonamide; MW = 639.83] was synthesized as described (33).

Affinity for Bcl-2 Family Members

The affinity of A-385358 (and its enantiomer) for Bcl-XL and Bcl-2 was measured with a fluorescence polarization assay (34). The constructs used for Bcl-2 and Bcl-XL as well as the labeled probes used in the competition assays were previously described (27, 34).

Cellular Uptake and Localization

Cellular uptake. FL5.12 cells suspended in EMB growth medium containing 4% fetal bovine serum (FBS) were incubated at 37°C for 1 hour in 10 μmol/L A-385358. Compound concentration was determined by high-performance liquid chromatography before and after the 1-hour incubation following brief centrifugation. To analyze membrane-bound fractions following compound incubation, cells were washed once with 10 volumes of cold PBS and lysed with 4 mL of water. A-385358 concentration was determined from aliquots of lysate before and after centrifugation.

Cellular localization. NCI-H460 lung carcinoma cells grown on glass coverslips were stained first with 20 nmol/L MitoTracker Green FM (Ex/Em 490 nm/510 nm), a mitochondria-specific fluorescent dye, for 15 minutes. The cells were washed once with PBS and incubated with fresh medium containing 50 μmol/L of compound 1 [62-[4-(4,4-difluoro-5-thiophen-2-yl)-3a,4a-diaza-4-bora-s-indacen-3-yl]-phenoxy]-acetylamino(hexanoic acid (SR-[4-(4,4-dimethyl-piperidin-1-yl)-benzylsulfanamoyl]-2-nitro-phenylamino)-6-(phenylsulfanyl-hexyl)-amide), a close structural analogue of A-385358 conjugated to the fluorescent probe BODIPY Texas red-X. FL5.12 cells were propagated in RPMI 1640 supplemented with 10% FBS) and medium was added to another set. [3H]Paclitaxel (5 μmol/L; 0.5 μCi/mL final concentration) was added to all wells and the cells were incubated at 37°C for various periods of time. For washing experiments, cells were exposed first to [3H]paclitaxel for 2 hours. The cells were washed once with medium and then incubated with fresh medium with or without 50 μmol/L A-385358 at 37°C for various periods of time.

In vitro Activity

All tumor cell lines were obtained from the American Type Culture Collection (Menassas, VA) and cultured according to their recommendations. Cell viability was analyzed with the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega Corp, Madison, WI) according to the instructions of the manufacturer. Cells were plated in 96-well plates at 5,000 per well 24 hours before treating with compound. For the UV-C assay, A549 cells (1 × 105) were plated in 96-well plates in medium for 24 hours. Cells were then plated at 1 × 104 per well and serial dilutions of compound were added. Cell viability was measured with a fluorescence polarization assay (34). The constructs used for Bcl-2 and Bcl-XL as well as the labeled probes used in the competition assays were previously described (27, 34).

Results

Affinity of A-385358 for Bcl-XL and Bcl-2. The binding affinities of A-385358 for Bcl-XL and Bcl-2 were determined with fluorescence polarization assays, which measure the ability to displace fluorescein-labeled BH3 peptides. Under serum-free conditions, the Ki’s of A-385358 for Bcl-XL and Bcl-2 were 0.8 ± 0.17 (n = 5) and 67 ± 7 (n = 6) nmol/L, respectively. For Bcl-XL, this binding is comparable to the Bad BH3 peptide (1.2 nmol/L), whereas for Bcl-2 the affinity is ~7-fold less than that of the peptide (9.1 nmol/L). The enantiomer, which bears the opposite configuration of the dimethylamino ethyl group, was 20- and 300-fold less active against Bcl-XL and Bcl-2, respectively, and was used as a negative control in cellular assays. In contrast, the affinity of ABT-737 for both Bcl-XL and Bcl-2 was well below the detection limit of 0.5 and 1 nmol/L, respectively (27).
Cellular uptake and localization of A-385358. To show that A-385358 effectively enters cells and associates with the appropriate cellular target, uptake and co-localization studies were conducted in FL5.12 and NIH-H460 cells, respectively. A-385358 was added to a preparation of FL5.12 cells at a concentration of 10 μM/L and the cells were incubated at 37°C for 1 hour. Following centrifugation, drug concentration was determined in both the cellular and extracellular fractions by high-performance liquid chromatography analysis. The cell/extracellular ratio of 51.1 ± 2.7 indicates significant association of A-385358 with cells. To determine the distribution of A-385358 within cells, purified cellular preparations were lysed and centrifuged with compound concentration determined in the pellet and supernatant fractions. Thirty percent of the compound associated with cells was released on lysis, indicating that the majority of A-385358 was bound to cellular membranes.

To evaluate intracellular localization, a fluorescent probe (compound 1) was prepared from a structurally similar analogue to A-385358, which could be visualized by confocal microscopy. H460 cells were first stained with the mitochondrial marker MitoTracker Green FM and then treated with 50 μM/L compound 1 before confocal imaging of the unfixed cells. Analysis of the merged image shows that compound 1 colocalizes with MitoTracker Green at the mitochondria (Fig. 1).

A-385358 restores interleukin-3 dependency to FL5.12/Bcl-X<sub>L</sub> cells. FL5.12 is a murine pro-B lymphocytic cell line that is dependent on interleukin-3 (IL-3) for survival (35). However, overexpression of anti-apoptotic proteins such as Bcl-X<sub>L</sub> and Bcl-2 can protect against apoptosis that accompanies cytokine withdrawal in this cell line (31). Treatment of IL-3-deprived FL5.12/Bcl-X<sub>L</sub> cells for 24 hours with A-385358 resulted in cell killing with an EC<sub>50</sub> of 0.47 ± 0.05 μM/L (n = 68). This effect was accompanied by an increase in caspase-3 activity (data not shown). Consistent with the greater affinity for the Bcl-X<sub>L</sub> versus Bcl-2 hydrophobic grooves, the EC<sub>50</sub> of A-385358 for IL-3-depleted FL5.12/Bcl-X<sub>L</sub> cells (1.9 ± 0.1 μM/L; n = 55) was 4-fold higher relative to the cytokine-deprived FL5.12/Bcl-X<sub>L</sub> cells. In addition, A-385358 was more effective at stimulating cytochrome c release from mitochondria isolated from FL5.12/Bcl-X<sub>L</sub> versus Bcl-2 cells (data not shown). The EC<sub>50</sub> of the enantiomer was ~10 μM/L in both cell lines. These data suggest that low nanomolar binding affinity to the requisite Bcl-2 family member may be needed for efficient killing of these cells, and this is consistent with the observation that ABT-737 exhibited highly potent killing of both IL-3-deprived FL5.12/Bcl-X<sub>L</sub> and FL5.12/Bcl-2 cells (0.03 ± 0.01 and 0.007 ± 0.001 μM/L, respectively).

A-385358 enhances the activity of cytotoxic agents in cancer cells in vitro. We have previously reported that ABT-737 exhibits potent cell killing of small-cell lung cancer and lymphoid cells but displays rather weak single-agent activity against most other tumor cell lines. This in vitro potency translated into robust in vivo single-agent activity, particularly in xenograft models of small-cell lung cancer wherein complete regression of established tumors was observed (27). Like ABT-737, A-385358 was most active against small-cell lung cancer and leukemia cells, although A-385358 was significantly less potent than ABT-737 (Table 1). The EC<sub>50</sub> for A-385358 was ~20-fold lower than that of ABT-737 in H146 cells under serum-free conditions and A-385358 also exhibited a greater loss of potency in the presence of 10% serum (Table 1). The monotherapy activity of A-385358 was also examined in the H146 xenograft small-cell lung cancer model. When administered at 100 mg/kg/d, i.p., for 21 days, only modest inhibition of tumor growth was observed (Table 1). In contrast, ABT-737 at equivalent plasma exposure significantly inhibited tumor growth, including complete regression of a majority of tumors (Table 1; ref. 27).

Although A-385358 did not show significant single-agent activity, we sought to determine whether it inhibited Bcl-2 family proteins sufficiently to potentiate the effect of other chemotherapies (as measured by shifting the EC<sub>50</sub> and/or enhancing the extent of cell killing). Human tumor cell lines were incubated for 48 hours with varying concentrations of cytotoxic agents with or without A-385358 at concentrations ranging from 2.5 to 10.0 μM/L. The cellular EC<sub>50</sub> was determined by MTS assay. A-385358 was able to enhance by a factor of 2 or more the cell killing activity of paclitaxel and doxorubicin in A549 cells, of etoposide in A549, SN12C, and 786-O cells, and of cisplatin in SN12C, MiaPaCa-2, NCI-H226, and NCI-H322M cells (Table 2; Fig. 2). Interestingly, the ability of A-385358 to enhance chemotherapeutic cytotoxicity seemed to be schedule dependent. When coincubated for 48 hours, A-385358 potentiated (i.e., shifted the EC<sub>50</sub>) the activity of paclitaxel in A549 cells by a factor of 3.5. Under these same conditions, ABT-737 shifted the EC<sub>50</sub> of paclitaxel by 4-fold in A549 cells (27). However, when paclitaxel was given 24 hours before addition of 10 μM/L A-385358 with a subsequent 48 or 72 hours of co-incubation, the potentiation factor was increased to 15- and 26-fold, respectively (Fig. 2). The enantiomer did not potentiate the activity of paclitaxel at concentrations as high as 20 μM/L (data not shown). One possible explanation for these results was that A-385358 might have indirectly enhanced cell killing due to increased uptake or reduced efflux of the cytotoxic drug. To address this possibility, A549 cells were incubated with [3H]paclitaxel in the presence or absence of 50 μM/L A-385358.

Figure 1. Compound 1 co-localizes with mitochondria in NCI-H460 cells. NCI-H460 cells were costained with MitoTracker Green FM and compound 1 (a close structural analogue of A-385358 labeled with BODIPY Texas red-X) and imaged by confocal microscopy. A, localization of MitoTracker Green FM. B, localization of compound 1. C, merged image of the two stains.
As shown in Figure 2C, the kinetics and extent of paclitaxel uptake and release were not altered by the presence of A-385358. To more explicitly rule out drug-drug interactions as a cause for the observed chemopotentiation, A-385358 was also examined in combination with UV radiation. A549 cells were exposed to different doses of UV-C irradiation followed by 48-hour incubation with A-385358 at various concentrations. As was observed with the chemotherapeutic drug combinations, A-385358 enhanced cell killing by UV-C by ~2-fold (Table 2). Treatment of A549 cells with the enantiomer produced no enhancement of UV-C-mediated cell killing at concentrations up to 20 μmol/L (data not shown). In addition, A-385358 treatment increased the extent of kill induced by UV irradiation. The percentage of viable A549 cells was not significantly different at the two highest doses of UV-C tested (35 ± 6% at 16 mJ/cm² versus 29 ± 3% at 32 mJ/cm²). However, in the presence of 1.25 μmol/L A-385358, the fraction of viable cells

### Table 1. Potency of A-385358 and ABT-737 in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound</th>
<th>A-385358</th>
<th>A-385358-e*</th>
<th>ABT-737</th>
<th>ABT-737-e*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H146</td>
<td>Serum-free</td>
<td>0.35 ± 0.03 (2)</td>
<td>6.1 ± 0.1 (2)</td>
<td>0.015 ± 0.006 (5)</td>
<td>0.43 ± 0.12 (4)</td>
</tr>
<tr>
<td>10% HS</td>
<td>31 ± 6 (5)</td>
<td>62.3 (1)</td>
<td>0.09 ± 0.04 (18)</td>
<td>12 ± 5 (6)</td>
<td></td>
</tr>
<tr>
<td>%TGI in vivo</td>
<td>29</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molt-4</td>
<td>Serum-free</td>
<td>0.74 ± 0.23 (3)</td>
<td>5.5 ± 1.3 (3)</td>
<td>0.004 ± 0.002 (3)</td>
<td>0.46 ± 0.03 (3)</td>
</tr>
<tr>
<td>10% HS</td>
<td>32.8 ± 10.3 (2)</td>
<td>&gt;50 (2)</td>
<td>0.71 ± 0.44 (7)</td>
<td>22.2 ± 3.2 (3)</td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>Serum-free</td>
<td>0.21 ± 0.12 (4)</td>
<td>2.7 ± 0.5 (4)</td>
<td>0.002 (1)</td>
<td>0.87 (1)</td>
</tr>
<tr>
<td>10% HS</td>
<td>&gt;30 (2)</td>
<td>&gt;50 (2)</td>
<td>0.3 ± 0.1 (4)</td>
<td>17 ± 6 (3)</td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>Serum-free</td>
<td>16 ± 1 (2)</td>
<td>&gt;20 (2)</td>
<td>5.2 ± 0.2 (2)</td>
<td>12 ± 0 (2)</td>
</tr>
<tr>
<td>10% HS</td>
<td>&gt;100 (4)</td>
<td>&gt;100 (4)</td>
<td>22.3 ± 5.0 (3)</td>
<td>&gt;100 (3)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data are given as mean EC₅₀ (μmol/L) ± SE (n).
*The enantiomer for A-385358.
†The enantiomer for ABT-737.
*Cells grown in 10% human serum.
*Percent tumor growth inhibition. H146 tumors were size matched at ~225 mm³ in scid mice and A-385358 or ABT-737 was administered at 100 mg/kg/d, i.p., for 21 days. %TGI reported using tumor measurements at the end of the dosing period.

### Table 2. A-385358 enhances cell killing of various cytotoxic agents in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>Chemotherapeutic</th>
<th>A-385358 (μmol/L)</th>
<th>Potentiation factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>NSCL</td>
<td>Etoposide</td>
<td>10</td>
<td>13 ± 1 (2)</td>
</tr>
<tr>
<td>SN12C</td>
<td>Renal</td>
<td>Doxorubicin</td>
<td>10</td>
<td>3.6 ± 0.7 (5)</td>
</tr>
<tr>
<td>786-O</td>
<td>Renal</td>
<td>Cisplatin</td>
<td>5</td>
<td>3.3 ± 0.1 (2)</td>
</tr>
<tr>
<td>MaPaCa</td>
<td>Pancreas</td>
<td>Etoposide</td>
<td>5</td>
<td>3.3 ± 0.1 (2)</td>
</tr>
<tr>
<td>NCI-H226</td>
<td>NSCL</td>
<td>Cisplatin</td>
<td>5.1</td>
<td>2.6 (1)</td>
</tr>
<tr>
<td>NCI-H322M</td>
<td>NSCL</td>
<td>Cisplatin</td>
<td>2.5</td>
<td>2.6 ± 0.7 (2)</td>
</tr>
<tr>
<td>DLD-1</td>
<td>Colon</td>
<td>Cisplatin</td>
<td>10</td>
<td>2.0 ± 0 (2)</td>
</tr>
<tr>
<td>A549</td>
<td>NSCL</td>
<td>UV-C</td>
<td>2.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>A549</td>
<td>NSCL</td>
<td>UV-C</td>
<td>5</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>A549</td>
<td>NSCL</td>
<td>UV-C</td>
<td>10</td>
<td>2.2 ± 0.2</td>
</tr>
</tbody>
</table>

*Serum-free conditions unless otherwise specified; 48-hour co-treatment.
†Ratio of EC₅₀ for chemotherapeutic alone to EC₅₀ of chemotherapeutic in combination with A-385358; mean ± SE (n).
‡3% FBS.
was given i.p. at 15 or 30 mg/kg/d (the maximum tolerated dose) given at either of these doses (Fig. 3). A-385358 significantly enhanced the activity of paclitaxel when given in combination with paclitaxel at 30 mg/kg/d and at 30 mg/kg/d plus paclitaxel at 15 mg/kg/d resulted in nearly complete inhibition of tumor growth during the majority of the A-385358 therapy period (Fig. 3C). After termination of therapy, the tumor growth rate increased and paralleled that observed for the paclitaxel monotherapy group. The efficacy observed in this combination was quite comparable to that observed for paclitaxel given at the maximum tolerated dose. In contrast, the combination of paclitaxel at 30 mg/kg/d plus A-385358 at 75 mg/kg/d led to regression of these established tumors (Fig. 3D). In the period following the last dose of paclitaxel, the average tumor size was reduced to ~200 mm³ from an initial peak of 400 mm³. Tumor growth then slowly increased to eventually parallel that observed for paclitaxel monotherapy. The enhanced inhibition of tumor growth as measured by ratios of tumor size (%T/C = 25) and tumor growth delay (%ILS = 180) were both highly significant compared with the effects observed with the maximum tolerated dose of paclitaxel given alone.

To interpret the significance of combination therapy in vivo, it is important to consider potential drug-drug interactions that could have affected the pharmacokinetic properties of the cytotoxic agent. Co-administration of A-385358 plus paclitaxel did not alter either the Cmax (30 μg/mL) or the AUC (13 μg h/mL) of paclitaxel relative to dosing with paclitaxel alone in scid mice (33). Thus, the improved efficacy observed with the combination of A-385358 plus paclitaxel was not the result of A-385358-mediated enhancement of paclitaxel exposure. The combination of A-385358 plus paclitaxel was also evaluated in the LX-1 squamous cell lung carcinoma model. Owing to the relatively high sensitivity of LX-1 tumors to paclitaxel therapy, A-385358 (at 100 mg/kg/d) was given in combination with paclitaxel at 5 mg/kg/d. Although the effect was less robust than that observed in the A549 model, A-385358 improved significantly the efficacy of monotherapy in LX-1-treated tumors (Fig. 3B). The 65% inhibition of tumor growth rate and 30% enhancement of tumor growth delay were both statistically significant relative to the results obtained for paclitaxel monotherapy. Previous work has shown that paclitaxel treatment of xenograft tumors results in a characteristic mitotic arrest followed by apoptosis. In breast and ovarian tumors, the peak mitotic arrest and apoptosis occur ~8 to 10 and 18 to 24 hours after a single dose of paclitaxel, respectively (36). To examine the in vivo cellular response to A-385358, established A549 tumor–bearing animals were treated with a single dose of A-385358, paclitaxel, or both, and tumors were harvested at various times after treatment (Fig. 4).
To analyze mitotic arrest, immunohistochemistry was done using an MPM-2 antibody that recognizes several phosphoproteins expressed specifically during mitosis (37). The apoptotic response was analyzed with an antibody specific for the activated form of caspase-3. For paclitaxel given alone, peak mitotic arrest was observed ~24 to 30 hours after treatment (Fig. 4B). When A-385358 was given in combination with paclitaxel, the timing of arrest was similar to that observed with paclitaxel monotherapy; however, the percentage of MPM-2-positive cells was increased by ~2-fold. Analysis of caspase-3 expression showed that the peak apoptotic index following paclitaxel treatment occurred at 30 hours (Fig. 4C). However, addition of A-385358 seemed to extend the apoptotic response. Whereas the apoptotic response had returned to basal levels by 36 hours after treatment with paclitaxel, significant numbers of caspase-3-positive cells were still observed at 36 hours in the combination treatment group (Fig. 4C).

**Discussion**

Although dysregulation of the Bcl-2 family of apoptosis regulators has long been implicated in the genesis and progression of cancer, the specific functions of these genes in this disease process remain poorly understood. Numerous studies have reported the overexpression of Bcl-2 in lymphoma, lung, and colon cancer (28, 38, 39). Furthermore, increased Bcl-2 expression is associated with disease progression including the emergence of metastatic disease and the development of hormone refractory breast and prostate cancer (40, 41). Both Bcl-2 and Bcl-X<sub>L</sub> are also implicated strongly in chemoresistance involving multiple classes of cytotoxic agents in numerous tumor types (2, 42, 43). Indeed, Amundson et al. (30) showed that Bcl-X<sub>L</sub> expression correlates with resistance to more than 100 standard chemotherapy agents. This correlation was p53 independent and was of higher statistical significance than the correlation with p53 mutational status.

It has now been established with ABT-737 that broad-spectrum Bcl-2 family inhibitors can elicit significant single-agent activity, both *in vitro* and *in vivo*, in addition to potentiating the activity of chemotherapeutic agents (27). The present study was undertaken to evaluate the biological activity of a compound with a more Bcl-X<sub>L</sub>-specific selectivity profile. The results presented here with A-385358 show that a compound with potent affinity for Bcl-X<sub>L</sub> is indeed sufficient to enhance the activity of a variety of chemotherapeutic agents in diverse cancer cell lines.

To show mechanism-based activity in cells, A-385358 was shown to co-localize to the mitochondrial membrane and to reverse Bcl-X<sub>L</sub>-mediated protection of FL5.12 cells from cytokine withdrawal (Fig. 1). Although A-385358 and ABT-737 exhibit similar patterns of sensitivity across a diverse panel of tumor cell lines, ABT-737 is
significantly more potent than A-385358 in all cell lines tested (Table 1; ref. 27). Furthermore, ABT-737 monotherapy of small-cell lung cancer xenografts resulted in highly significant inhibition of tumor growth whereas A-385358 had only modest single-agent activity (Table 1; ref. 27). As ABT-737 is a more potent inhibitor of both Bcl-2 and Bcl-X<sub>L</sub>, it is possible that the stronger biological activity in human tumor cell lines associated with ABT-737 is attributable, at least in part, to the stronger affinity for Bcl-X<sub>L</sub>.

The analysis of A-385358 plus cytotoxic agents <i>in vitro</i> showed an especially strong potentiation of paclitaxel in A549 cells (Fig. 2). This effect was most pronounced when cells were preincubated with paclitaxel before addition of the Bcl-X<sub>L</sub> inhibitor. To determine whether this potentiation could translate to enhanced antitumor efficacy <i>in vivo</i>, a series of experiments were conducted in xenograft models (Fig. 3). These results indicate that A-385358 can indeed potentiate the efficacy of paclitaxel on both an early-treatment schedule as well as on established tumors. In the early-treatment model, complete inhibition of tumor growth was observed during treatment with A-385358, whereas in the staged model tumor regression was observed (Fig. 3). Interestingly, ABT-737 and A-385358 showed a similar extent of potentiation when coinubated with paclitaxel in A549 cells <i>in vitro</i>, although ABT-737 is a more potent inhibitor of both Bcl-2 and Bcl-X<sub>L</sub>. ABT-737 also significantly enhances the <i>in vivo</i> efficacy of numerous cytotoxic agents in xenograft models, including antimitotic agents (27).

Although paclitaxel is efficacious in the A549 xenograft tumor model, this activity is rather weak relative to that observed in many other models. Furthermore, A549 tumors seem to be generally chemoresistant as they show little or no response to a variety of other chemotherapeutic agents, despite the fact that these cells express functional p53 (44). Although A549 cells express relatively low levels of Bcl-2, expression of Bcl-X<sub>L</sub> is among the highest reported for the NCI-60 cell line panel, suggesting that Bcl-X<sub>L</sub> may be a key component of drug resistance in this tumor (30, 45). There does not seem to be a direct correlation between absolute levels of Bcl-X<sub>L</sub> expression and extent of potentiation of other cytotoxic agents. However, it will be important to characterize drug-induced changes in Bcl-X<sub>L</sub> expression in each of the other tumor lines examined. A recent report has suggested that a key component of resistance to paclitaxel therapy in tumors with activated RAS (such as A549) may involve targeted degradation of the proapoptotic protein Bim (46). An additional link between the activity of paclitaxel and the function of Bcl-2 family members involves the finding that reduced activity of Bcl-2 and/or Bcl-X<sub>L</sub> may result from paclitaxel-mediated phosphorylation of these proteins (47–50). Thus, the combination of paclitaxel and A-385358 may have cooperative effects on the inhibition of prosurvival Bcl-2 family proteins and the consequences of these effects may be especially profound in RAS mutant tumors such as A549. Efficacy in the RAS mutant LX-1 model showed that the ability of A-385358 to potentiate paclitaxel is not unique to A549 tumors. It will be of interest to determine if the correlation between RAS status and sensitivity to A-385358 extends beyond potentiation of paclitaxel. Preliminary studies have shown a significant but transient enhancement of gemcitabine activity by...
A-385358 in the \textit{Ras} mutant MiaPaCa-2 xenograft model (data not shown).

When studying the biological activity of two compounds given in combination, it is important to eliminate the possibility of contributions that are the result of drug-drug interactions. Through several lines of experimentation, we have shown that the activity of A-385358 is not the result of such indirect effects. First, the examination of $^{3}H$-paclitaxel in A-549 cells showed that A-385358 does not influence either the uptake or efflux of paclitaxel in these cells (Fig. 2C). Second, analysis of drug levels in mice showed that paclitaxel plasma concentration was not significantly altered by co-dosing with A-385358 (33). This result is important to rule out the possibility of metabolic interactions and/or inhibition of drug transporters. Finally, in vitro analysis of A-385358 in combination with UV-C irradiation showed potentiation similar to that observed in combination with various cytotoxic drugs (Table 2).

Immunohistochemical analysis of A-549 tumors showed that A-385358 enhances the apoptotic activity of paclitaxel (Fig. 4). Milas et al. (36) showed that treatment with paclitaxel leads to significant apoptosis that is preceded by mitotic arrest. Addition of A-385358 seemed to extend the apoptotic response with significant caspase-3 activity observed from 24 to 36 hours after a single treatment with both paclitaxel and A-385358. Interestingly, although the timing of mitotic arrest (as observed with MPM-2 staining) was similar with cotreatment, the absolute level of MPM-2 staining was increased following treatment with A-385358 (Fig. 4). These results suggest that MPM-2 staining may detect cells in the transition from mitotic arrest to apoptosis or that alteration of proapoptotic Bcl-X\textsubscript{L}/Bcl-2 function by treatment with A-385358 may in itself influence the onset and/or duration mitotic arrest following treatment with paclitaxel.

We have described here a small-molecule inhibitor of Bcl-X\textsubscript{L} that potentiates the activity of cytotoxic agents both in vitro and in vivo. These results extend our previous findings reported with ABT-737 and show that potent inhibition of Bcl-X\textsubscript{L} alone is sufficient to significantly potentiate the activity of chemotherapeutic agents both in vitro and in vivo. The future development of agents with varying selectivity profiles for inhibition of Bcl-2 family proteins will augment our understanding of the functions of these proteins in cancer development and progression and facilitate the development of effective proapoptotic cancer therapies.

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References


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Alex R. Shoemaker, Anatol Oleksijew, Joy Bauch, et al.


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