CXCR4 Inhibition Synergizes with Cytotoxic Chemotherapy in Gliomas

Navid Redjal, Jennifer A. Chan, Rosalind A. Segal, and Andrew L. Kung

Abstract

Purpose: The chemokine receptor CXCR4 is expressed in many different cancers. In malignant brain tumors, CXCR4 signaling has been implicated in tumor growth, survival, and migration, and pharmacologic inhibition of CXCR4 results in decreased tumor growth in preclinical models. To understand how CXCR4 inhibitors may be incorporated into clinical therapy, we examined determinants of responsiveness to CXCR4 inhibition. Because optimal use of CXCR4 inhibition will likely be a part of multimodality therapy, we also investigated the efficacy of CXCR4 inhibition combined with conventional cytotoxic chemotherapy.

Experimental Design: CXCR4 protein levels and responsiveness to the CXCR4 inhibitor AMD3100 were determined in a panel of glioblastoma multiforme cell lines. The effects of AMD3100, alone or in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), on cell growth were determined for several of these cell lines in vitro. We used an orthotopic model of glioblastoma multiforme to evaluate the antitumor efficacy of AMD3100 combined with BCNU in vivo.

Results: The level of CXCR4 protein expression in glioblastoma multiforme cells predicts the dose at which there is a response to AMD3100; cells that express higher levels of CXCR4 protein require higher doses for equivalent response. In all cell lines tested, treatment of glioblastoma multiforme cells with BCNU followed by AMD3100 results in synergistic antitumor efficacy in vitro. This synergy can also be seen in an orthotopic glioblastoma multiforme model. Treatment with subtherapeutic doses of BCNU in combination with AMD3100 results in tumor regression in vivo, and this reflects both increased apoptosis and decreased proliferation following combination therapy.

Conclusion: These studies support testing CXCR4 inhibitors in patients with glioblastoma multiforme and establish that inhibition of CXCR4 synergizes with conventional cytotoxic therapies in a clinically relevant combinatorial strategy.

The chemokine receptor CXCR4 is expressed in a diverse number of cancers, including lung, prostate, breast, renal, and ovarian disease (1, 2). We and others have shown that CXCR4 is highly expressed in a variety of brain tumors (2–9) and that activation of CXCR4 by its ligand CXCL12 (SDF-1) promotes survival, proliferation, and migration of medulloblastoma- and glioblastoma multiforme–derived tumor cells (5). Furthermore, systemic administration of AMD 3100, a bicyclam noncompetitive CXCR4 inhibitor (10), decreases growth of intracranial glioblastoma multiforme and medulloblastoma xenografts, establishing in vivo proof of concept for targeting CXCR4 as a therapeutic strategy in brain tumors (5).

There is a pressing need for more effective treatments for patients with malignant brain tumors. The median survival for patients with glioblastoma multiforme is 9 to 14 months, in spite of maximal treatment with surgery, chemotherapy, and focal radiotherapy (11, 12). Novel approaches in development for glioblastoma multiforme include radiation sensitizers, gene therapy, vaccines, and drugs that target tumor-specific molecular changes (13). Optimized use of existing and novel therapies will likely be achieved through combinations of agents with different mechanisms of action that have additive or synergistic antitumor efficacy, hopefully without additive toxicity. Our prior studies showed that treatment of animals with AMD3100 resulted in decreased activation of the mitogen-activated protein kinase and AKT pathways in xenograft brain tumors. Because these pathways provide critical antiapoptotic signals, we reasoned that inhibition of CXCR4 may potentiate the effects of conventional cytotoxic therapies. Here, we investigate the determinants of responsiveness to CXCR4...
inhibition in glioblastoma multiforme cells and evaluate the antitumor efficacy of CXCR4 inhibition in combination with cytotoxic therapy in vitro and in vivo.

Materials and Methods

Cell culture. The human glioma cell lines U87, HS683, and A172 were obtained from American Type Culture Collection (Manassas, VA). The cell lines LN308, LN428, and LN827 were obtained from Dr. Erwin Van Meir (Emory University, Atlanta, GA). The U87, LN308, LN428, and LN827 cells were engineered to express luciferase as previously described (5).

Western blotting. Lysates were prepared as previously described (14). Equivalent amounts of protein were fractionated by SDS-PAGE, transferred onto Immobilon-P membranes, and were subsequently immunoblotted with a rabbit polyclonal antibody against CXCR4 (Chemicon, Temecula, CA) or a monoclonal antibody against β-tubulin (Sigma-Aldrich, St. Louis, MO). After application of horseradish peroxidase–labeled secondary antibody (Amersham Pharmacia, Piscataway, NJ), chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL) was quantified using ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA). One-way ANOVA and Turkey’s post hoc test were used to assess statistical significance.

Drug treatment and cell survival. Cells were plated at a density of 5,000 per well in a 96-well plate 1 day before drug treatment. AMD3100 (Sigma-Aldrich) or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; Bristol-Myers Squibb, Princeton, NJ) was added to achieve their indicated concentrations. After 24 hours, the medium was replaced with fresh medium containing drugs at the indicated concentrations. Cell numbers were assayed as either luciferase activity in luciferase-expressing cells (Bright-Glo, Promega, Madison, WI) or with a colorimetric assay (CellTiter 96 Aqueous One Solution, Promega) according to the instructions of the manufacturer. For all cell lines used in these studies, we have previously established a linear relationship between cell number and the colorimetric or luminescence read-outs of these assays. As such, data in survival assays are expressed relative to the control well values. Statistical significance was determined by one-way ANOVA and Turkey’s post hoc test.

Tumor xenografts, in vivo treatment, and bioluminescence imaging. Establishment of intracranial orthotopic xenografts and in vivo bioluminescence imaging were done as previously described (5). Mice were imaged at least twice after implantation of cells to identify those in which tumor burden increased over time. Ten to 12 days after implantation of U87 cells, mice with approximately equivalent tumor bioluminescence were divided into control and treatment groups (n = 6–8 in each group). As indicated, mice were treated with BCNU 13.3 mg/kg injected i.p., AMD3100 10 mg/kg/d through a s.c. osmotic pump (Alzet, Palo Alto, CA), AMD3100 10 mg/kg injected s.c. twice a day, or vehicle. Bioluminescence through standardized regions of interest was quantified using Living Images (Xenogen, Alameda, CA) and data were normalized to bioluminescence at the initiation of treatment for each animal. Student’s t test was used to determine the significance of bioluminescence differences between treatment cohorts. All animal procedures were approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

Histology and immunohistochemistry. Animals were perfused and their brains were subsequently fixed and frozen following standard protocols. Coronal sections, 14 μm thick, were stained with cresyl violet to detect the presence of tumor. Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (Roche Molecular Biochemicals, Indianapolis, IN). For assessment of the mitotic index, a standard protocol using Tris-HCl containing 1 mmol/L EDTA (pH 8) was used to unmask antigens. The slides were then incubated with phospho-histone H3 (Ser10) monoclonal antibody (Cell Signaling, Beverly, MA) followed by a secondary

Fig. 1. CXCR4 protein expression levels correlate with dose dependence of AMD3100 response. A, Western blot analysis with a CXCR4-specific antibody shows protein expression in seven glioma cell lines compared with Saos-2 cells, which have negligible CXCR4 expression. Blots were stripped and reprobed with β-tubulin antibody as a loading control. B, levels of CXCR4 protein were quantified and were normalized to β-tubulin protein levels. Columns, mean of three independent experiments; with results expressed as relative to U87, which was set to 1; bars, SE; * P < 0.001 by one-way ANOVA and Tukey’s post hoc test. C, dose dependence of AMD3100 response in LN308 and U87 cell lines. Treatment for 48 hours with low doses of AMD3100 (0.01 and 0.05 ng/mL) decreased cell numbers in U87 cells as measured by 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Similar effects were seen with the LN428 cell line at higher doses of AMD3100 (10 and 100 ng/mL). Arrows, concentration of AMD3100 that causes 50% of the maximal response. Points, mean of triplicates; bars, SE. D, the relative levels of CXCR4 protein expression are plotted against the concentration of AMD3100 that causes 50% maximal response, with a Pearson correlation coefficient of R = 0.9976.
antibody conjugated to Alexa 546. DNA was counterstained with 4',6-diamidino-2-phenylindole (Sigma). Fluorescent and bright-field images were obtained using a CCD camera on a Nikon Eclipse microscope (Tokyo, Japan). Images were processed with Spot Camera software (Diagnostic Instruments, Sterling Heights, MI). 4',6-Diamidino-2-phenylindole, phospho-histone H3, and terminal deoxynucleotidyltransferase – mediated dUTP nick end labeling– positive cells were counted by an investigator who was blind to the experimental groups. Statistical significance was determined by one-way ANOVA and Turkey’s post hoc test.

**Flow cytometry.** Cells were collected, fixed, stained with propidium iodide, and analyzed by flow cytometry using standard protocols. The relative percentage of cells in each cell cycle compartment was estimated using CellQuest Pro (BD Biosciences).

**Results**

**CXCR4 expression and response to AMD3100 in glioblastoma multiforme cell lines.** Activating mutations of CXCR4 have not been identified in tumors, and therefore receptor signaling is likely dictated by the presence of ligand in the microenvironment and expression of wild-type CXCR4 on tumor cells. To determine if there is a correlation between the level of CXCR4 expression and sensitivity to AMD3100, we used immunoblotting to determine CXCR4 protein expression across a number of glioblastoma multiforme cell lines. CXCR4 protein was detected in all glioblastoma multiforme cell lines at the expected molecular weight of 48 kDa (Fig. 1A). The level of CXCR4 protein, normalized to tubulin, was analyzed in triplicate for each cell line, and the highest level of expression was found in the LN428 cell line (Fig. 1B). Whereas all other glioblastoma multiforme cell lines had comparatively reduced levels of CXCR4, the presence of CXCR4 protein was quantifiable by comparison to Saos-2 cells (Fig. 1A and B), which have previously been shown to have negligible CXCR4 expression (15). Using quantitative reverse transcription-PCR, we confirmed that CXCR4 RNA is expressed in all of these glioma cell lines (data not shown), which is consistent with prior studies (6).

We previously showed that inhibition of CXCR4 signaling with AMD3100 in the U87 cell line inhibits proliferation and is proapoptotic (5). Given the differences in CXCR4 protein expression among the glioblastoma multiforme cell lines (Fig. 1A and B), we asked whether receptor expression level correlates with response to AMD3100. We analyzed the effects of AMD3100 on the number of viable cells in U87, LN827, LN308, and LN428 cells. In LN428 cells, which have the highest levels of CXCR4, statistically significant reduction in cell numbers was observed at doses \( \geq 10 \) ng/mL of AMD3100 (Fig. 1C and D). In contrast, LN827 and U87 cells, which have lower expression levels of CXCR4, had statistically significant reductions in cell number beginning at 0.05 ng/mL AMD3100. We find that there is a linear relationship between the level of CXCR4 expression and the log concentration of AMD3100 that results in a half maximal response \( (R = 0.9976) \). As a negative control for these assays, AMD3100 had no effects on Saos-2 cells (data not shown). Taken together, these data indicate that, in vitro, glioma cells respond to a CXCR4 antagonist in a dose-dependent manner that correlates with the level of the CXCR4 receptor.
Synergy of AMD3100 and BCNU in cultured glioma cell lines. The effects of AMD3100 alone on cell number were statistically significant and reproducible, but were modest in magnitude of effect (Fig. 1). Because we have previously shown that AMD3100 inhibits signaling pathways downstream of CXCR4 that might provide key antiapoptotic signals (e.g., AKT; ref. 5), we hypothesized that combining AMD3100 with a cytotoxic agent might result in additive or synergistic cell killing. We analyzed the effect of combining CXCR4 inhibition by AMD3100 at 0.05 ng/mL (the dose that gives half maximal killing; Fig. 1C and D) with BCNU, a DNA-alkylating agent, at doses that have minimal effects as monotherapy. Statistically significant synergy was found between AMD3100 and BCNU in doses that have minimal effects as monotherapy. Because we have previously shown that AMD3100 inhibits signaling pathways downstream of CXCR4 that might provide key antiapoptotic signals (e.g., AKT; ref. 5), we hypothesized that combining AMD3100 with a cytotoxic agent might result in additive or synergistic cell killing. We analyzed the effect of combining CXCR4 inhibition by AMD3100 at 0.05 ng/mL (the dose that gives half maximal killing) with BCNU, a DNA-alkylating agent, at doses that have minimal effects as monotherapy. Statistically significant synergy was found between AMD3100 and BCNU in doses that have minimal effects as monotherapy. Because we have previously shown that AMD3100 inhibits signaling pathways downstream of CXCR4 that might provide key antiapoptotic signals (e.g., AKT; ref. 5), we hypothesized that combining AMD3100 with a cytotoxic agent might result in additive or synergistic cell killing. 

In vivo efficacy of AMD3100 and BCNU combination therapy. Based on the in vitro results indicating synergistic cytotoxicity with AMD3100 and BCNU, we hypothesized that this combination therapy might be an effective treatment for gliomas in vivo. To test this hypothesis, we used intracranial orthotopic xenografts of U87 cells that were engineered to express luciferase, so that tumor burden could be assessed using in vivo bioluminescence imaging (5, 16). Mice with established orthotraft tumors were separated into four treatment groups. To evaluate for synergy, we used doses of BCNU and AMD3100 that produced no antitumor efficacy when given alone (Fig. 3A, and data not shown). Animals in group 1, the control group, were treated with vehicle (PBS); animals in group 2 were treated with two s.c. doses of AMD3100 spaced 12 hours apart; animals in group 3 received a single i.p. dose of BCNU; and animals in group 4 received both AMD3100 and BCNU. After one course of treatment (on treatment day 1), tumor growth was monitored for 15 days without further treatments. Treatment of animals with either BCNU alone or AMD3100 alone had no significant effects on tumor growth (Fig. 3). In contrast, the animals treated with AMD3100 and BCNU exhibited regression in tumor size after a single course of combination therapy. As expected, following a single course of treatment, after a delay of ~1 week, the residual tumor cells returned to a normal rate of growth (Fig. 3A). Similar results were obtained in an independent experiment in which AMD3100 was delivered by osmotic pump, as described in Materials and Methods (data not shown). These results show that combination therapy with AMD3100 and BCNU results in synergistic antitumor efficacy and tumor regression in an in vivo orthotraft glioma model.

AMD3100 and BCNU combination therapy causes increased apoptosis and decreased proliferation in vivo. Given the tumor regression that we observed with the combination therapy, we examined whether the antitumor effects were caused by changes in cell growth, apoptosis, or both. In a third independent in vivo experiment, animals were separated into four groups and were administered one course of treatment, as described above. Approximately 24 hours following treatment, animals were sacrificed and tumors were examined histologically. Significant differences were seen when apoptosis was assessed in the tumors using terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling staining. In comparison with tumors from animals in the control group, animals from both the AMD3100-treated group (P < 0.001) and the combination therapy–treated group (P < 0.01) showed statistically significant increases in apoptosis (Fig. 4A and B), with 1.47% in the AMD3100-treated group and 0.54% in the combination therapy–treated group, as compared with 0.037% in the control group. The tumors from animals treated with

\[ \text{Relative bioluminescence} = \frac{\text{Bioluminescence at time} \times \text{Dose}}{\text{Bioluminescence at baseline} \times \text{Dose}} \]

\[ \text{Vehicle} \rightarrow \text{AMD} \rightarrow \text{BCNU} \rightarrow \text{BCNU+AMD} \]

**Fig. 3.** In vivo synergy of AMD3100 and BCNU in an orthotopic glioma model. Animals with established intracranial U87 orthografts were treated with a single course of therapy on treatment day 1. Treatments consisted of either vehicle only, BCNU 13.3 mg/kg i.p. x 1 dose, AMD3100 10 mg/kg s.c. x 2 doses (12 hours apart), or a combination of BCNU and AMD3100. A, effects of treatment were followed by bioluminescence imaging. Points, mean (n = 6 animals per group); bars, SE. **, P < 0.001, compared with the control groups by Student’s t test. B, bioluminescent images from representative control and treated animals on treatment days 1, 4, and 8. Animals chosen had changes in luminescence similar to the mean of the groups as indicated in (A).
BCNU had similar levels of apoptosis (0.054%) as the control group (Fig. 4B).

The mitotic index was assessed by phospho-histone H3 staining to visualize mitotic cells and with 4',6-diamidino-2-phenylindole to visualize nuclei. Compared with animals in the control group, there was a decrease in the mitotic index observed in tumor tissue from animals treated only with BCNU ($P < 0.001$) and in animals treated with both AMD3100 and BCNU ($P < 0.001$; Fig. 4C and D). Whereas the mitotic index in tumors from control animals was 1.73%, the index in tumors from the BCNU only group was 0.84% and in animals treated with combination therapy, 0.80% (Fig. 4D). In contrast, the mitotic index in tumors from animals treated with only AMD3100 was actually somewhat increased compared with the control group. Based on in vitro studies with pulse treatment with BCNU with or without AMD3100, this decrease in the number of mitotic cells likely reflects arrest of cells in G2 due to BCNU-induced DNA damage (Fig. 5).

These data indicate that of the four treatments, only the combination of AMD3100 and BCNU both decreases tumor cell division and increases tumor cell apoptosis in vivo. Because tumor burden (total cell number) is a balance between cellular proliferation and apoptosis, these results suggest that the synergistic antitumor efficacy of combining AMD3100 with BCNU results from a concomitant reduction in cell production and an increase in cell destruction with combination therapy.

**Discussion**

The data presented here add to a growing body of literature indicating that CXCR4 is important in a variety of cancers, and specifically indicate that this receptor can be a propitious target in treating glioblastoma multiforme (2–9). We show that all glioma cell lines tested express CXCR4 and that there is a correlation between the level of CXCR4 expression and response to CXCR4 inhibition. Moreover, we show increased antitumor efficacy as a result of combining CXCR4 inhibition with a cytotoxic chemotherapeutic agent. In vitro, AMD3100 and BCNU produce synergistic cytotoxicity in an order-dependent manner. Strikingly, in vivo, we find that combining subtherapeutic doses of AMD3100 and BCNU produces tumor regression, resulting from both a decrease in proliferation and
an increase in apoptosis of tumor cells. These results suggest that concomitant inhibition of CXCR4 synergizes with conventional cytotoxic therapy, a strategy that may have clinical use.

The optimum use of novel therapies that are currently in development will likely result from their incorporation into multimodality combinatorial treatment regimens. In the case of glioblastoma multiforme, many therapeutic approaches have antimitogenic effects and may also be useful as adjuvants to conventional cytotoxic therapies.

As with other tumor types, there is growing evidence that brain tumors contain only a small subpopulation of cells with self-renewal capacity (18). Because the ability to regenerate a tumor after cytoreduction may be confined to this rare population, it is possible that therapies that specifically target these self-renewing “cancer stem cells” may have heightened efficacy. To this end, it is of interest that there is growing evidence that glioblastoma multiforme may arise from a common neural-glial stem cell, rather than from differentiated astrocytes (19). CXCR4 is highly expressed in neural precursor cells and is enriched in glioma-derived stem cells (20). Strategies that target multiple signaling pathways known to be important in the normal progenitor or stem cells from which glioblastoma multiforme arises, including CXCR4, may provide additional rational combinations for future exploration.

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