The Small Organic Compound HA14-1 Prevents Bcl-2 Interaction with Bax to Sensitize Malignant Glioma Cells to Induction of Cell Death

Florence Manero,1 Fabien Gautier,1 Tristan Gallenne,1 Nicolas Cauquil,2 Danielle Grée,3 Pierre-François Cartron,1 Olivier Geneste,2 René Grée,3 François M. Vallette, and Philippe Juin1

1Institut National de la Santé et de la Recherche Médicale U601, Département de Recherche en Cancérologie, Nantes, France; 2Cancer Research, Institut de Recherches Servier, Croissy/Seine, France; and 3Université de Rennes 1, Synthèse et Electrosynthèse Organiques, Centre National de la Recherche Scientifique UMR 6510, Rennes, France

Abstract
A functional imbalance between proapoptotic Bax and antiapoptotic Bcl-2 is likely to participate in the resistance of cancer cells to therapy. We show here that ethyl 2-amino-6-bromo-4-[1-cyano-2-ethoxy-2-oxoethyl]-4H-chromene-3-carboxylate (HA14-1), a small organic compound recently proposed to function as an inhibitor of Bcl-2, increases the sensitivity of human glioblastoma cells to radiotherapy and chemotherapy. This sensitizing effect is lost if Bcl-2 expression, but not Bcl-xL expression, is knocked down or if cells only express a mutant of Bax that does not interact with Bcl-2. This points to a specific Bcl-2 inhibitory function of HA14-1 and implies that it selectively involves hindrance of Bcl-2 binding to Bax, which HA14-1 inhibits in cell-free assays and in cells in receipt of an apoptotic stimulation. Moreover, HA14-1, in combination with a cytotoxic treatment, slows down the growth of glioblastoma in vivo. Thus, the inhibition of Bcl-2 achieved by HA14-1 might improve treatment outcome. (Cancer Res 2006; 66(5): 2757-64)

Introduction
WHO grade 4 glioblastoma multiforme are the most frequent tumors in the central nervous system (1). Their prognosis is extremely unfavorable, and the therapeutic failure often observed might be associated, at least in part, to the intrinsic radioresistance and/or chemoresistance of these tumors (2, 3). Resistance to induction of apoptosis, a form of cell death critically regulated by members of the proto-oncogene Bcl-2 family, constitutes one major obstacle to radiotherapy and chemotherapy in many cancer cells (4). The expressions of Bcl-2 and its homologue Bcl-xL are elevated in human glioblastoma tumors compared with nonneoplastic glial cells (5), although, more specifically, an increase in the expression of Bcl-2 itself from low-grade astrocytoma (WHO grade 2 astrocytoma) to glioblastoma multiforme has been reported (6). Antisense approaches have implicated Bcl-2 and Bcl-xL in the survival of human malignant gliona cells to apoptotic stimuli (5, 7, 8).

A significant part of the survival function of Bcl-2 in cancer cells seems to reside in its ability to counterbalance the detrimental effects of its proapoptotic counterpart Bax, a multidomain protein that, on its activation by an apoptotic stimulus, acquires the ability to directly perturb mitochondrial membrane permeability and to promote the release of apoptogenic proteins from this organelle (9). Bax plays a major role in the apoptotic response of glioblastoma multiforme cells: the expression of a highly apoptogenic variant of Bax, BaxΔc, was shown to correlate with an increased survival of glioblastoma multiforme patients (10), whereas cells derived from the very rare glioblastomas in which Bax expression is silenced exhibit a marked resistance to multiple cell death inducers (11).

Current evidence indicates that the functional inhibition of Bax by Bcl-2 relies, in part, in the ability of a hydrophobic cleft formed by the BH1, BH2, and BH3 domains of the latter to bind to the conserved BH3 domain of proapoptotic Bcl-2 members (12). This type of interaction is proposed to allow Bcl-2 to (a) engage a group of proapoptotic Bcl-2 family members, the BH3-only proteins, preventing the BH3 domains of a functional subset of these proteins (e.g., Bid and Bim) by physically sequestering them from directly activating Bax, and (b) interact with Bax directly, thereby preventing it from exerting its deleterious effect on its activation by an apoptotic stimulus (13–15). Thus, molecules that would interfere with the ability of Bcl-2 to interact with BH3-only proteins, Bax or both, might help to overcome the mechanisms of survival conferred by Bcl-2 to cancer cells.

More than a dozen compounds that may occupy the hydrophobic cleft of Bcl-2 (or regions close to it) and interfere with the binding properties of this survival protein have been reported (16–18). Of these, ethyl 2-amino-6-bromo-4-[1-cyano-2-ethoxy-2-oxoethyl]-4H-chromene-3-carboxylate (HA14-1), a small organic compound of relatively simple chemical structure, was the first to be identified as a putative Bcl-2 inhibitor from in silico screens (19). Although HA14-1 was reported to sensitize leukemia cells to induction of death by diverse apoptotic stimuli (20–22), its in vitro effects on the apoptotic sensitivity of cells derived from highly resistant tumors, such as glioblastoma multiforme, and its ability to affect the growth of these solid tumors in vivo have, however, not been investigated to date. Moreover, the aforementioned studies did not show that the reported sensitizing effects of HA14-1 were mediated through Bcl-2 inhibition, leaving the question of whether HA14-1 specifically inhibits Bcl-2, and at which level, unanswered.

We provide evidence in this study that low concentrations of HA14-1 sensitize glioblastoma multiforme cells to induction of cell death by radiation and chemotherapy. The sensitizing activity of
HA14-1 requires Bcl-2 expression to manifest and involves the selective inhibition of the interaction between Bcl-2 and Bax. Moreover, HA14-1 synergizes with a chemotherapeutic agent to slow down the growth of glioblastoma multiforme cells in a xenograft mouse model. Thus, HA14-1 exerts Bcl-2 inhibitory activity in glioblastoma multiforme cells, and this compound or analogues thereof might be of particular utility in combination with a cytotoxic treatment to interfere with the growth of solid tumors that display high levels of this antiapoptotic protein.

Materials and Methods

Reagents and antibodies. Unless otherwise stated, all reagents used in this study were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Etoposide was from Teva Classics (Paris, France) and was used from a stock solution at 20 mg/mL in physiologic saline. Commercial antibodies used in this study are described in the figure legends. The polyclonal anti-Bax TL41 antibody was raised against the BH3 domain of Bax (residues 57-72; ref. 23). Horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence reagents were obtained from Amersham Biosciences (Orsay, France).

HA14-1 synthesis. The synthesis of HA14-1 was done following the method published by Yu et al. (24). For biological tests, HA14-1 was systematically prepared freshly in DMSO before dilution to appropriate concentrations in the indicated buffers.

Peptides and recombinant proteins. High-pressure liquid chromatography–purified BH3-peptides (EDIIRNARHLAQVGDSMDR) and BH3 (MRPEIQGLRRIDEFNA) peptides, unlabeled or labeled at their N terminal with fluorescein, were obtained from Neosystem (Strasbourg, France). The preparation of N-terminal histidine-tagged human recombinant Bcl-2 has been described (14). Recombinant glutathione S-transferase (GST)-fusion proteins were prepared as described in ref. 23.

Cell-free assays. Fluorescence polarization assays were done essentially as described in ref. 13. Briefly, fluorescent peptides (15 nmol/L BH3 and BH3) and GST-Bcl-2 (1 μmol/L) were mixed in binding buffer (20 mmol/L NaH2PO4, (pH 7.4), 50 mmol/L NaCl, 1 mmol/L EDTA, 0.05% pluronic acid) with titrations of HA14-1 or of the indicated nonfluorescent peptides (10-5-10-3 M). Fluorescence polarization was then measured with a Fusion-Packard equipment. mP units were determined after subtraction of background polarization (i.e., fluorescent peptide mixed with the competitor in the absence of Bcl-2 protein).

For cell-free, pull-down assays, protein complexes containing ~4 fmol radiolabeled Bax bound to histidine-tagged Bcl-2 immobilized on nickel-Sepharose were prepared as described in ref. 25 using histidine-tagged Bcl-2 (14) instead of histidine-tagged Bcl-xL. These complexes were incubated with the indicated concentration of HA14-1 for 1 hour at 30°C in 40 μL buffer [250 mmol/L sucrose, 80 mmol/L KCl, 10 mmol/L MgCl2, 10 mmol/L malate, 8 mmol/L succinate, 1 mmol/L ATP-Mg2+; 20 mmol/L MOPS (pH 7.5)]. Histidine-bound complexes were then isolated by centrifugation at 5,000 × g for 10 minutes at 4°C, and the amount of free radiolabeled Bax present in the resulting supernatant was quantified with IPlab gel program (Signal Analytics, Rockville, MD). following separation by SDS-PAGE and analysis by scanning with a PhosphorImager (Amersham Biosciences). (Saint-Quentin-Fallavier, France).

Cancer Research 2006; 66: (5). March 1, 2006 2758 www.aacrjournals.org

Cell culture. All glioma cells used in this study were cultured in RPMI 1640 supplemented with 10% FCS, penicillin (100 units/mL), streptomycin (100 μg/ mL), and 2 mmol/L glutamine. Bax-expressing and Bax-deficient cells derived from human glioblastoma multiforme tumors (BeGBM and BdGBM, respectively) have been described previously (14). Recombinant glutathione S-transferase (GST)-fusion proteins were prepared as described in ref. 23.

Results

HA14-1 increases sensitivity of human glioblastoma cells to radiotherapy-induced apoptosis and chemotherapy-induced apoptosis. We first analyzed the effects of HA14-1 on the response of human malignant glioma cells (BeGBM cells) to radiation injury in vitro. Time-lapse analysis revealed that a low but significant number of cells exhibiting morphologic changes typical of apoptosis were observed in populations irradiated with γ-rays (20 Gy; Fig. 1A). Consistent with this, significant caspase activity was detected in cell lysates 48 hours following irradiation (Fig. 1B). Pretreatment of glioblastoma cells with HA14-1 at 20 μmol/L, a dose that, by itself, exerted neither an effect on cell viability (Fig. 1A) nor on caspase activity (Fig. 1B), increased both the amount of apoptotic cell deaths (Fig. 1A) and caspase activity (Fig. 1B) in irradiated cells.

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The observation that pretreatment with HA14-1-enhanced radiation-induced cell death, as judged by a more classic cell viability assay, was in agreement with these kinetical analysis (Fig. 1B). Importantly, pretreatment with HA14-1 (10-20 μmol/L) also enhanced cell death and caspase activity in cells treated for 16 hours with the DNA-damaging agent etoposide (50 μg/mL; Fig. 1C). Thus, HA14-1 has an effect on the sensitivity of glioblastoma multiforme cells to induction of apoptosis by radiotherapy and chemotherapy.

The sensitizing activity of HA14-1 is Bcl-2 dependent. To investigate whether HA14-1 enhances the efficiency with which the mitochondrial apoptotic pathway is activated at early stages of apoptosis, we did immunocytochemical analysis of glioma cells treated with etoposide for 6 hours in the presence or absence of HA14-1. HA14-1 enhanced the low but significant rates of etoposide-treated cells exhibiting active caspase-3, and it also significantly increased the percentage of cells exhibiting cytochrome c release (Fig. 2A).

We reasoned that if the sensitizing activity of HA14-1 involves the mitochondrial apoptotic pathway, then this activity should require the expression of Bax, a proapoptotic multidomain protein of the Bcl-2 family that plays a major role in the activation of this pathway in human malignant glioma cells (11). We used glioma cells in which Bax expression was knocked down using a small-interfering RNA, pSilencer2.1-hygro-Bax construct (Fig. 2B). Bax knockdown (pSilBax) cells were significantly more resistant to cell death induced by irradiation or etoposide than cells transfected with a control construct (pSilSCR cells), and HA14-1 treatment did not have any effect on this feeble apoptotic response (Fig. 2C).

We then investigated whether HA14-1 exerts its sensitizing activity by interfering with Bcl-2 function. For this purpose, we analyzed whether endogenous Bcl-2 is involved in restraining cell....
death and in HA14-1-induced sensitization to apoptosis using glioma cells in which endogenous Bcl-2 was knocked down by a pSilencer2.1-puro-Bcl-2 construct (Fig. 2B). Bcl-2 knockdown (pSilpBcl-2) cells were more sensitive to cell death induced by irradiation or etoposide than control (pSilpSCR) cells, confirming that endogenous Bcl-2 provides a rate-limiting hindrance to induction of apoptosis by these stimuli (Fig. 2D). Moreover, pretreatment with HA14-1 did not have any effect on cell death induced by irradiation or etoposide in Bcl-2 knockdown cells (Fig. 2D), indicating that endogenous Bcl-2 is required for the sensitizing effect of HA14-1 to be manifest.

To analyze whether the Bcl-2 homologue Bcl-xL is also involved in the sensitizing property of HA14-1, we used cells in which its expression was knocked down by a pSilencer2.1-puro-Bcl-xL construct (Fig. 2B). The down-regulation of Bcl-xL achieved by this RNA interference approach was sufficient to sensitize glioma cells to irradiation or etoposide-induced cell death (Fig. 2D), indicating that endogenous Bcl-xL, akin to Bcl-2, plays a role in the resistance of these cells to apoptotic stimuli. Bcl-xL knockdown cells were nevertheless further sensitized to the effects of these insults by HA14-1 treatment (Fig. 2D).

To confirm that the inconsequence of Bcl-xL knockdown on the sensitizing activity of HA14-1 relies on the inability of this compound to interfere with the survival function of this protein in the conditions used, recombinant Bcl-xL was microinjected in Bcl-2 knockdown cells before their treatment with etoposide in the presence or absence of HA14-1. Microinjected Bcl-xL protected Bcl-2 knockdown cells against etoposide-induced cell death, but HA14-1 was unable to interfere with this protective activity (Fig. 2E). Importantly, in similar experiments, HA14-1 overcame the resistance conferred by microinjected recombinant Bcl-2 to Bcl-2 knockdown cells. This indicates that the lack of effect of HA14-1 in Bcl-2 knockdown cells is a direct consequence from Bcl-2 being absent in these cells. Taken together, these results show that the sensitizing activity of HA14-1 is critically determined by the cellular amounts of Bcl-2, but not by these of Bcl-xL, strongly supporting a model in which HA14-1 exerts its sensitizing effect by preferentially interfering with Bcl-2 function. **HA14-1 sensitizes to apoptosis by preferentially inhibiting the interaction of Bcl-2 with Bax.** Because Bcl-2 exerts a significant part of its survival function by physically interacting with proapoptotic members of its family (29), we analyzed in cell-free assays the effect of HA14-1 on Bcl-2 interactions with Bax and with two synthetic peptides derived from the BH3 domains of Bid and Bim (BH3\textsuperscript{Bid} and BH3\textsuperscript{Bim}, respectively) that reportedly function as direct "death agonists" of Bax (13, 14, 30). Using a previously described pull-down assay in which in vitro translated radiolabeled Bax was shown to interact with histidine-tagged recombinant Bcl-2 (14), we found that HA14-1 displaced radiolabeled Bax from Bcl-2 in a dose-dependent manner (1-100 μmol/L; Fig. 3A). In sharp contrast, in a fluorescence polarization assay, concentrations as high as 100 μmol/L HA14-1 were unable to compete with fluorescent BH3\textsuperscript{Bid} or BH3\textsuperscript{Bim} for binding to Bcl-2, whereas the corresponding nonfluorescent peptides were potent competitors (Fig. 3B).

The above observations indicate that HA14-1 is a more potent inhibitor of Bax/Bcl-2 interactions than it is an inhibitor of Bcl-2...
null interactions with death agonists BH3 domains and suggest that Bax/Bcl-2 interactions are the preferential target for the sensitizing activity of HA14-1. To support this view, Bax/Bcl-2 interactions were analyzed by coimmunoprecipitation experiments using lysates from glioma cells that were treated with etoposide for 6 hours in the presence or absence of HA14-1. Cell lysis was done in the presence of CHAPS, a detergent that does not induce overt modifications of Bax conformation by itself (31). Although some Bax coimmunoprecipitated with Bcl-2 in untreated cells, etoposide treatment significantly enhanced the amount of Bax bound to Bcl-2 (Fig. 4), without leading to any detectable increase in Bax expression in itself (data not shown). HA14-1 treatment did not affect Bax/Bcl-2 interactions in untreated cells, but it significantly prevented Bax/Bcl-2 interactions in etoposide-treated cells (Fig. 4). Thus, HA14-1 prevents the interaction between Bax and Bcl-2 that is dynamically induced by etoposide treatment.

To confirm, in an independent assay, that the ability of HA14-1 to enhance cell death involves the inhibition of the interaction between Bax and Bcl-2, we analyzed whether this sensitizing effect was still manifest in cells that express a mutant of Bax that does not interact with Bcl-2. We used, for this purpose, BdGBM cells that ectopically express the BaxL63E mutant, a mutant that does not interact with antiapoptotic proteins, such as Bcl-2 and Bcl-xL (14), but that can still be activated by the death agonist Bid in cell-free assays (32). Whereas BdGBM cells expressing wild-type Bax (Baxx) were sensitized to induction of cell death by irradiation by pretreatment with HA14-1, BdGBM cells expressing BaxL63E were not (Fig. 5). Thus, an interaction between Bax and Bcl-2 is required for the ability of HA14-1 to enhance cellular sensitivity to apoptosis. Of note, both BdGBM/Baxx and BdGBM/BaxL63E cells expressed similar levels of Bcl-2 (data not shown), ruling out that their differing responses to HA14-1 pretreatment resulted from different expression levels of Bcl-2.

HA14-1 increases the effect of the DNA-damaging agent etoposide on glioblastoma growth in vivo. In the final series of experiments, we investigated whether HA14-1 might have some effect on the growth of glioblastoma in vivo. Swiss nude mice were challenged with BeGBM cells (10⁵ injected s.c.), HA14-1 (400 nmol) in 100 μL free RPMI 1640-50% DMSO, or the carrier alone, was given at the site of injection once weekly from day 2 following cell injection. As shown in Fig. 6, HA14-1 treatment did not have any significant effect on the growth of glioblastoma tumors in immunodeficient mice as monitored twice weekly by measuring the volume of the expanding tumors. Moreover, no gross organ toxicity or weight loss could be detected in mice receiving such treatment (data not shown). To analyze whether HA14-1 treatment might enhance the efficiency of another antitumoral treatment, Swiss nude mice challenged with human glioblastoma multiforme cells were also given i.p. low doses of etoposide (2.5 mg/kg in 200 μL of 0.9% NaCl 5 days a week from day 2 following cell injection) together with HA14-1 or mock treatment. Whereas etoposide treatment was insufficient by itself to restrain the growth of glioblastoma cells, its combination with HA14-1 lead to a significant restrain on tumor growth as judged by the ability of the combined treatment to increase the doubling time of the tumor volume (Fig. 6).

Discussion

The data presented herein show that low concentrations of the small organic compound HA14-1 increase the efficiency with which
either γ-irradiation or etoposide induce caspase activity and apoptosis in human glioblastoma cells. Of note, whereas γ-irradiation induced profound block of glioma cells in the G2-M phase of the cell cycle, etoposide treatment, in the conditions used in this study, failed to do so in the presence or absence of HA14-1 (data not shown). These observations argue that the sensitizing activity of HA14-1 is unlikely to be cell cycle phase dependent. They suggest rather that HA14-1 acts at some point of the decision phase of apoptosis. Our observation that the sensitizing activity of HA14-1 requires the expression of Bax, a major mediator of the mitochondrial apoptotic pathway in glioma cells (11), further supports this view and indicates that HA14-1 acts at a point upstream of or proximal to the mitochondrial apoptotic pathway. We show, moreover, that endogenous Bcl-2 is required for the sensitizing effect of HA14-1 to be manifest. Bcl-2 knockdown in glioma cells enhances their apoptotic response to radiotherapy and chemotherapy, arguing that endogenous Bcl-2 is rate limiting for the response of these cells to these insults, but it abrogates the sensitizing properties of HA14-1. These results rule out the possibility that HA14-1 functions by amplifying death signals upstream of Bcl-2 and point to an effect of HA14-1 on some of the protective functions of Bcl-2.

We show that HA14-1 exerts an inhibitory effect on Bax/Bcl-2 interactions in cell-free assays. At concentrations at which it does so, it does not, however, prevent the binding of Bcl-2 to peptides derived from the BH3 domains of the death agonists BH3-only proteins Bid and Bim. This observation, together with a recent report that showed that HA14-1 inhibits the interaction between Bim and Bcl-2 only at concentrations way above those used in this study (33), indicates that HA14-1 does not compete well with the binding of such BH3-only proteins to Bcl-2 and implies that

**Figure 3.** Effect of HA14-1 on Bcl-2 binding to Bax and BH3 peptides in cell-free assays. A, binding of in vitro translated Bax to Bcl-2. Radiolabeled Bax/histidine-tagged Bcl-2 complexes were incubated with the indicated concentrations of HA14-1, and the amount of radiolabeled Bax released from histidine-bound complexes was analyzed and quantified as described in Materials and Methods. Points, mean of two independent experiments; bars, SE. Top, autoradiogram illustrating one representative experiment. Where indicated, 1 fmol radiolabeled Bax was loaded for illustrative purposes. B, binding of BH3 peptides to Bcl-2. Fluorescence polarization–based competitive binding assays using a fluorescein-labeled BH3<sup>Bim</sup> peptide (left) or BH3<sup>Bid</sup> peptide (right) in complex with Bcl-2 for HA14-1 and respective nonfluorescent peptides at the indicated concentration (C).

**Figure 4.** Effect of HA14-1 on Bax/Bcl-2 interactions in vitro. BeGGM cells were treated or not with etoposide (50 μg/mL) in the presence or absence of HA14-1 for 6 hours. Cellular extracts were then immunoprecipitated (IP) with an anti-Bcl-2 antibody (Calbiochem, La Jolla, CA) as described in Materials and Methods, and the presence of Bcl-2 and Bax in the immunoprecipitated fractions was analyzed by immunoblotting [anti-Bcl-2 antibody and anti-Bax antibody (TL41)]. Histograms show quantification by densitometric analysis of the amount of Bax immunoprecipitated with anti-Bcl-2 antibodies. Data were normalized to the amount of Bax bound to Bcl-2 in etoposide-treated cells in each experiment. Columns, mean of three independent experiments; bars, SE.

Cancer Res 2006; 66: (5). March 1, 2006 2762 www.aacrjournals.org

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the inhibition of such interaction(s) is unlikely to account for the sensitizing activity of HA14-1. We suspect that the lack of effect of HA14-1 on BH3Bid or BH3Bim binding to Bcl-2 stems from a more general inability of this compound to occupy tightly the BH3 binding site of Bcl-2. Indeed, HA14-1 was also unable, in our hands, to significantly prevent the binding of Bcl-2 to a peptide derived from the BH3 domain of Bax itself (data not shown).

Because binding of the BH3 domain of Bax within the hydrophobic pocket of antiapoptotic Bcl-2 family members is essential for the formation of stable heterodimers (12), the differential effects of HA14-1 on Bax/Bcl-2 versus BH3Bax/Bcl-2 interactions raise some intriguing questions about the exact mechanism through which HA14-1 inhibits the former. Some mutations in the hydrophobic groove of Bcl-2 were shown to prevent its binding to full-length Bax without affecting its binding to the sole BH3 domain of Bax (34). Moreover, regions distinct from that encompassing the Bax BH3 domain and from these forming the BH3 binding site of Bcl-2 homologues have recently been reported to be involved in the regulation of Bax binding to antiapoptotic Bcl-2 family members (35, 36). The interaction between Bcl-2 and Bax might therefore be subject to more stringent conformational constraints than that between Bcl-2 and a sole BH3 domain, and HA14-1 might interact with regions within Bcl-2 that are more critical for its interaction with the Bax protein than for its binding to BH3 domains. On that aspect, the interaction of HA14-1 with Bcl-2 would be reminiscent to that of 2-methoxyantimycin A3 with Bcl-xL, because this compound binds to Bcl-xL, exerts Bcl-xL inhibitory activity, but does not efficiently displace BH3 peptides from this antiapoptotic protein (37). It should also be mentioned that, because data actually showing a physical interaction between HA14-1 and Bcl-2 are currently lacking, it is also formally possible that HA14-1 only acts on Bax/Bcl-2 complexes.

Regardless of the molecular mechanisms involved, our observations lead us to propose a novel model to account for the biological activity of HA14-1 in which HA14-1 interferes with Bcl-2 function not by equally inhibiting its binding to multiple proapoptotic BH3 domains but by preferentially affecting its interaction with Bax during apoptosis. This model is supported by two lines of evidence: (a) HA14-1 rapidly inhibits Bax/Bcl-2 interactions induced by an apoptotic stimulation in cells in vitro and (b) glioblastoma cells, which only express a mutant of Bax that does not interact with antiapoptotic Bcl-2 members (BaxL63E), are exempt from HA14-1-induced sensitization to induction of cell death. Importantly, the effects reported here for HA14-1 are selective for Bcl-2, as HA14-1 does not seem to interfere with the protective function exerted by the structural homologue of Bcl-2 Bcl-xL in glioma cells. Endogenous Bcl-xL is indeed dispensable for the sensitizing activity of HA14-1, whereas HA14-1 is unable to overcome the resistance conferred by exogenous Bcl-xL to glioma cells devoid of Bcl-2. Further experiments are required to elucidate whether the consequence of HA14-1 treatment on Bcl-xL function relies on the fact that Bcl-2 and Bcl-xL exert their antiapoptotic functions through different mechanisms in glioma cells or on the fact that HA14-1 is unable to interfere with any of the binding properties of Bcl-xL.

Because HA14-1 can efficiently sensitize glioblastoma cells to apoptosis by selectively interfering with Bcl-2 and with its ability to interact with Bax in particular, one important implication from our studies is that a significant part of the survival function Bcl-2 exerts in these cancer cells is to directly titrate Bax molecules. The expression of Bcl-2 was reported to increase in parallel with that of Bax from low-grade to high-grade tumors of glial origin (6).
Expression levels of Bcl-2 that mirror these of Bax might therefore suffice to promote the resistance of high-grade tumors to radiotherapy or chemotherapy, and this resistance might be relieved using a chemical inhibitor, such as HA14-1.

Finally, we found that the local administration of HA14-1 albeit inefficient in itself to restrain the s.c. growth of glioblastoma multiforme tumors; in fact, its delivery to the central nervous system may in itself be a challenge. Despite these limitations, however, our data provide some proof of concept that HA14-1 or functional analogues with increased affinity for their target and/or enhanced pharmacokinetical properties and whose biological activity can be characterized by the methodologic approach described here can be helpful to improve the treatment of glioblastoma multiforme tumors.

Acknowledgments

Received 6/15/2005; revised 12/14/2005; accepted 1/5/2006.

Grant support: Association pour la Recherche contre le Cancer grant 3316 (P. Juin) and Ligue Nationale contre le Cancer postdoctoral fellowship (F. Manero).

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We thank A. Maisonneuve and S. Minault for the help with animal experiments, Dr. L. Oliver for invaluable help with time-lapse microscopy and for fruitful discussion throughout this work, and Drs. F. Paris and J. Hickman for invaluable comments.

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