Phosphorothioate oligodeoxynucleotides and G3139 induce apoptosis in 518A2 melanoma cells

Johnathan C. Lai,2 Luba Benimetskaya,1 Anastasia Khvorova,2 Sijian Wu,1 Emily Hua,1 Paul Miller,4 and C.A. Stein1

1Department of Oncology, Montefiore Medical Center, Albert Einstein-Montefiore Cancer Center, Bronx, New York; 2Department of Biomedical Engineering, Columbia University, New York, New York; 3Dharmacon Research, Lafayette, Colorado; and 4Johns Hopkins University School of Public Health, Baltimore, Maryland

Abstract

In a previous study, we showed that G3139, an antisense phosphorothioate oligonucleotide that down-regulates the expression of Bcl-2 protein, did not cause chemosensitization of 518A2 melanoma cells. In this work, we show that G3139, and the 2-base mismatch, G4126, can initiate apoptosis in this and other melanoma cell lines as shown by increased cell surface Annexin V expression, typical nuclear phenotypic changes as assessed by 4′,6-diamidino-2-phenylindole staining, activation of caspase-3 (but not caspase-8) and Bid, appearance of DEVDase (but not IETDase) activity, and cleavage of poly(ADP-ribose)-polymerase 1. Depolarization of the mitochondrial membrane occurs as a relatively late event. All of these processes seem to be substantially, but perhaps not totally, Bcl-2 independent as shown by experiments employing an anti-Bcl-2 small interfering RNA, which as shown previously down-regulated Bcl-2 protein expression but did not produce apoptosis or chemosensitization in melanoma cells. In fact, these G3139-induced molecular events were not dramatically altered in cells that forcibly overexpressed high levels of Bcl-2 protein. Addition of irreversible caspase inhibitors (e.g., the pan-caspase inhibitor zVAD-fmk) to G3139-treated cells almost completely blocked cytotoxicity. Examination of the time course of the appearance of caspase-3 and cleaved poly(ADP-ribose)-polymerase 1 showed that this could be correlated with the release of cytochrome c from the mitochondria, an event that begins only ~4 hours after the end of the oligonucleotide/LipofectAMINE 2000 5-hour transfection period. Thus, both G3139 and cytotoxic chemotherapy activate the intrinsic pathway of apoptosis in these cells, although Bcl-2 expression does not seem to contribute strongly to chemo-resistance. These findings suggest that the attainment of G3139-induced chemosensitization in these cells will be difficult. [Mol Cancer Ther 2005;4(2):305–15]

Introduction

The cellular expression of the Bcl-2 protein has been associated at least in some instances with resistance to cytotoxic chemotherapeutic agents (1–5). The generation of resistance could occur due to the ability of Bcl-2 [which may be located on either the outer (6, 7) or the inner mitochondrial membrane (8, 9)] to inhibit the oligomerization of the proapoptotic proteins Bax and Bak (10, 11). Such inhibition would be predicted to limit the ability of Bax and Bak to form channels in the mitochondrial membrane (12). Lack of channel formation should prevent the permeabilization of the outer mitochondrial membrane to cytochrome c, which is one of the initiators of caspase activation (13–15).

The down-regulation of Bcl-2 protein expression has been an important and popular objective in anticancer drug development (16–20). G3139, an 18mer phosphorothioate oligonucleotide that successfully targets the initiation region of the Bcl-2 mRNA (16, 21–28), has been evaluated in combination with dacarbazine in a phase II study (29, 30). Despite this, a 771-patient randomized phase III trial of dacarbazine with or without G3139 in advanced melanoma produced equivocal results.5 However, the mechanism of action of G3139 is actually quite complex (23, 31) and depending on the model employed may be predominately due to non-sequence-specific effects (31–35). In additional studies done in 518A2 melanoma cells (the line that formed the basis of the original phase II trial of dacarbazine with or without G3139; ref. 30), down-regulation of Bcl-2 protein and mRNA expression by >90% by an anti-Bcl-2 small interfering RNA (siRNA; refs. 36, 37) did not lead to either cytotoxicity or chemosensitization (38). Furthermore, down-regulation of Bcl-2 protein expression by the siRNA before G3139 treatment did not alter the cytotoxic properties of G3139. This was true even in cells forced to markedly overexpress Bcl-2 protein; G3139 produced cellular cytotoxicity (in the absence of any significant Bcl-2 protein down-regulation) to the same extent in these Bcl-2-overexpressing cells as it produced in mock-transfected cells. In addition, forced overexpression of Bcl-2 also produced only a relatively small

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increment in chemoresistance. Thus, the value of Bcl-2 as a target in 518A2 melanoma cells does not, given the current data, seem to be clear as it also does not in other cell lines (39).

Indeed, the value of Bcl-2 as a target in clinical melanoma is also questionable. The protein is expressed in normal melanocytes (40, 41) and in early-stage malignant lesions, but some data do not support a difference in Bcl-2 expression as a function of disease progression, whereas other studies suggest that Bcl-2 levels actually decrease (42). On the other hand, one study has shown that patients whose lymph node metastases express Bcl-2 (43) have a poorer prognosis than those whose lymph node metastases do not express Bcl-2. However, the number of samples evaluated in this study (melanoma cells from only 15 patients of 42 sampled expressed Bcl-2 protein) is too small to draw any firm conclusions. Thus, actual function of Bcl-2 in melanomas of any stage must currently be considered unknown.

In this work, we show that G3139 and G4126, as members of the class of phosphorothioate oligonucleotides, induce apoptosis in 518A2 and other melanoma cells. This induction of apoptosis is associated with release of cytochrome c from the mitochondrion (which at early times after treatment occurs in the absence of mitochondrial membrane depolarization), activation of caspase-3 (but not caspase-8), and cleavage of Bid to tBid. In addition, our data strongly suggest that the reason that it is difficult to observe chemosensitization in 518A2 melanoma cells treated with phosphorothioate oligonucleotides is because these cells are “destined” for apoptosis relatively early via activation of the intrinsic pathway, which is also activated by cytotoxic chemotherapy.

Materials and Methods

**Cells**

The *Mycoplasma*-free human melanoma cell line 518A2 was a kind gift of Dr. Volker Wacheck (University of Vienna, Vienna, Austria). Cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin G sodium and 100 μg/mL streptomycin sulfate. 346.1, 201.2, and 333.1 melanoma cells were obtained directly from patients by Dr. John Kirkwood (University of Pittsburgh, Pittsburgh, PA), expanded, and frozen. These lines were grown in RPMI (Invitrogen) supplemented as above and with the inclusion of 1% of both sodium pyruvate and nonessential amino acids. Stock cultures of all cells were maintained at 37°C in a humidified 5% CO₂ incubator. 518A2 cells were transfected with the neomycin-selectable pSFFV/Bcl-2 plasmid (courtesy of S. Korsmeyer, Dana-Farber Cancer Center, Boston, MA) or with the control, neomycin-resistant expression vector pSFFV. Aliquots containing 10 μg plasmid and 5 μg/mL LipofectAMINE 2000 in serum-free Opti-MEM were added to the cultured cells. The transfection medium was replaced 5 hours later with complete medium. Individual colonies were selected from the plates after ~3 to 4 weeks of routine maintenance in complete medium containing 0.6 μg/mL G418 sulfate (Invitrogen).

**Reagents**

The anti-Bcl-2 monoclonal antibody was purchased from DAKO (Carpinteria, CA), and the anti-Cip, anti-Kip, anti-β-gal (ADP-ribosyl)polymerase 1 (PARP-1), anti-lamin B, anti-caspase-3, anti-caspase-8, and anti-Bid antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-α-tubulin monoclonal antibody and thapsigargin were from Sigma-Aldrich (St. Louis, MO). LipofectAMINE 2000 was from Invitrogen. SUPERase In is a product of Ambion (Austin, TX). The Annexin V-FITC apoptosis detection kit was from BD Biosciences (San Diego, CA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetracyethylbenzimidazolocarbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR). Phosphorothioate oligo-nucleotides were kindly supplied by Genta (Berkeley Heights, NJ). All siRNAs were supplied by Dharmacon Research (Lafayette, CO).

**Oligonucleotide Transfections**

Cells were seeded the day before the experiment in six-well plates at a density of 15 × 10⁴ cells per well at 60% to 70% confluence on the day of the experiment. All transfections were done in Opti-MEM medium (Invitrogen) plus complete medium without antibiotics as described previously (23). The incubation time for oligonucleotide/LipofectAMINE 2000 complexes was 5 hours. The total incubation time before cell lysis and protein isolation was between 24 and 72 hours at 37°C.

**siRNA Transfections**

Cells were seeded in complete medium without antibiotics the day before the experiment in six-well plates at a density of 15 × 10⁴ cells per well at 60% to 70% confluence on the day of the experiment. All transfections were done in Opti-MEM medium plus complete medium without antibiotics as described previously (21).

**Western Blot Analysis**

Cells treated with oligonucleotide-lipid complexes were extracted in lysis buffer at 4°C for 1 hour. Aliquots of cell extracts, containing 25 to 40 μg of protein, were resolved by SDS-PAGE and then transferred to Hybond enhanced chemiluminescence filter paper (Amersham, Arlington Heights, IL). After treatment with appropriate primary and secondary antibodies, enhanced chemiluminescence was done as described previously (23). The typical margin of error for a Western blotting is at least 20% to 25%.

**Determination of Rate of Cell Proliferation in Complete Medium**

Briefly, 0.4 × 10⁴ cells were seeded in 96-well plates and allowed to attach overnight. Cells were then treated with the appropriate concentrations of drugs. After 2 to 3 days of incubation at 37°C, 5-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis was done.
Absorbance was determined at 570 nm by a Benchmark plus Microplate spectrophotometer (Bio-Rad, Hercules, CA). Experiments were done in quadruplicate, and data are presented as averages ± SD.

**Annexin V Cell Surface Binding**

Cells treated for 48 hours with oligonucleotides were harvested after trypsinization, and apoptotic cells were assayed by flow cytometry as described previously (23) using the Annexin V-FITC apoptosis detection kit (Becton Dickinson, San Jose, CA). Early apoptotic cells bound to Annexin V-FITC but excluded propidium iodide. Cells in late apoptotic stages were labeled with both Annexin V-FITC and propidium iodide.

**Flow Cytometric Analysis of Mitochondrial Membrane Potential**

Mitochondrial membrane potential was determined by JC-1 fluorescence and analyzed in FL-1 and FL-2 channels of a FACScan (Becton Dickinson) flow cytometer equipped with a single 488 nm argon laser. Briefly, cells were trypsinized, washed in PBS, and resuspended in phenol red–free DMEM containing JC-1 at 10 μg/mL for 10 minutes at room temperature in the dark. Stained cells were washed once with PBS before the flow cytometric analysis. A minimum of 10,000 cells per sample were acquired using a log scale, and data were analyzed using CellQuest software (Becton Dickinson).

**Immunohistochemical Staining of Cytochrome c and 4′,6-Diamidino-2-Phenylindole Staining of Nuclei**

Cells were grown and treated in four-chamber slides. At indicated times, the cells were fixed for 15 minutes in 4% paraformaldehyde (Electron Microscopy Services, Fort Washington, PA), permeabilized with 0.5% Triton X-100/PBS for 5 minutes, and incubated for 1 hour in a 1% bovine serum albumin/PBS blocking solution. Cytochrome c was detected using a mouse monoclonal anti–cytochrome c IgG (Santa Cruz Biotechnology, 1:200 dilution), the binding of which was detected with a goat anti-mouse FITC-conjugated secondary antibody (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). The secondary antibody was used at a dilution of 1:200 with incubation for 1 hour. To visualize nuclei, cells were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (Molecular Probes). Cells were visualized with a BX60 Olympus fluorescence microscope (Olympus, Melville, NY), and all images captured with a SPOT RT charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI).

**Subcellular Fractionation**

Cells were harvested by trypsinization and washed with cold PBS. Cell pellets were resuspended in 300 μL buffer A [250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EGTA, 50 μg/mL Pefabloc, and 15 μg/mL leupeptin, aprotinin, and pepstatin]. Cells were then homogenized on ice in a Dounce homogenizer until ~90% of cells were disrupted as judged by trypan blue staining. Crude lysates were centrifuged at 1,000 × g for 10 minutes at 4°C twice to remove nuclei and unbroken cells. The supernatant was collected in a new tube and was subjected to a 10,000 × g centrifugation for 30 minutes at 4°C. The supernatant was collected as the cytosolic fraction, and the mitochondrial pellets were resuspended in 30 μL buffer A.

**Caspase-3/7 and Caspase-8 Activity Assay**

Caspase-3/7 and caspase-8 activity was measured colorimetrically using the spectrophotometric detection of the p-nitroaniline chromophore after its cleavage by caspases from the labeled caspase-specific substrates according to the manufacturer’s protocol (BD Biosciences). Caspase-3 is specifically inhibited irreversibly by the DEVD-fmk and caspase-8 by IETD-fmk. These inhibitors were used as the negative controls.

**Results**

**G3139, Somewhat More Rapidly than G4126, Induces Apoptosis in 518A2 Melanoma Cells after Transfection with LipofectAMINE 2000**

When 518A2 melanoma cells were treated with 40 μmol/L G3139 in the absence of carrier, no changes in cellular viability as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were observed. On the other hand, if 100 nmol/L G3139 were transfected into these cells in a complex with LipofectAMINE 2000 (1.9 μg/mL, 5 hours in Opti-MEM), substantial apoptosis was observed. To show apoptosis, we analyzed levels of Annexin V cell surface binding by flow cytometry 2 days after treatment for 5 hours with G3139 or G4126 (100 nmol/L/1.9 μg/mL LipofectAMINE 2000). The number of apoptotic cells (61%; Fig. 1A) treated with G3139 is ~2 times that of G4126 (29%), which in turn is ~3-fold that of untreated cells (11%). By 3 days, however, the differences between G3139 and G4126 almost disappear and the vast majority of the cells are either in early or late apoptosis (data not shown). Similar experiments with the phosphodiester analogue of G3139 or lipid alone showed little change from the control, untreated cells, demonstrating that the apoptosis is not a transfection or lipid artifact.5

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4′,6-Diamidino-2-stained cells treated with 100 nmol/L G3139/1.9 μg/mL LipofectAMINE 2000 showed hyperchromatic nuclei after 2 days, which to a lesser extent was also seen in G4126-treated cells (Fig. 1B). The apoptosis induced by G3139 at 2 days is associated with significant mitochondrial membrane depolarization as assessed by the green (FL-1 channel) shifting of JC-1 fluorescence (Fig. 2). This shift was somewhat more pronounced with G3139 versus G4126, the 2-base mismatch, and was not observed at all with PO-G3139.

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5 E. Anderson et al. Signatures of patterns of gene expression in prostate cancer cells after treatment with anti-bcl-2 and related phosphorothioate oligonucleotides and siRNAs, submitted for publication.
Induction of Apoptosis by G3139 Occurs via Activation of the Intrinsic Pathway

Apoptosis of 518A2 melanoma cells leads to cleavage of PARP-1 (producing an 85-kDa cleavage fragment) and cleavage of pro-caspase-3 to caspase-3 (17- and 19-kDa fragments) and Bid to tBid (16- and 14-kDa fragments) as shown by Western blotting (Fig. 3). The Bid-to-tBid cleavage is usually thought to be effected by activated caspase-8 (extrinsic pathway), but we have found no evidence for the cleavage of procaspase-8 to caspase-8 by Western blotting and no evidence of IEHDase activity in cell lysates by colorimetric assay (data not shown). Examination of the kinetics of PARP-1 cleavage, pro-caspase-3 activation, and Bid cleavage (Fig. 3) showed simultaneous appearance of the 85-kDa cleavage fragment of PARP-1, caspase-3, and tBid as rapidly as 9.5 hours after beginning of the transfection (i.e., 4 hours after its end). We also did these experiments in 346.1, 201.2, and 333.1 melanoma cells. In each cell line, virtually identical to what we observed in the 518A2 cell line, treatment with 200 nmol/L G3139 or G4126 led to cleavage of PARP-1 and caspase-3 after 7 to 9 hours in the absence of any down-regulation of Bcl-2 expression. Furthermore, in each case, the cells were morphologically apoptotic after 24 hours (data not shown).

In 518A2 cells, congruent with the Annexin V binding and mitochondrial membrane depolarization studies above, cleavage or activation of these proteins by G4126 was delayed relative to G3139, not appearing until 14 hours. One explanation for this delay could be that the cellular uptake of G3139/LipofectAMINE 2000 led to cleavage of pro-caspase-3 to caspase-3 and cleavage of PARP-1 after 7 to 9 hours in the absence of any down-regulation of Bcl-2 expression. Furthermore, in each case, the cells were morphologically apoptotic after 24 hours (data not shown).

Figure 1. A, flow cytometric analysis of oligonucleotide-treated 518A2 melanoma cells after fluoresceinated anti Annexin V monoclonal antibody binding (FL1-H) and propidium iodide uptake (FL3-H). Cells were treated with 100 nmol/L G4126, G3139, or the phosphodiester analogues of G4126 and G3139, complexed with LipofectAMINE 2000 (1.9 μg/mL) for 5 h, and then incubated for a further 43 h in complete medium without the complexes before the analysis. Representative of three separate experiments in which 10,000 cells were analyzed in each. Upper right quadrant, late apoptosis/early necrosis; lower right quadrant, early apoptosis; upper left quadrant, necrosis. B, 4’,6-diamidino-2-phenylindole staining of nuclei in cells treated with 100 nmol/L G3139 or G4126 complexed with LipofectAMINE 2000 (1.9 μg/mL) as described previously. Extensive hyperchromatic staining of the nuclei was observed in G3139-treated cells and to a lesser extent in G4126-treated cells.
The concentration dependence of the effects of G3139 on Bid, pro-caspase-3, and Cip-1 are shown in Fig. 4. For G3139, 100 nmol/L is the minimum concentration required for apoptosis in 518A2 cells. We were concerned that G3139 might be acting as a “prodrug” in the sense that 3'–nuclease activity could have released, albeit slowly, physiologically significant quantities of 3'–terminal deoxyriboadenosine monothiophosphate. However, experiments done with G4320, which is G3139 containing a highly nuclease-resistant 3'-3' inverted T, show little or no concentration-dependent differences between the two species.

We then colorimetrically measured the appearance of DEVDase activity (to evaluate the production of caspase-3/7) as a function of time (Fig. 5) and compared this with the percentage of cells with depolarized DC<sub>m</sub>. Similar to our related, previous data, there is a relative delay in the increase in DEVDase activity for G4126 versus G3139, but the two are essentially equivalent by ~24 hours and remain so until at least 48 hours after transfection. The appearance of DEVDase activity precedes mitochondrial membrane depolarization, which cannot be readily detected until ~18 hours by JC-1 staining, and is maximal only after 48 hours.

**Early Appearance of Cytochrome** c **in the Cytoplasm of Cells Treated with G3139**

Our data therefore indicate that after transfection of 518A2 cells with G3139 (and to a lesser extent G4126) procaspase-3 and Bid are cleaved, at least at the earlier time points, in the absence of mitochondrial membrane depolarization. To further examine this, we then studied the appearance of cytochrome c in the cytoplasm by immunohistochemistry (Fig. 6A) and by immunoblotting in cellular lysates (Fig. 6B) as a function of time. After treatment with G3139, but not as readily with G4126, cytochrome c can be observed by 9 hours, but not at 5 hours, both by fluorescence microscopy and by Western blotting in cellular lysates. By 24 hours, G3139-treated cells are extensively stained, and significant cytoplasmic staining is observed in G4126-treated cells. In addition, the cell nuclei are shrunken and in some cases fragmented, typically for cells undergoing apoptosis. These results were confirmed by immunoblotting of the lysates from three different cell lines (518A2, 201.2, and 333.1; Fig. 6B and C). The 346.1 cells seem to be exceptionally fragile and the production of uncontaminated subcellular fractions from them has been problematic. Cytochrome c (up to ~8-fold increase relative to untreated cells by 9 and 18 hours after the beginning of the transfection as measured by laser scanning densitometry) could be observed in the cytoplasmic fractions of both G3139-treated cells and, to a lesser extent, G4126-treated cells. However, the loss of cytochrome c from the mitochondria was relatively small as a proportion of the total present and could not be detected by immunoblotting of the mitochondrial fraction.

**Figure 2.** Analysis of mitochondrial membrane depolarization in 518A2 melanoma cells treated with G4126 and G3139 after 2 d. Percentage of cells (of 10,000 analyzed) with depolarized ΔΨ<sub>m</sub> was quantitated 2 d after oligonucleotide treatment. Cells were treated with 100 nmol/L G4126, G3139, or the phosphodiester analogues of G4126 and G3139, complexed with LipofectAMINE 2000 (1.9 µg/mL) for 5 h, and then incubated for a further 43 h in complete medium, without the complexes, before the analysis. Flow cytometric analysis of the shift in JC-1 fluorescence emission from red (FL2 channel) to green (FL1 channel) was done as described in Materials and Methods. Representative of three separate experiments. Columns, average cell number; bars, SD.

**Figure 3.** Representative Western blot analyses showing the time course of protein processing by G4126 and G3139 in 518A2 cells as shown by cleavage of PARP-1, procaspase-3, and Bid. Cells were treated with 100 nmol/L G4126 or G3139 complexed with LipofectAMINE 2000 (1.9 µg/mL) for 5 h. Total cellular proteins was subjected to Western blotting as described in Materials and Methods at the indicated time points. Average error in Western blot measurements is ~20–25%.
Release of Cytochrome c from the Mitochondria in G3139-Treated Cells Is Bcl-2 Independent

The release of cytochrome c from the mitochondria, which is probably responsible for initiation of the events that lead to caspase-3 cleavage, occurs 9 hours after the start of the transfection and cannot be related to Bcl-2 down-regulation (see Fig. 6C). As seen in Fig. 7A, in total cellular lysates of 518A2 cells, Bcl-2 protein expression is only ∼34% down-regulated by G3139 after 24 hours as opposed to 91% by 48 hours. However, mitochondrial levels of Bcl-2 are only minimally reduced by G3139 after 24 hours and by 40% after 48 hours. In contrast, in 518A2 cells treated with D6, an anti-Bcl-2-siRNA, mitochondrial-associated Bcl-2 protein is reduced by 90% by 3 days, although no apoptosis is observed (38). In neither case are mitochondrial levels of Bax protein altered (data not shown). These data show that the down-regulation of Bcl-2 expression, by itself, is not related to cellular apoptosis.

We then examined several other proteins in cells treated with G3139 or G4126 that are associated with the apoptosis process. As shown in Fig. 8A and B, levels of expression of Cip-1 and Kip-1 decrease after G3139 treatment, as does Bcl-xL, after 1 day (it recovers after 2 days). However, levels of total cellular Bax, Akt, and phospho-Akt are unchanged or only slightly diminished. No evidence of the cleavage of pro-caspase-3 to caspase-3 was observed.

Caspase Inhibitors Block G3139-Induced Cytotoxicity

We then examined the ability of the cell-permeable, irreversible caspase-3/7 inhibitor DEVD-fmk and the pan-caspase inhibitor zVAD-fmk to alter protein processing after treatment with G3139 and G4126. As shown in Fig. 9, DEVD-fmk (100 μmol/L) cannot reverse cleavage of PARP-1 or pro-caspase-3 or down-regulation of Cip-1. It does, however, prevent down-regulation of Kip-1 expression and most of the cleavage of Bid to tBid. As expected, the processing of pro-caspase-3 is not altered. These results show that in this cell line Bid processing is downstream of caspase-3/7 and not necessarily of caspase-8. This is confirmed in cells treated with cisplatin, an activator of pro-caspase-3 and Bid but not of caspase-8 (data not shown). Kip-1 down-regulation is also downstream of caspase-3, but we did not observe cleavage of either Kip-1 or Cip-1. On the other hand, after treatment with 100 μmol/L zVAD-fmk, no processing or down-regulation of these proteins was observed. In addition, treatment of 518A2 cells with either DEVD-fmk or zVAD-fmk almost completely maintains cellular viability, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 2 days after transfection of either G3139 or G4126 and significantly increases it after 3 days. In fact, it is likely that the decreases in viability after 3 days, even in the presence of the caspase inhibitors, are due to their instability in serum or to non-caspase-mediated induction of apoptosis (e.g., release of apoptosis-inducing factor or endonuclease G).

Bcl-2 Overexpression Does Not Block G3139-Induced Apoptosis in 518A2 Melanoma Cells

In previous experiments (38), we showed that in 518A2 cells that forcibly overexpress Bcl-2 only a relatively small amount of chemoresistance to cisplatin, gemcitabine, and thapsigargin can be observed. Furthermore, these cells are not resistant to G3139-induced cytotoxicity, although total cellular levels of Bcl-2 are not changed by G3139 treatment. In Fig. 10, we show that two 518A2-derived clones, B3 and B8, which express >1,000 and >100-fold the amount of Bcl-2 as in mock-transfected cells, also undergo PARP-1, pro-caspase-3, and Bid cleavage after G3139 treatment to almost the same extent as the wild-type and mock-transfected cells. However, Bcl-2 overexpression does seem to delay the initiation of pro-caspase-3 cleavage from 9.5 hours after the initiation of the transfection in the mock-transfected cells to ~15 to 18 hours in the B3 clone.
Discussion

Taken together, our data strongly indicate that in 518A2 melanoma cells G3139 relatively rapidly (within 9 hours) begins to activate the intrinsic pathway of apoptosis, resulting in cytochrome c release from the mitochondria, activation of caspase-3, and cellular demolition. These results are virtually identical in 346.1, 201.2, and 333.1 melanoma cells, which have not been passaged nearly as extensively as the 518A2 cells, and thus probably represent a closer relationship to clinical metastatic melanoma. This activation of caspase-3 occurs at much earlier time points than activation induced by cytotoxic chemotherapy, which may have to wait for progression through the cell cycle and may take as long as 24 hours.

Figure 6. A, immunohistochemical staining of cytochrome c in 518A2 cells treated with 100 nmol/L G4126 or G3139 complexed with LipofectAMINE 2000 (1.9 μg/mL). At the indicated times posttransfection, cells were costained with anti–cytochrome c antibody and 4’,6-diamidino-2-phenylindole and analyzed by fluorescence microscopy as described in Materials and Methods. Cytochrome c release could be detected as early as 9 h posttransfection in G3139-treated cells (white arrows). By 24 h, marked diffuse cytoplasmic staining of cytochrome c was evident in both G4126- and G3139-treated cells.

B, G3139 and G4126 induce mitochondrial release of cytochrome c as determined by cellular fractionation. 518A2 untreated cells (lane a) and cells treated with 100 nmol/L G4126 (lane b) or G3139 (lane c) complexed with LipofectAMINE 2000 (1.9 μg/mL), as described previously, were analyzed at indicated time points. Western blot analyses were done on mitochondrial pellets and cytosolic supernatants prepared as described in Materials and Methods. C, G3139 and G4126 induce the cleavage of PARP-1 and pro-caspase-3 as early as 9 h posttransfection in 201.2 and 333.1 melanoma cell lines. Accumulation of cytochrome c could be detected in the cytosolic supernatants of G3139-treated cells and to a lesser extent G4126-treated cells, in the absence of any changes in Bcl-2 protein expression after 9 h. Cells were either untreated (lane a) or treated with 200 nmol/L G4126 (lane b) or G3139 (lane c) complexed with LipofectAMINE 2000 (3.8 μg/mL) as described in the text.
Our data also indicate that there is a delay in induction of pro-caspase-3 and Bid cleavage for G4126 versus G3139, which can be correlated, respectively, with relatively diminished mitochondrial cytochrome c release, Annexin V cell surface binding, and depolarized $\Delta \Psi_m$. However, it seems unlikely, based on the experiments in which we employed the D6 siRNA to down-regulate Bcl-2 expression, that this delay is in fact due to any down-regulation of Bcl-2 expression. As shown, the D6 siRNA can cause down-regulation of 93% to 99% of the mitochondrial Bcl-2 without inducing apoptosis (or chemosensitization; ref. 38). (One third of cellular Bcl-2 protein are found on the outer mitochondrial membrane, with the remainder is located on the endoplasmic reticulum and nuclear envelope; refs. 44, 45). In contrast, G3139 can cause significant apoptosis when only ~40% of the mitochondrial Bcl-2 protein have been lost (Fig. 7).

The ability of caspase-3 to cleave Bid to tBid was first described by Slee et al. (46). Similar to caspase-8, cleavage by caspase-3 occurs at Asp59 and produces a COOH-terminal fragment (tBid) that inserts into the outer mitochondrial membrane (47, 48), a process that can result in the mitochondrial release of cytochrome c. Thus, a proapoptotic feedback loop may be established: release of cytochrome c leads to Apaf-1-mediated activation of caspase-9, its cleavage of procaspase-3 to caspase-3, and the latter’s cleavage of Bid to tBid, which then inserts in the mitochondrial membrane and promotes additional release of cytochrome c. Our data strongly suggest that a very similar process is occurring in 518A2 melanoma cells as well as in the other melanoma lines. It has also been suggested (49) that the cleavage of Bcl-2 by caspase-3 could cause mitochondrial release of cytochrome c. However, we have never been able to detect any Bcl-2 cleavage products by Western blotting.

The mechanism by which tBid can induce release of mitochondrial cytochrome c is not fully understood and is likely to be complex. Release may be caused by several factors, including induction of Bax (50) or Bak (51) oligomerization and its insertion into the mitochondrial membrane (50, 52) and interactions with Bax and lipids to form large openings in the outer mitochondrial membrane (53). However, we could not show insertion of Bax into mitochondria at any time after G3139 treatment, indicating that in 518A2 cells this process and the subsequent is probably Bax independent (54). Nevertheless, tBid can also cause cytochrome c release in Bax-deficient mitochondria (55). Others have suggested that the proapoptotic action of caspase-3 and caspase-9 could be potentiated by the presence of tBid.
of tBid is due to a direct interaction with the adenine nucleotide translocase, a member of the mitochondrial permeability transition pore complex (56), although this view has been challenged (55, 57).

tBid-induced cytochrome c release can also occur without loss of $\Delta \Psi_m$ (57, 58), and it seems that in general the release of mitochondrial cytochrome c does not require mitochondrial membrane depolarization (15). Indeed, because mitochondrial membrane depolarization can be blocked by caspase inhibitors (15, 57), it seems to be a caspase-dependent, and hence secondary, event.

Our findings in 518A2 melanoma cells are entirely consistent with these previous data. The appearance of tBid only 9 hours after the initiation of transfection with G3139 correlates with the synchronous appearance of caspase-3 and is probably a consequence of it. Cytochrome c release from the mitochondria, as shown by both immunohistochemical fluorescence and Western blotting of cytoplasmic lysates, also seems to begin at approximately this time. As suggested above, and as our data confirm, loss of $\Delta \Psi_m$ is a relatively late-occurring event and is probably a result of amplification of caspase activity that itself may originate from Bid-to-tBid cleavage by caspase-3 or other downstream caspases. This process, as we have shown, is independent of caspase-8 and hence independent of the activation of death receptors (48). Strikingly, forced overexpression of Bcl-2, while providing some apparent molecular protection (see Fig. 10), does not protect 518A2 cells against the phenotypic changes of apoptosis also probably because this process is amplified as a function of time once threshold levels of pro-death mediators are reached.

This early activation of the “intrinsic” pathway by G3139 (and to a lesser extent by G4126), relative to its rate of activation by cytotoxic chemotherapy, provides an answer as to why chemosensitization is not seen in these cells. In fact, it may be impossible to detect it because both cytotoxic chemotherapy and G3139 activate the intrinsic pathway, and after treatment with the latter, the 518A2 cells are already “marked” for death. Furthermore, although it may be possible that coactivation of the intrinsic and extrinsic pathways (by, for example, TRAIL or an anti-Fas antibody) could lead to chemosensitization, the extrinsic pathway terminates in Bid, which is already activated by G3139 treatment.

Finally, it should be noted that at this point these experiments cannot be extrapolated to other nonmelanoma cell lines, and data do not exist to make any statements about similar processes in vivo. Further extensive experimentation will be required to address these complex issues.

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**Figure 9.** A, representative Western blot analyses demonstrating the effects of the caspase-3/7 inhibitor DEVD-fmk and the pan-caspase inhibitor zVAD-fmk on protein expression in 518A2 cells treated with G4126 or G3139 (100 nmol/L). B, inclusion of either the caspase-3/7 inhibitor DEVD-fmk (100 nmol/L) or the pan-caspase inhibitor zVAD-fmk (100 nmol/L) to oligonucleotide (100 nmol/L)/LipofectAMINE 2000 (1.9 μg/mL) –treated cells can maintain cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 2 and 3 days. Columns, average ($n = 3$); bars, SD.

**Figure 10.** Analysis of induction of protein processing in a mock-transfected and Bcl-2-overexpressing 518A2 melanoma cells. Wild-type (Wt), mock-transfected (M4), and Bcl-2-overexpressing clones (B3 and B8, with >1,000 and >100-fold increase in Bcl-2 protein expression, respectively) were treated with 100 nmol/L G4126 or G3139 complexed with LipofectAMINE 2000 (1.9 μg/mL) for 5 h and then for a further 43 h in complete medium without the complexes before the analysis. Western blotting was done on total cellular protein as described in Materials and Methods.
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