Gallium-induced cell death in lymphoma: role of transferrin receptor cycling, involvement of Bax and the mitochondria, and effects of proteasome inhibition

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Abstract

Gallium nitrate is a metallodrug with clinical efficacy in non-Hodgkin’s lymphoma. Its mechanisms of antineoplastic action are not fully understood. In the present study, we investigated the roles of transferrin receptor (TfR) targeting and apoptotic pathways in gallium-induced cell death. Although DoHH2 lymphoma cells displayed a 3-fold lower number of TfRs than CCRF-CEM lymphoma cells, they were 3- to 4-fold more sensitive to gallium nitrate. Despite a lower TfR expression, DoHH2 cells had greater TfR cycling and iron and gallium uptake than CCRF-CEM cells. In other lymphoma cell lines, TfR levels per se did not correlate with gallium sensitivity. Cells incubated with gallium nitrate showed morphologic changes of apoptosis, which were decreased by the caspase inhibitor Z-VAD-FMK and by a Bax-inhibitory peptide. Cells exposed to gallium nitrate released cytochrome c from mitochondria and displayed a dose-dependent increase in caspase-3 activity. An increase in active Bax levels without accompanying changes in Bcl-2 or Bcl-XL was seen in cells incubated with gallium nitrate. The endogenous expression of antiapoptotic Bcl-2 was greater in DoHH2 cells than in CCRF-CEM cells, suggesting that endogenous Bcl-2 levels do not correlate with cell sensitivity to gallium nitrate. Gallium-induced apoptosis was enhanced by the proteasome inhibitor bortezomib. Our results suggest that TfR function rather than TfR number is important in gallium targeting to cells and that apoptosis is triggered by gallium through the mitochondrial pathway by activating proapoptotic Bax. Our studies also suggest that the antineoplastic activity of combination gallium nitrate and bortezomib warrants further investigation. [Mol Cancer Ther 2006;5(11):2834–43]

Introduction

Although there have been advances in the treatment of lymphoma, a significant number of patients succumb to this disease every year (1). Hence, there is a great need to develop agents for the treatment of lymphoma and to understand their basic mechanisms of action so that they may be used optimally in the clinic. The group IIIA metal salt gallium nitrate was first found to have antineoplastic activity in animal tumor models ~3 decades ago and was deemed a National Cancer Institute investigational drug (2). Subsequent phase 2 clinical trials showed gallium nitrate to be an active agent in the treatment of lymphoma (3–7), whereas a more recent multicenter phase 2 trial confirmed its efficacy in this disease (8).

Although gallium nitrate has shown antineoplastic activity in the clinic, its mechanisms of cytotoxic action and, in particular, its intracellular targets have only been partly elucidated. Several investigations have indicated that, in the circulation, gallium binds avidly to transferrin (Tf), the iron transport protein, to form Tf-gallium complexes that can home in on Tf receptor (TfR) on the surface of lymphoma cells (9–14). However, the presence of TfRs on these cells in vivo has been shown primarily by immunohistochemical staining of tissue samples taken from patients with lymphoma. Hence, it has never been established whether the cytotoxicity of gallium correlates with the density of TfR expression per se or whether additional factors, such as receptor function, are of equal, greater, or lesser importance. Moreover, whereas TfR targeting seems to be an important first step in the delivery of gallium to lymphoma cells, the subsequent downstream events leading to gallium-induced cell death are more complex and are incompletely understood.

In prior studies, we have shown that Tf-gallium interferes with the cellular uptake of Tf-iron and the intracellular release of iron from the endosome to cellular compartments (15). The resultant gallium-induced iron deprivation coupled with a direct effect of intracellular gallium on the iron-dependent R2 subunit of ribonucleotide reductase results in a decrease in the synthesis of deoxyribonucleotides and a block in DNA synthesis (16–18). Gallium has
also been shown to inhibit membrane tyrosine phosphatase in Jurkat and HT-29 cells and to interfere with the polymerization of tubulin in a cell-free system; however, the extent to which these effects contribute to the antitumor activity of gallium is not clear (19, 20). Although our earlier studies showed that the blockade of cellular iron uptake by Tf-gallium led to DNA fragmentation (21), a finding suggestive of apoptosis, the events leading to this process have never been defined.

In the present study, we have attempted to advance our understanding of the pathways involved in gallium-induced cell death in T-cell CCRF-CEM and B-cell DoHH2 lymphoma cell lines. Our results suggest that the cytotoxicity of gallium is linked to TfR cycling rather than cellular receptor density and that gallium-induced cell death results from the activation of Bax and the mitochondrial release of cytochrome c with subsequent caspase-3 activation, without alterations in Bcl-2 or Bcl-XL levels. Furthermore, we show that gallium-induced apoptosis can be enhanced by inhibition of proteasome activity.

Materials and Methods

Materials

Gallium nitrate was obtained from Genta, Inc. (Berkely Heights, NJ). Hoechst dye and Z-VD-FMK were purchased from Calbiochem (San Diego, CA). Human Tf and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Bortezomib was obtained from Millenium Pharmaceuticals (Cambridge MA). 125I-Na and 59FeCl3 were obtained from Amersham (Arlington Heights, IL). 59Fe-Tf was prepared as described by Bates and Schlabach (22), whereas 125I-Tf was prepared by the chloramine T method (23). 67Ga citrate was obtained from NycoMed-Amersham (Milwaukee, WI). Bax-inhibitory peptide was used as reported previously (24, 25). Polyclonal rabbit antibody to Bax, murine 6A7 monoclonal antibody (mAb) to Bax, mAbs to Bcl-2, and cytchrome c were purchased from BD Biosciences (San Diego, CA). Murine mAb to Bcl-X1 was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Cells

Human CCRF-CEM, Jurkat, and Raji lymphoma cell lines were obtained from American Type Cell Collection (Manassas, VA). Human B-cell DoHH2, HBL-2, JVM-2, and Z-138C cell lines were obtained from the British Columbia Cancer Agency and have been described previously (26, 27). All cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in an atmosphere of 5% CO2.

Cell Proliferation Assay

The effects of gallium nitrate and bortezomib on cellular proliferation were examined by a colorimetric MTT assay as described previously (28, 29). Cell growth was measured after 24 to 72 hours of incubation. The absorbance of wells was determined spectrophotometrically at dual wavelength 570/630 nm. Wells containing gallium nitrate or bortezomib were compared with wells, in which these additives were omitted (control). Cells were also counted with a hemocytometer.

TF Binding Assay

TfRs exist as TfR1 or TfR2. However, because only TfR1 is expressed in lymphoid cells, reference to TfR in this study indicates TfR1. Cell surface TfR density was determined by 125I-Tf binding to intact cells as described previously (15). Cells were harvested and washed with ice-cold PBS containing 0.1% bovine serum albumin and assayed for 125I-Tf binding at 4°C. Maximal Tf binding to cell surface TfR was determined according to the method of Scatchard (30).

TF-TfR Cycling

The kinetics of internalization of cell surface TfR-bound 125I-Tf and the release of internalized 125I-Tf was examined in ligand pulse-chase experiments in cells as described previously (31). For the Tf internalization experiments, 107 cells were harvested, washed with PBS containing 1 mg/mL bovine serum albumin, and then incubated at 4°C for 60 minutes in 200 μL of the same buffer with 276 ng 125I-Tf (to allow for ligand binding to cell surface TfR). Cells were then washed by centrifugation with ice-cold PBS-bovine serum albumin and resuspended in 1 mL serum-free medium prewarmed to 37°C. The cell suspension was maintained at 37°C in a water bath. For the pulse/internalization phase, 100 μL aliquots were removed from the cell suspension at 2.5 minutes intervals for the initial 15 minutes and added to 1 mL ice-cold 10 mmol/L acetic acid/150 mmol/L NaCl (pH 3) buffer (acid wash) to remove 125I-Tf on the cell surface. Cells were centrifuged in a microfuge centrifuge for 1 minute at full speed and the supernatant was carefully removed. The radioactivity in the cell pellet and supernatant was counted to determine the amount of 125I-Tf internalized by cells (acid-resistant cpm). For the 125I-Tf exocytosis (chase) phase, 1 mg nonradioactive Tf-Fe was added to the cell suspension after the initial 15 minutes of ligand internalization, and 100 μL aliquots were removed and centrifuged in acid wash buffer. The radioactivity in the cell pellet and supernatant was counted as described above.

Assay for Cellular Iron and Gallium Uptake

The cellular uptake of iron or gallium was examined using 59Fe or 67Ga described previously (31, 32). Cells were harvested, washed with medium, and replated (106 per mL) in 24-well plates in fresh medium. For iron uptake studies, 59Fe-Tf (75 pmol Fe) was added to each well at the start of the incubation. For gallium uptake studies, cells were incubated with 0.5 μCi 67Ga citrate/well. After 4 to 5 hours of incubation at 37°C, cells were removed from the wells and washed by centrifugation with ice-cold PBS. The radioactivity in the cell pellet was counted to determine the amount of 59Fe or 67Ga taken up by cells.

Morphologic Analysis for Apoptosis and Effects of Inhibitors

Cells exposed to gallium nitrate were examined by light microscopy at different time points for the presence of apoptosis based on previously defined morphologic
criteria (33). Gallium nitrate was added to CCRF-CEM or DoHH2 cells that had been plated 24 hours previously in fresh medium in 1-mL wells. One hour before the addition of gallium nitrate, the pancaspase Z-VDAD-FMK (100–400 μmol/L), Bax-inhibitory peptide (100–400 μmol/L), or a negative control peptide (100–400 μmol/L) was added to some wells to determine the effects of these inhibitors on gallium-induced cell death. Cells were stained with Hoechst dye and examined by light microscopy at various times of incubation for morphologic changes consistent with apoptosis. Apoptotic and nonapoptotic cells were counted and the fraction of the total number of cells undergoing apoptosis was determined.

**Caspase-3 Activity**

The effect of gallium nitrate on caspase-3 activity in cells was measured using an assay based on the enzymatic cleavage of the fluorogenic caspase-3 substrate Ac-DEVD-AFC (Calbiochem). CCRF-CEM and DoHH2 cells were harvested after 24 hours of incubation with gallium nitrate and lysed in 50 μL buffer consisting of 150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), and 1% CHAPS. Cellular debris was removed by centrifugation and the protein content of the supernatant was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL). Caspase-3 activity was measured in a reaction carried out in black plastic well plates containing 50 μg protein from cell lysates, 1 μg Ac-DEVD-AFC, and reaction buffer [50 mmol/L Tris-HCl (pH 7.2), 100 mmol/L KCl, 10% sucrose, 0.1% CHAPS, 10 mmol/L DTT] to make a total volume of 100 μL/well. The generation of fluorescent product in each reaction was measured at 405/510 nm over 5 minutes in a spectrofluorimeter and expressed as arbitrary relative fluorescent units.

**Detection of Cytochrome c Released from Mitochondria**

CCRF-CEM and DoHH2 cells were harvested by centrifugation following incubation with gallium nitrate. Cells were resuspended in 200 μL homogenization buffer [250 mmol/L sucrose, 20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride] and disrupted in a Dounce homogenizer (60 strokes) at 4°C. The cytosol and heavy membrane (containing mitochondria and endoplasmic reticulum) fractions were separated by centrifugation as described previously (34), and the cytosolic fraction was analyzed for the presence of cytochrome c by Western blotting as described below.

**Immunoprecipitation of the Active Form of Bax**

Cells that had been incubated with gallium nitrate were harvested and lysed in CHAPS buffer [150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), 1% CHAPS]. Insoluble cell fractions were removed by centrifugation (14,000 rpm × 30 minutes) and the supernatant was collected and assayed for protein content by bicinchoninic acid protein assay. The cell supernatant (200 μL containing 1,880 μg protein) was then mixed for 2 hours at 4°C with conformation-specific anti-Bax mAb 6A7 bound to protein G-Sepharose beads. The latter were prepared by mixing 2 μg 6A7 antibody with 40 μL of a 50% suspension of Sepharose G beads overnight on rotating disk at 4°C. The antibody beads with bound Bax protein were then washed by centrifugation in CHAPS buffer, resuspended in 2× Laemmli buffer, and used in the Western blotting studies described below.

**Western Blotting**

Cytosolic fractions prepared from cells exposed to gallium nitrate and samples from the mitochondria release and immunoprecipitation experiments were analyzed for Bcl-2, Bcl-X, cytochrome c, and Bax by Western blotting using an enhanced chemiluminescence Western blotting detection system (Amersham). SDS-PAGE of the samples was done as described (35). Proteins were transferred from the gel onto a nitrocellulose membrane as described (36), using a Transblot system (Bio-Rad, Richmond, CA). Membranes were first incubated in blocking buffer (PBS with 0.1% Tween 20 containing 10% nonfat dry milk) followed by sequential washes in PBS-Tween 20. Membranes were then incubated in PBS-Tween 20 containing a primary antibody specific to the protein of interest. Polyclonal rabbit antibody to Bax was used for immunoblotting. Membranes were washed with PBS-Tween 20 and incubated in the same buffer containing the appropriate secondary antibody (sheep anti-mouse Ig, mouse anti-hamster Ig, or donkey anti-rabbit Ig) conjugated to horseradish peroxidase. Membranes were immersed in enhanced chemiluminescence detection solution and exposed to XAR-5 film for autoradiography.

**Results**

**Inhibition of Cell Growth by Gallium Nitrate**

The effects of gallium nitrate on the growth of T-cell lymphoma CCRF-CEM and B-cell lymphoma DoHH2 cells were measured by MTT assay after 24 to 72 hours of incubation. As shown in Fig. 1, gallium nitrate inhibited the proliferation of both cell lines in a concentration- and time-dependent manner. DoHH2 cells were found to be more sensitive to growth inhibition by gallium nitrate than CCRF-CEM cells. In the experiment shown in Fig. 1A, for DoHH2 cells, the IC50 of gallium nitrate was approximately 60 and 40 μmol/L after 48 and 72 hours of incubation, respectively. For CCRF-CEM cells, the IC50 was approximately 280 and 120 μmol/L after 48 and 72 hours of incubation, respectively (Fig. 1B). In contrast to the inhibitory effects of gallium nitrate on cell proliferation after 48 and 72 hours of incubation, cell proliferation after 24 hours of incubation was only minimally inhibited by higher concentrations of gallium nitrate and was slightly stimulated at lower gallium nitrate concentrations (Fig. 1A and B). These findings suggest that the induction of cell death by gallium nitrate requires a minimum of 24 hours of continuous exposure of cells to this drug. To confirm that the inhibitory effects of gallium nitrate on cell proliferation assessed by MTT assay represented an actual decrease in cell number, cell counts were done after 72 hours of incubation. As shown in Fig. 1C and D, gallium nitrate produced a reduction in cell number, consistent with the results of the MTT assay.
Comparison of TfR Expression, Tf-TfR Cycling, and Iron and Gallium Uptake in CCRF-CEM and DoHH2 Cells

Prior studies have shown the importance of the TfR in cellular gallium uptake and have indicated that gallium and iron share common uptake pathways (10, 11, 37). Hence, cells with higher numbers of TfRs might be expected to be more sensitive to gallium nitrate. To examine whether the 3- to 4-fold difference in sensitivity to gallium nitrate between DoHH2 and CCRF-CEM cells could be related to differences in TfRs, cells were analyzed for TfR expression by Tf binding assay. As shown in Fig. 2A, Scatchard analysis of Tf binding to TfR on intact cells revealed that CCRF-CEM cells displayed an ~3-fold greater number of Tf binding sites than DoHH2 cells. As shown in Fig. 2A, the maximal Tf bound was 8.1 ng Tf/10^9 cells for DoHH2 cells versus 23.9 ng Tf/10^9 cells for CCRF-CEM cells. Hence, contrary to expectation, DoHH2 cells that were more sensitive to gallium nitrate than CCRF-CEM cells actually expressed a lower density of TfRs.

The cycling of Tf bound to the TfR is a two-phase process that involves endocytosis of the TfR-Tf-Fe complex from the cell surface to an intracellular acidic endosome where iron is released from Tf. This is followed by an exocytosis phase, in which TfR-apoTf (Tf without iron) cycles back to the cell surface where Tf is released to the exterior (38). Pulse-chase experiments were conducted to compare the cycling kinetics of a single cohort of cell surface TfR-bound 125I-Tf in CCRF-CEM and DoHH2 cells. As shown in Fig. 2B, despite having a lower number of Tf binding sites than CCRF-CEM cells, DoHH2 cells internalized a greater amount of cell surface TfR-bound 125I-Tf than CCRF-CEM cells over the initial 10 minutes of the ligand uptake phase. This suggests that the proportion of cell surface TfRs that are actively involved in cycling is greater in DoHH2 cells than in CCRF-CEM cells. If this were the case, DoHH2 cells would also be expected to incorporate a greater amount of iron and gallium. To test this hypothesis, cells were examined for their uptake of 59Fe and 67Ga. As shown in Fig. 2C, iron uptake was ~1.8-fold greater in DoHH2 cells than in CCRF-CEM cells (P = 0.0005, t test). Similarly, as shown in Fig. 2D, gallium uptake was ~1.5-fold greater in DoHH2 cells than in CCRF-CEM cells (P = 0.01, t test). Regardless of the duration of incubation, DoHH2 cells incorporated greater amounts of gallium than CCRF-CEM cells. After 48 hours of incubation, gallium uptake was ~1.7-fold greater in DoHH2 cells than in CCRF-CEM cells (data not shown).

Discordance between Cellular TfR Number and Growth Inhibition by Gallium Nitrate in Other Lymphoma Cell Lines

Because the above studies with DoHH2 and CCRF-CEM cells indicated that cellular TfR expression per se did not correlate with the growth-inhibitory effects of gallium nitrate, the relationship between cellular TfR levels and the cytotoxicity of gallium was further explored in a panel of human lymphoma cell lines. As shown in Fig. 3A, TfR
density (measured as Tf binding) varied significantly among the cell lines examined. Jurkat and Z-138C cells displayed the highest and lowest numbers of TfRs, respectively, whereas the other cell lines (JVM-2, HBL-2, and Raji cells) displayed intermediate levels of TfRs. No correlation between the growth-inhibitory effects of gallium nitrate and cellular TfR expression was noted. Jurkat cells that were least sensitive to gallium nitrate (IC50, 125 μmol/L) had the highest TfR density. In contrast, Z-138C, JVM-2, HBL-2, and Raji cells that had different levels of TfRs were more sensitive to gallium nitrate (IC50s, 68–40 μmol/L) than Jurkat cells (Fig. 3B). Tf internalization by Z-138C and Jurkat cells was then examined because these cells displayed the lowest and highest levels of TfRs. As shown in Fig. 3C, Z-138 cells that were more sensitive to gallium nitrate internalized a greater amount of Tf than Jurkat cells. These findings are consistent with the results obtained with CCRF-CEM and DoHH2 cells and support the notion that, in some cells, TfR function rather than TfR density correlates with the cytotoxicity of gallium nitrate.

**Induction of Apoptosis by Gallium Nitrate and Effects of Inhibitors of Caspase and Bax**

To examine whether the growth-inhibitory effects of gallium nitrate were due to induction of apoptosis, CCRF-CEM and DoHH2 cells were incubated with gallium nitrate and examined at various time points by Hoechst dye staining for changes in nuclear morphology consistent with apoptosis. Morphologic changes of apoptosis could be detected after ~24 hours of continuous incubation of cells with gallium nitrate. At that time point, 48% of DoHH2 cells incubated with 70 μmol/L gallium nitrate and 68% of CCRF-CEM cells incubated with 250 μmol/L gallium nitrate displayed changes consistent with apoptosis. In contrast, <5% of cells incubated without gallium nitrate showed such changes (Fig. 4A and B).

To confirm that the morphologic changes seen with gallium nitrate were indeed the result of caspase activation and induction of apoptosis, cells were preincubated with the pancaspase inhibitor Z-VAD-FMK for 1 hour before the addition of gallium nitrate to the incubation. This resulted in a marked decrease in cells showing gallium-induced apoptotic changes. In the presence of Z-VAD-FMK, only 14% of DoHH2 cells and 27% of CCRF-CEM cells incubated with gallium nitrate showed apoptotic changes (Fig. 4A and B). It is known that the proapoptotic protein Bax plays an important role in apoptosis (39, 40). Therefore, in additional incubations, the effects of a Bax-inhibitory peptide or a negative control peptide on gallium-induced apoptosis were examined. This strategy has been used by others to investigate the involvement of Bax in apoptosis (41, 42). Bax-inhibitory peptide was added to the incubation 1 hour before the addition of gallium nitrate. In the presence of Bax-inhibitory peptide, morphologic changes of apoptosis occurred in 16% of DoHH2 cells and 36% of

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**Figure 2.** TfR expression, Tf-TfR cycling, and cellular iron uptake studies. A, Scatchard analysis of 125I-Tf binding to cells. 125I-Tf binding studies were done on intact DoHH2 and CCRF-CEM cells at 4°C as described in Materials and Methods. Representative experiment. B, Tf-TfR cycling. Pulse-chase studies in DoHH2 and CCRF-CEM cells were conducted to examine the endocytosis and exocytosis of 125I-Tf bound to cell surface TfR. Cells were allowed to internalize surface TfR-bound 125I-Tf at 37°C. Nonradioactive Tf was added to cells after 15 min (chase) as described in Materials and Methods. Representative of three experiments. C, 59Fe uptake. DoHH2 and CCRF-CEM cells were incubated with 59Fe-Tf (76 pmol Fe/106 cells) for 5 h. Cells were washed and radioactivity in the cell pellet was counted to determine total cellular 59Fe uptake. Columns, mean (n = 3); bars, SE. D, 67Ga uptake. DoHH2 and CCRF-CEM cells were incubated with 0.5 μCi 67Ga citrate for 4 h. Cells were washed and radioactivity in the cell pellet was counted to determine total cellular 67Ga uptake. Columns, mean (n = 4); bars, SE.
In contrast, no protection from gallium-induced apoptosis occurred with the negative control peptide; 50% of DoHH2 cells and 66% of CCRF-CEM cells displayed apoptotic changes, similar to that seen with gallium nitrate alone (Fig. 4A and B).

**Activation of Caspase-3 by Gallium Nitrate**

Because a decrease in the number of cells undergoing gallium-induced apoptosis was seen in the coincubation experiments with the caspase inhibitor Z-VAD-FMK, further studies were conducted to measure caspase-3 enzymatic activity in CCRF-CEM and DoHH2 cells that had been incubated with gallium nitrate. Figure 4C and D shows the kinetics of caspase-3 enzymatic activity in DoHH2 cells incubated with 70 and 110 μmol/L gallium nitrate and CCRF-CEM cells incubated with 250 or 400 μmol/L gallium nitrate. These data show that gallium nitrate produced a dose-dependent increase in caspase-3 activity in both cell lines, consistent with induction of apoptosis shown in Fig. 4A and B.

**Gallium Nitrate Induces the Release of Cytochrome c from Mitochondria but Does Not Affect Bcl-2 or Bcl-X\textsubscript{L} Levels**

The triggering of apoptosis through the mitochondrial pathway involves activation of Bax and subsequent Bcl-2-dependent and Bcl-2-independent interactions at the mitochondrial level leading to the release of cytochrome c from the mitochondrion (39, 40). To investigate this pathway further, cells were incubated with gallium nitrate and the mitochondria-free cytosolic extracts were examined for cytochrome c by Western blotting. As shown in Fig. 5A, cytochrome c could be detected in the cytosol of cells exposed to gallium nitrate but not in cells incubated without gallium nitrate. These results strongly suggest that gallium induces apoptosis via a pathway that involves the mitochondrial release of cytochrome c leading to the downstream activation of caspase-3.

Additional experiments were conducted to determine whether the mechanism of gallium-induced apoptosis could be linked to changes in the levels of antiapoptotic Bcl-2 or Bcl-X\textsubscript{L}. As shown in Fig. 5B, DoHH2 cells displayed higher basal levels of Bcl-2 and lower levels of Bcl-X\textsubscript{L} when compared with CCRF-CEM cells. These levels did not change with exposure of cells to gallium nitrate in either cell line (Fig. 5B).

**Activation of Bax by Gallium Nitrate**

An initial step in the activation of proapoptotic Bax involves a conformation change in its structure, which results in the exposure of epitopes that can be specifically recognized by mAb 6A7 (43). Western blotting of cytosolic extracts with polyclonal antibody to Bax revealed no change in total Bax levels in cells exposed to gallium nitrate (Fig. 5C, top row). In contrast, Western blotting of mAb 6A7 immunoprecipitates from gallium-treated cells showed an increase in active Bax levels with increasing concentrations of gallium nitrate (Fig. 5C, bottom row).

**Proteasome Inhibition Enhances Gallium-Induced Apoptosis**

Because recent studies have shown the proteasome inhibitor bortezomib to have clinical activity in the treatment of non-Hodgkin’s lymphoma (44), additional experiments were conducted to explore the effects of bortezomib and gallium nitrate in combination on apoptosis. The effect of bortezomib alone on cell growth was first
examined to determine the concentrations with low cytotoxicity to be used for coincubation with gallium nitrate. These experiments revealed that the growth of CCRF-CEM cells was inhibited by 2% and 32% with 3 and 5 nmol/L bortezomib, respectively, following 24 hours of incubation (Fig. 6A). These bortezomib concentrations were therefore added to cells along with increasing concentrations of gallium nitrate and the effects on cytotoxicity were measured by MTT and caspase assays. As shown in Fig. 6B, although 3 nmol/L bortezomib alone had a minimal effect on cell growth, it increased cell growth inhibition by 250 μmol/L gallium nitrate from 24% to 38%. With 5 nmol/L bortezomib, the enhancement of gallium cytotoxicity was even greater; growth inhibition by 250 μmol/L gallium nitrate was increased from 24% to 73% (Fig. 6B). In parallel with the effect on cell proliferation, 5 nmol/L bortezomib when combined with gallium nitrate produced an increase in caspase-3 activation beyond that seen with 250 μmol/L gallium nitrate alone (Fig. 6C). This suggests that the enhanced cytotoxicity with the combination of the two agents is due to an increase in apoptosis.

Discussion

The mechanisms of the antineoplastic activity of gallium in lymphoma can be viewed as a two-step process: the first step involves the trafficking and uptake of gallium by cells through TfR-dependent (and, possibly, TfR independent) pathways, whereas the second step involves action of gallium on intracellular targets resulting in cell death. In the first set of studies, we attempted to clarify the role of the TfR in the cytotoxicity of gallium by investigating whether a direct correlation exists between receptor density and gallium-induced cell death. Although, logically, such an association might be assumed to exist, it has never been critically examined. Contrary to expectation, our results showed that, whereas the number of TfRs on DoHH2 cells

Figure 4. Induction of apoptosis and activation of caspase-3 by gallium nitrate. A and B, induction of apoptosis and effects of inhibitors. DoHH2 and CCRF-CEM cells were incubated in 1-mL wells for 24 h with or without gallium nitrate, (Ga) respectively. Caspase inhibitor Z-VAD-FMK (z-VAD; 400 μmol/L), Bax inhibitory peptide (BIP; 400 μmol/L), or a negative control peptide (NCP; 400 μmol/L) was added to selected wells 1 h before the addition of gallium nitrate. A, DoHH2 cells incubated with or without 70 μmol/L gallium nitrate. B, CCRF-CEM cells incubated with or without 250 μmol/L gallium nitrate. Con, control cells without gallium nitrate added. Columns, mean of a representative experiment done in duplicate; bars, range. C and D, induction of caspase-3 activity. Lysates prepared from DoHH2 and CCRF-CEM cells that had been incubated for 2 h with gallium nitrate (GaN) were assayed for caspase-3 enzymatic activity using the fluorogenic caspase-3 substrate Ac-DEVD-AFC as described in Materials and Methods. C, caspase-3 activity in DoHH2 cells incubated with 0, 70, or 110 μmol/L gallium nitrate. D, caspase-3 activity in CCRF-CEM cells incubated with 0, 250, and or 400 μmol/L gallium nitrate. Values shown are from a representative experiment. Two additional experiments produced similar results.
was one third that of CCRF-CEM cells, DoHH2 cells were three to four times more sensitive than CCRF-CEM cells to growth inhibition by gallium nitrate. This discrepancy between the TfR number and cell sensitivity to gallium nitrate was confirmed by additional experiments in a panel of lymphoma cell lines where no correlation was noted between cellular TfR expression and growth inhibition by gallium nitrate.

Because the level of TfR expression in cells did not seem to correlate with their sensitivity to gallium nitrate, we examined the role of TfR function in this process by comparing TfR cycling and gallium and iron uptake in DoHH2 and CCRF-CEM cells. Although the intracellular trafficking of gallium is not well understood, prior studies have shown that gallium resembles iron with respect to its initial uptake via TfR-dependent and TfR-independent cellular uptake pathways (10, 11, 37). Within the cell, however, gallium seems to follow a somewhat different trafficking pathway than iron and does not seem to incorporate into ferritin, the iron storage protein (45). In the present study, DoHH2 cells, despite having a lower number of TfRs, displayed more efficient Tf-TfR cycling and greater iron and gallium uptake than CCRF-CEM cells. This difference in TfR and iron kinetics likely means that DoHH2 cells require a greater amount of iron for viability and proliferation than CCRF-CEM cells. Importantly, it also means that DoHH2 cells take up a greater amount of gallium than CCRF-CEM and that this in turn contributes to their higher sensitivity to gallium nitrate. Collectively, these studies suggest that in some cells TfR function rather than TfR number is the more important factor in the initial step of the antineoplastic action of gallium (gallium uptake). The clinical implication from this study is that lymphomas with high TfR expression in vivo (as recognized by immunohistochemical staining of tissues) may not necessarily be more sensitive to gallium nitrate than those with lower TfR staining, and, vice versa.

In the second set of studies, we investigated the pathways involved in gallium-induced cell death and show for the first time that gallium nitrate induces apoptosis in
lymphoma cells through the mitochondrial pathway and that the proapoptotic protein Bax plays a role in this process. The ability of gallium to trigger apoptosis was suggested by (a) the changes in morphology that were observed in cells exposed to gallium nitrate and (b) the ability of the caspase inhibitor Z-VAD-FMK to abrogate these changes. Confirmation of caspase activation in these cells was provided by the demonstration of a dose-dependent increase in caspase-3 activity with gallium nitrate in CCRF-CEM and DoHH2 cells.

The activation of caspase-3, an effector caspase in apoptosis, may be triggered through the Fas (death) receptor pathway that involves activation of caspase-8 or through the mitochondrial pathway (39, 40). The latter involves the release of cytochrome c from mitochondria resulting in the generation of an apoptosome (consisting of caspase-9 and Apaf-1) that activates caspase-3. The proapoptotic protein Bax plays an important role in the mitochondrial pathway via its translocation from the cytoplasm to the mitochondria where antiapoptotic Bcl-2 resides (39, 40). Bcl-2 and Bax antagonize each other’s action by heterodimerization-dependent and heterodimerization-independent mechanisms. The balance of Bcl-2 and Bax (and their homologues) are known to determine the sensitivities of cells to apoptosis. In our studies, evidence for involvement of the mitochondrial pathway in gallium-induced apoptosis was provided by the following results. First, the presence of Bax-inhibitory peptide during incubation of cells with gallium nitrate reduced the number of cells undergoing apoptosis, an effect consistent with interference with the proapoptotic action of Bax. Second, cells incubated with gallium nitrate displayed an increase in 6A7-immunoprecipitable Bax, indicating that cytosolic Bax had been activated to undergo a conformational change that precedes its translocation to the mitochondria. Third, incubation of cells with gallium nitrate resulted in the release of cytochrome c from mitochondria, consistent with activation of caspase-3 via this pathway. Studies are now in progress to define the upstream events that lead to Bax activation by gallium compounds.

It has been shown that increased levels of Bcl-2 are associated with an increase in tumor cell resistance to a variety of chemotherapeutic drugs (26, 46). In this regard, it was of interest to consider whether endogenous Bcl-2 expression per se correlated with cell sensitivity to gallium nitrate. Although gallium nitrate inhibited the growth of both DoHH2 and CCRF-CEM cells, there was a 3- to 4-fold difference in their sensitivity to gallium (based on IC50s), thus providing an opportunity to address this question. We found that, although DoHH2 cells expressed greater levels of Bcl-2, they were relatively more sensitive to apoptosis induction by gallium nitrate than CCRF-CEM cells. In addition, the difference in the sensitivity of CCRF-CEM and DoHH2 cells to gallium nitrate did not seem to be due to a differential effect of gallium on Bcl-2 or Bcl-XL because the levels of either antiapoptotic protein were unaffected by gallium nitrate. Collectively, our results suggest that measurement of Bcl-2 levels alone in lymphoma cells is unlikely to be of value in predicting clinical response to gallium nitrate and that gallium-induced apoptosis is triggered by an increase in the ratio of Bax/Bcl-2 that is due primarily to an increase in active Bax levels.

An important strategy in cancer treatment has been to attempt to enhance tumor cell kill by using combination chemotherapy involving the simultaneous administration of two or more drugs with nonoverlapping toxicities. In the final set of studies, we explored whether the ubiquitin-proteasome pathway, which has been shown recently to be a potentially important target for cancer therapy, might be exploited to enhance the antineoplastic activity of gallium nitrate. The proteasome inhibitor bortezomib has shown clinical activity in the treatment of lymphoma and myeloma (44), and it seemed logical to investigate it in combination with gallium nitrate. Our studies showed that gallium-induced apoptosis could be enhanced by minimally cytotoxic concentrations of bortezomib. Although the mechanisms involved remain to be determined, these results suggest a novel therapeutic drug combination that warrants further investigation for the treatment of lymphoma.

In conclusion, the present investigation expands our knowledge of the role of the cellular targets for gallium and provides new insights into its mechanisms of antitumor action. Further studies are in progress to identify molecular determinants of sensitivity and resistance to gallium compounds. Such information may be useful in selecting lymphoma subtypes more likely to respond to treatment with gallium nitrate and in designing logical strategies to improve its clinical efficacy.

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