

Development of Gallium Compounds for Treatment of Lymphoma: Gallium Maltolate, a Novel Hydroxypyrrone Gallium Compound, Induces Apoptosis and Circumvents Lymphoma Cell Resistance to Gallium Nitrate

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ABSTRACT

Clinical studies have shown gallium nitrate to have significant antitumor activity against non-Hodgkin's lymphoma and bladder cancer, thus indicating that gallium-based drugs have potential for further development as antineoplastic agents. In this study, we compared the cytotoxicity of gallium maltolate, a novel gallium compound, with gallium nitrate in lymphoma cell lines, including p53 variant and unique gallium nitrate-resistant cells. We found that gallium maltolate inhibited cell proliferation and induced apoptosis through the mitochondrial pathway at lower concentrations and more rapidly than gallium nitrate. Gallium maltolate produced an increase in intracellular reactive oxygen species (ROS) within 2 h of incubation with cells; this effect could be blocked by mitoquinone, a mitochondria-targeted antioxidant. The role of the transferrin receptor (TfR) in gallium maltolate's action was examined using monoclonal an-

tibody (MoAb) 42/6 to block TfR function. However, although MoAb 42/6 reduced gallium maltolate-induced caspase-3 activity, it had only a minor effect on cell growth inhibition. Importantly, gallium maltolate induced apoptosis in cells resistant to gallium nitrate, and, unlike gallium nitrate, its cytotoxicity was not affected by cellular p53 status. Cellular gallium uptake was greater with gallium maltolate than with gallium nitrate. We conclude that gallium maltolate inhibits cell proliferation and induces apoptosis more efficiently than gallium nitrate. Gallium maltolate is incorporated into lymphoma cells to a greater extent than gallium nitrate via both TfR-independent and -dependent pathways; it has significant activity against gallium nitrate-resistant cells and acts independently of p53. Further studies to evaluate its antineoplastic activity in vivo are warranted.

Despite recent advances in the treatment of non-Hodgkin's lymphoma, a significant number of patients succumb to this disease each year. It is estimated that in 2007, there will be 63,190 new cases of non-Hodgkin's lymphoma and 18,660 deaths from this disease in the United States (Jemal et al., 2007). Hence, there remains a great need to develop novel agents for the treatment of this malignancy. In the late 1970s, the group IIIa metal salt gallium nitrate was discovered to have antitumor activity in animal tumor models, and in subsequent clinical trials, it proved to have significant antineoplastic activity against non-Hodgkin's lymphoma and

urothelial malignancies (Warrell et al., 1983; Weick et al., 1983; Keller et al., 1986; Chitambar et al., 1997; Einhorn, 2003; Chitambar, 2004). A more recent clinical trial confirmed the efficacy of gallium nitrate in patients with non-Hodgkin's lymphoma whose disease had relapsed following treatment with conventional chemotherapeutic agents (Pro et al., 2004).

The mechanisms responsible for the cytotoxicity of gallium are only partly understood. In the circulation, gallium binds to the iron transport protein transferrin (Tf) and may be incorporated into cells via Tf receptors (TfRs) present on the cell surface (Larson et al., 1980; Rasey et al., 1982; Harris and Pecoraro, 1983; Chitambar and Seligman, 1986; Chitambar and Zivkovic, 1987); Tf-independent pathways may also contribute to the uptake of gallium by certain cells (Chitambar and Zivkovic, 1987). Although much remains to be

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ABBREVIATIONS: Tf, transferrin; TfR, transferrin receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; FITC, fluorescein isothiocyanate; PI, propidium iodide; MoAb, monoclonal antibody; mito-Q, mitoquinone; ROS, reactive oxygen species; DCF, 2',7'-dichlorofluorescein; z, benzyloxy-carbonyl; fmk, fluoromethyl ketone; 6-carboxy-DCF-AM, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di-acetoxymethyl ester.

learned about the intracellular trafficking pathways for gallium, it is known that the steps leading to the inhibition of cellular proliferation by gallium involve a disruption of iron homeostasis and a diminution in the activity of iron-dependent ribonucleotide reductase (Chitambar and Seligman, 1986; Chitambar et al., 1988, 1991). More recently, it was shown that gallium nitrate induces apoptosis through a pathway that includes the activation of proapoptotic Bax, release of cytochrome *c* from the mitochondrion and downstream activation of effector caspases (Chitambar et al., 2006). Gallium may also act on other cellular process; however, the extent to which those actions contribute to its cytotoxicity remains to be determined (Collery et al., 2002).

Although gallium nitrate is a clinically active drug in lymphomas, a number of patients fail to respond to treatment, suggesting that their malignant cells have developed resistance to this drug. Tumor resistance to chemotherapy, whether to gallium nitrate or any other antineoplastic drug, is a major challenge to the successful treatment of malignancy. There is only a partial understanding of how cells manage to evade gallium's cytotoxicity. Using CCRF-CEM lymphoma cell lines made resistant to gallium nitrate, we have previously shown that these cells display a decrease in their uptake and intracellular accumulation of gallium and iron (Chitambar and Wereley, 1997, 1998). More recently, we reported that these gallium-resistant cells display a marked increase in the expression of metallothionein-2A, a low-molecular weight metal binding protein, and ZnT-1, a cell membrane-based protein involved in the efflux of zinc (Yang et al., 2007). The specific mechanisms by which these proteins modulate cell sensitivity to gallium are under investigation; however, current evidence suggests that the development of tumor cell resistance to gallium nitrate includes changes in gallium transport into cells as well as processes involved in intracellular metal sequestration.

The ability of gallium nitrate, a simple metal salt, to inhibit tumor cell growth *in vitro* and *in vivo* provides a strong rationale for continued research into the development of new gallium compounds with improved therapeutic application. One new candidate compound of interest is gallium maltolate, which consists of gallium bound to maltol (3-hydroxy-2-methyl-4-pyrone) and whose chemical structure has been previously reported (Bernstein et al., 2000). This gallium compound was recently shown to be cytotoxic in hepatocellular carcinoma cell lines (Chua et al., 2006). The hydroxypyrones are a family of chelators with high affinity for a range of metal ions (Thompson et al., 2006). An important property of these chelators is that they are capable of not only mobilizing metal ions from cells but also delivering metals to cells. In the present study, we show that gallium maltolate is a more efficient inducer of apoptosis than gallium nitrate; its cytotoxicity is independent of p53 and extends to cells resistant to the growth-inhibitory effects of gallium nitrate. Induction of apoptosis by gallium maltolate is mediated through the mitochondria and includes an increase in the generation of reactive oxygen species. Our studies show that cellular gallium uptake is more efficient with gallium maltolate than with gallium nitrate and suggest that strategies to enhance the incorporation of gallium into cells may be an important approach to circumventing gallium resistance.

Materials and Methods

Materials. Gallium nitrate was obtained from Genta Incorporated (Berkley Heights, NJ), whereas gallium maltolate was obtained from Titan Pharmaceuticals (South San Francisco, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), annexin V-FITC, propidium iodide (PI), Tf, and maltol were purchased from Sigma Chemical Co. (St. Louis, MO). Ga-67 citrate and ¹²⁵I-Na were purchased from GE Healthcare (Piscataway, NJ). JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide) was obtained from Cell Technology Inc. (Mountain View, CA). 6-Carboxy-2',7'-dichlorodihydrofluorescein diacetate, di-acetoxymethyl ester (6-carboxy-DCF-AM) was purchased from Invitrogen (Carlsbad, CA). Monoclonal antibody (MoAb) 42/6 against the human TfR was a generous gift from Dr. Ian Trowbridge (Salk Institute for Biologic Studies, La Jolla, CA). Mitoquinone (mito-Q) was generously provided by Dr. B. Kalyanaraman (Medical College of Wisconsin, Milwaukee, WI).

Cell Lines. Gallium-sensitive CCRF-CEM cells were obtained from American Type Culture Collection (Manassas, VA). Gallium-resistant cells were developed from this cell line by incubation of cells with incremental concentrations of gallium nitrate for several months, as described previously (Chitambar and Wereley, 1997). Lymphoid leukemic cell lines WTK1 (containing mutant p53, methionine to isoleucine substitution at codon 237 of the p53 gene) and TK6 (containing wt p53) and NH32 cells (p53 null) were provided by Dr. Lyuba Varticoski (National Cancer Institute, Bethesda, MD) and have been described previously (Little et al., 1995; Xia et al., 1995). Mantle cell lymphoma cell lines HBL-2, Granta, JVM-2, NCEB-1, and Z138C were obtained from the British Columbia Cancer Agency (Vancouver, Canada) and have been described previously (Tucker et al., 2006). All cells were grown in an atmosphere of 5% CO₂ in RPMI 1640 supplemented with 10% bovine serum.

Cell Proliferation. The effects of gallium nitrate or gallium maltolate on cell proliferation were assessed by MTT assay as described previously (Mosmann, 1983). Cells (2×10^5 /ml) were plated in 96-well microwell plates (100 μ l/well) in the presence of increasing concentrations of either gallium compound. After 24 to 72 h of incubation at 37°C, 10 μ l of MTT (5 mg/ml stock) was added to each well, and the incubation continued for an additional 4 h. One hundred microliters of 0.04 N HCl in isopropyl alcohol was then added to each well to solubilize the cells, and the absorbance of each well was determined by spectrophotometry at dual wavelengths of 570 and 630 nm using an EL808 microplate autoreader (Biotech Instruments, Winooski, VT). The absorbance of the wells containing additives was compared with wells in which the additives were omitted (control cells).

Caspase-3 Assay. Cells incubated with increasing concentrations of gallium maltolate or gallium nitrate for various periods of time were analyzed for caspase-3 activity using an Apo-One Homogeneous Caspase-3/7 assay (Promega, Madison, WI), which is based on the enzymatic cleavage of the fluorogenic caspase-3 substrate rhodamine 110, bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide). In selected incubations, the pan-caspase inhibitor z-VAD-fmk (R&D Systems, Minneapolis, MN) or the anti-TfR MoAb 42/6 was added to the incubation 1 h before the addition of gallium compounds. After incubation with gallium compounds, cells were counted, and equal numbers of cells were transferred to microwell plates (20,000 cells/well) for assay. The fluorescence from each well was measured by fluorometer using an excitation wavelength of 485 nm and emission wavelength of 530 nm. The fluorescence from cells treated with gallium compounds was expressed relative to that of cells not exposed to gallium.

Annexin V Staining. After 12 to 24 h of incubation with various concentrations of gallium maltolate, cells were washed with PBS, stained with annexin V FITC, and propidium iodide for 10 min, and analyzed by flow cytometry as recommended by the manufacturer.

Mitochondrial Membrane Potential. The effect of gallium maltolate on mitochondrial permeability transition was examined using JC-1 dye. Cells that had been incubated with increasing concentrations of gallium maltolate were washed, stained with JC-1 reagent, and then analyzed by two parameter flow cytometry, according to the manufacturer's recommendation.

Measurement of Reactive Oxygen Species in Cells. Intracellular reactive oxygen species (ROS) was measured as described previously (Said Ahmed et al., 2000). CCRF-CEM cells were plated (10^6 cells/ml) in 25-cm² tissue culture flasks and loaded with 10 μ M 6-carboxy-DCF-AM by incubation with this compound for 1 h. In selected flasks, 10 μ M of the mitochondria-targeted antioxidant mito-Q was added, and the incubation continued for an additional hour. Gallium maltolate (0, 50, or 200 μ M) was then added to each flask and after 2 or 3 h of incubation, 1 ml of cell suspension (10^6 cells) was removed and centrifuged. Cell pellets were resuspended in 400 μ l of PBS and transferred to 96-well assay plates for fluorometry analysis. 2',7'-Dichlorofluorescein (DCF) fluorescence in each well was measured using an excitation wavelength of 495 nm and emission wavelength of 525 nm in a PerkinElmer 96-well plate reader (PerkinElmer Life and Analytical Sciences, Boston, MA).

Gallium Uptake. Gallium nitrate-sensitive and -resistant CCRF-CEM cells were plated (5×10^5 cells/ml) in 1-ml multiwell plates and incubated with increasing concentrations of either gallium nitrate or gallium maltolate containing Ga-67 as a tracer (1 μ Ci of Ga-67/1 mM gallium nitrate or gallium maltolate). After 24 h of incubation, the number of cells in each well was counted with a hemocytometer, and the cells were harvested and washed with PBS by centrifugation. Ga-67 radioactivity in the cell pellet was counted in a gamma counter, and the amount of Ga-67 incorporated per 10^6 cells was determined.

Tf Binding Studies. CCRF-CEM cells were harvested, washed with ice-cold PBS containing 0.1% bovine serum albumin, and assayed for Tf binding at 37°C in the presence or absence of anti-TfR MoAb 42/6 using a previously described ¹²⁵I-Tf binding assay (Chitambar and Seligman, 1986).

Results

Comparison of the Growth-Inhibitory Effects of Gallium Maltolate and Gallium Nitrate. The effects of gallium compounds on cell proliferation were examined in human leukemic CCRF-CEM cells. As shown in Fig. 1A, both gallium nitrate and gallium maltolate inhibited cellular proliferation; however, the inhibitory effects of gallium nitrate were seen after 48 h and not after 24 h of incubation of cells with this drug. In contrast, a significant dose-dependent inhibition of cell growth was noted with gallium maltolate after the 24-h incubation. At all time-points examined, cell growth inhibition was greater with gallium maltolate than with gallium nitrate. Following 72-h incubation, the IC₅₀ concentrations of gallium maltolate and gallium nitrate were 29 and 103 μ M, respectively. Because maltol has metal binding properties that could potentially contribute to the growth-inhibitory effects of gallium maltolate, cell proliferation in the presence of 0 to 450 μ M maltol was also examined. No effect of maltol alone on cell proliferation was seen, thus indicating that growth-inhibitory effects of gallium maltolate were produced by gallium and not by maltol (data not shown).

To determine whether the differential effects of gallium maltolate and gallium nitrate extended to other lymphoma cells, the growth-inhibitory effects of these compounds were examined in a panel of mantle cell lymphoma cell lines. As shown in Table 1, the IC₅₀ of gallium nitrate for these cell lines varied over a considerably wide range; Granta and NCEB-1 cells displayed the lowest sensitivity to gallium nitrate, whereas JVM-2 cells displayed the greatest sensitivity. In contrast, the proliferation of all these cell lines was uni-

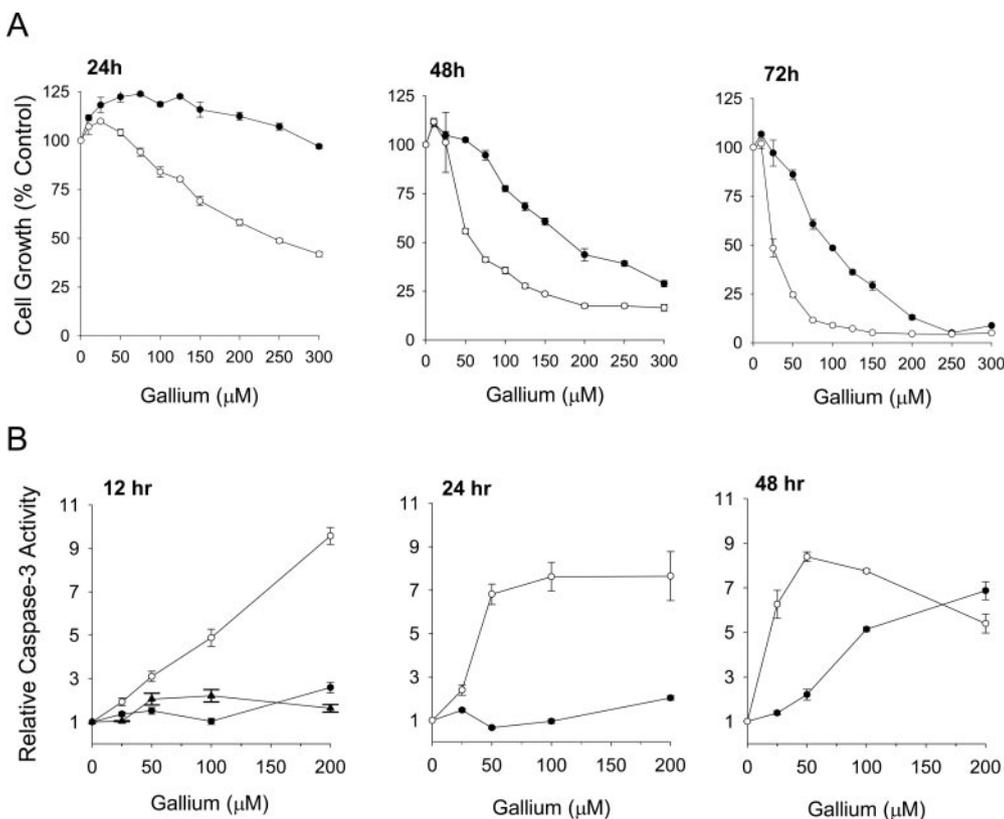


Fig. 1. Gallium maltolate and gallium nitrate inhibit cell proliferation and activate caspase-3. A, cell proliferation. CCRF-CEM cells were plated in 96-well plates (0.2×10^6 cells/ml) in the presence of increasing concentrations of gallium maltolate (open circles) or gallium nitrate (closed circles). Cell growth was measured by MTT assay after 24- to 72-h incubation. Values shown represent the means \pm S.E. ($n = 3$). B, induction of caspase-3 activity. CCRF-CEM cells incubated with increasing concentrations of gallium maltolate (open circles) or gallium nitrate (closed circles) were analyzed for caspase-3 activity at the times shown. For the 12-h incubation, cells were also incubated with 50 μ M z-VAD-fmk (triangles) before the addition of gallium maltolate. Values shown are the means \pm S.E. of an experiment performed in triplicate. Similar results were obtained in three separate experiments.

TABLE 1

Comparison of the growth-inhibitory effects of gallium nitrate and gallium maltolate in mantle cell lymphoma cell lines

Cells were plated in 96-well plates (0.2×10^6 cells/ml) in the presence of increasing concentrations of gallium nitrate or gallium maltolate. Cell growth was measured by MTT assay after a 72-h incubation.

Cell Lines	IC ₅₀	
	Gallium Nitrate	Gallium Maltolate
	μM	
Granta	295	35
Z138C	80	19
HBL-2	165	15
JVM-2	37	22
NCEB-1	300	23

formly inhibited by gallium maltolate at much lower gallium concentrations.

Gallium Maltolate Activates Caspase-3. To examine whether the cytotoxicity of gallium maltolate was the result of caspase activation, effector caspase-3 activity was measured in CCRF-CEM cells exposed to gallium compounds for various times. As shown in Fig. 1B, a concentration-dependent increase in caspase-3 activity was noted in cells incubated with gallium maltolate for 12 h. This activity was inhibited by the pancaspase inhibitor z-VAD-fmk, thus confirming that caspase-3 was indeed being activated (Fig. 1B). In contrast to gallium maltolate, gallium nitrate at similar concentrations did not produce a significant increase in caspase-3 activity after 12 and 24 h of incubation with cells. These results are consistent with the findings of the MTT assay (Fig. 1A) in which an inhibition of cell proliferation was not seen during the first 24-h incubation of cells with gallium nitrate. After 48 h, however, cells incubated with gallium nitrate displayed a concentration-dependent increase in caspase-3 activity (Fig. 1B). In additional experiments, caspase-3 activity was measured in cells incubated with 0 to 200 μM gallium maltolate for 3 and 6 h. Although an increase in caspase-3 activity was not detected at 3 h, a 2- and 4-fold increase in caspase-3 activity was seen after 6 h of incubation with 100 and 200 μM gallium maltolate, respectively (not shown). Hence, gallium maltolate inhibits cell growth and

activates caspase-3 at earlier time-points and at lower concentrations than gallium nitrate.

Gallium Maltolate Induces Apoptosis through the Mitochondrial Pathway. Early cellular changes in apoptosis are characterized by the translocation of phosphatidylserine to the external surface of the plasma membrane where it can be detected by binding to annexin V-FITC. As cell membranes are further compromised and cell death ensues, cellular DNA becomes accessible for staining with PI. In the study shown in Fig. 2A, cells that had been incubated with gallium maltolate for 12 h were dual-stained with annexin-FITC and PI and analyzed by flow cytometry. As shown in Fig. 2A, with increasing concentrations of gallium maltolate, there was a progressive increase in the population of cells staining positive for annexin V, a finding that is consistent with the induction of apoptosis in these cells. With 200 μM gallium maltolate, there was also an increase in the population of PI-stained cells, indicating that a smaller population of cells had progressed to a more advanced stage of cell death.

The signaling of cell death through the mitochondrion involves an increase in mitochondrial permeability transition that results in the release of cytochrome *c* and downstream activation of effector caspases (Zimmermann et al., 2001; Green and Kroemer, 2004). The increase in mitochondrial permeability transition is accompanied by a collapse in mitochondrial membrane potential that can be measured by JC-1 dye staining. In healthy nonapoptotic cells, JC-1 is taken up by mitochondria to produce a red fluorescence; however, with the loss of mitochondrial membrane potential, the dye remains in the cytoplasm to produce a green fluorescence. As shown in Fig. 2B, a progressive increase in green fluorescence and a loss of red fluorescence were noted in cells incubated with increasing concentrations of gallium maltolate for 12 h. The gallium-induced loss of mitochondrial membrane potential was even greater with longer incubation. After 24 h of incubation with 200 μM gallium maltolate, the population of cells with red fluorescence decreased from 92 to 51%, whereas the population of cells with green fluorescence increased from 4 to 46% (Fig. 2C).

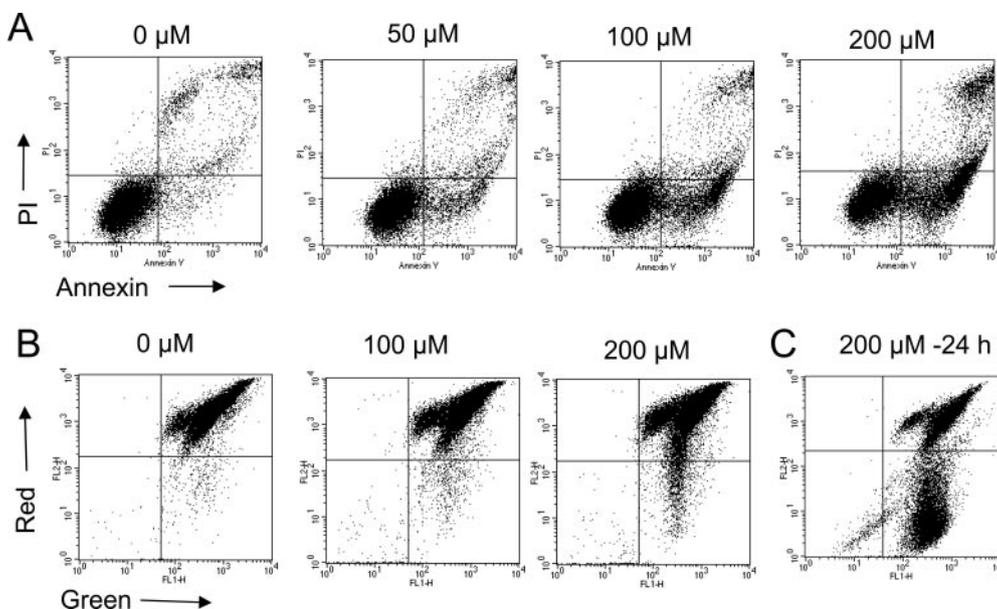


Fig. 2. Gallium maltolate induces apoptosis through the mitochondrial pathway. A, flow cytometry analysis of annexin V-FITC and PI staining. Cells were incubated for 12 h with increasing concentrations of gallium maltolate and analyzed for annexin V-FITC and PI fluorescence. Cells positive for annexin V-FITC are in the lower right quadrant of the dot plot, whereas cells staining positive for PI are in the upper right quadrant of the plot. B and C, mitochondrial staining using JC-1 dye. Cells were analyzed after incubation with gallium maltolate for 12 (B) or 24 (C) h as described under *Materials and Methods*. Loss of mitochondrial membrane potential results in the loss of red fluorescence (upper right quadrant on the dot plot) and an increase in green fluorescence (lower right quadrant on the dot plot).

Gallium Maltolate Increases Mitochondrial ROS Production. To examine whether the cytotoxicity of gallium maltolate was associated with oxidative stress, studies were conducted using 6-carboxy-DCF-AM, a nonfluorescent compound that can be incorporated into cells and oxidized to yield a detectable fluorescent product DCF. As shown in Fig. 3, in the absence of gallium maltolate, CCRF-CEM cells displayed a basal level of intracellular DCF fluorescence indicating the existence of endogenously generated ROS in these cells. This fluorescence increased significantly with exposure of cells to gallium maltolate and was consistent with a gallium-induced increase in intracellular ROS. As shown in Fig. 3, in the presence of 200 μM gallium maltolate, a significant increase in intracellular ROS above baseline was noted after 2 h of incubation. In contrast, in the presence of 50 μM gallium maltolate, an increase in intracellular ROS was seen after 3 h of incubation. To examine whether this ROS originated from the mitochondria, cells were incubated with the mitochondria-targeted antioxidant mito-Q before exposure to gallium maltolate. This approach has been used by other investigators to study mitochondria-generated ROS (Kelso et al., 2001; Dhanasekaran et al., 2004). As shown in Fig. 3, mito-Q completely blocked the gallium-induced increase in intracellular ROS. Interestingly, mito-Q produced a decrease in DCF fluorescence to a level below that seen in cells incubated with gallium maltolate alone, suggesting that this antioxidant blocked both gallium-induced and basal ROS production by the mitochondrion.

Role of the Tfr in Gallium Maltolate-Induced Apoptosis. Prior studies have demonstrated a role for Tfr-mediated endocytosis in the cellular uptake and cytotoxicity of gallium (Larson et al., 1980; Rasey et al., 1982; Harris and Pecoraro, 1983; Chitambar and Seligman, 1986; Chitambar and Zivkovic, 1987); however, the relevance of this pathway to gallium maltolate-induced apoptosis is not known. To examine this, experiments were conducted to determine whether blockade of cell surface Tfr function with MoAb 42/6 would affect the activation of caspase-3 by gallium maltolate.

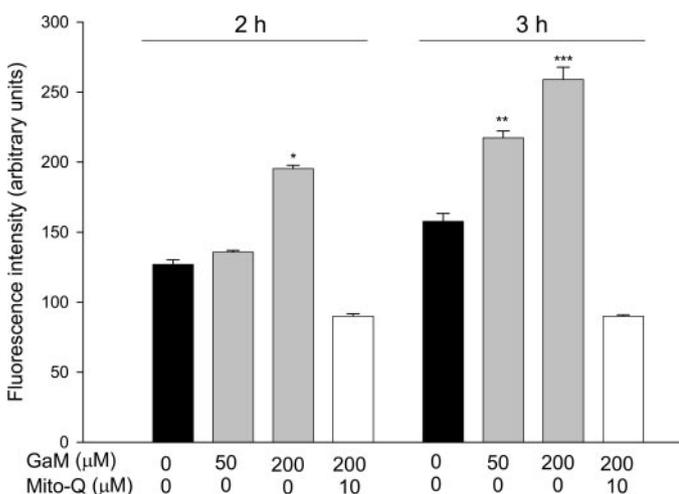


Fig. 3. Gallium maltolate increases mitochondrial ROS production. CCRF-CEM cells were assayed for DCF fluorescence indicative of ROS production as described under *Materials and Methods*. Cells were analyzed after 2 or 3 h of incubation with or without gallium maltolate. Values shown represent means \pm S.E. ($n = 3$). Differences between control and gallium maltolate-treated cells are significant. *, $p = 0.0004$; **, $p = 0.00021$; and ***, $p = 0.0032$ by paired Student's t test.

Binding studies were first conducted to confirm that 42/6 did indeed interfere with the interaction of Tf with its receptor. As shown in Fig. 4A, specific ^{125}I -Tf binding to cells was markedly reduced by MoAb 42/6, consistent with previous reports that show that this MoAb blocks Tfr function (Trowbridge and Lopez, 1982; Taetle et al., 1986). The next set of experiments examined whether Tfr blockade by MoAb 42/6 affected gallium maltolate-induced apoptosis. As shown in Fig. 4B, 30 $\mu\text{g/ml}$ MoAb 42/6 (a concentration sufficient to saturate cell surface Tfrs) reduced gallium maltolate-induced caspase-3 activity; however, a significant level of caspase-3 activity still remained. This residual caspase-3 activity was due to gallium maltolate and not Tfr blockade because 30 $\mu\text{g/ml}$ MoAb 42/6 alone did not induce caspase-3 activity over a 24-h incubation (data not shown). Although MoAb 42/6 reduced caspase-3 activity, it had only a small effect on preventing cell growth inhibition by gallium maltolate. As shown in Fig. 4C, MoAb 42/6 reduced the growth-inhibitory effect of 100 μM gallium maltolate by approximately 15% ($p = 0.01$ by Student's t test) but had no impact on cell growth inhibition by higher concentrations of gallium maltolate.

Gallium Maltolate Cytotoxicity Is Independent of p53 Status. The role of p53 in gallium-induced apoptosis is not known. To examine this, lymphoma cell lines TK-6 (wild-type p53), NH-32 (p53 null), and WTK-1 (mutant p53) were examined for their sensitivity to growth inhibition by gallium compounds. As shown in Fig. 5, all three cell lines were sensitive to gallium maltolate. TK-6 and NH-32 cells were also sensitive to gallium nitrate, albeit to a lesser extent than gallium maltolate. The IC_{50} for gallium maltolate was 18 and 40 μM for TK-6 and NH-32 cells, respectively, whereas the IC_{50} for gallium nitrate was 145 and 150 μM for the same cell lines (Fig. 5). WTK-1 cells were markedly less sensitive to gallium nitrate; 450 μM gallium nitrate inhibited the growth of these cells by approximately 25% (Fig. 5). In contrast, WTK-1 cells were highly sensitive to gallium maltolate (IC_{50} , 45 μM ; Fig. 5). Hence, the growth of these p53 variant cell lines was inhibited uniformly by gallium maltolate but not by gallium nitrate.

Gallium Maltolate Inhibits the Growth of Gallium Nitrate-Resistant CCRF-CEM Cells. CCRF-CEM cells resistant to gallium nitrate (GnR cells) have previously been reported by us (Chitambar and Wereley, 1997). As shown in Fig. 6A, gallium nitrate inhibited the proliferation of gallium-sensitive CCRF-CEM cells, whereas it failed to inhibit the growth of GnR cells. In contrast, the proliferation of GnR cells could be inhibited by increasing concentrations of gallium maltolate (Fig. 6, B and C). However, even though GnR cells were sensitive to gallium maltolate, their growth was not inhibited to the same extent as gallium-sensitive CCRF-CEM cells; the IC_{50} of gallium maltolate was 25 and 72 μM for gallium-sensitive and GnR cells, respectively (Fig. 6B). Consistent with the differential effects of these gallium compounds on GnR cell growth, gallium maltolate produced an increase in caspase-3 activity, whereas gallium nitrate failed to do so (Fig. 6D).

Cellular Gallium Uptake Is More Efficient with Gallium Maltolate. To examine whether differences in the cytotoxicity of gallium maltolate and gallium nitrate could be related to differences in their ability to transport gallium into cells, cellular gallium uptake was examined using Ga-67 as a

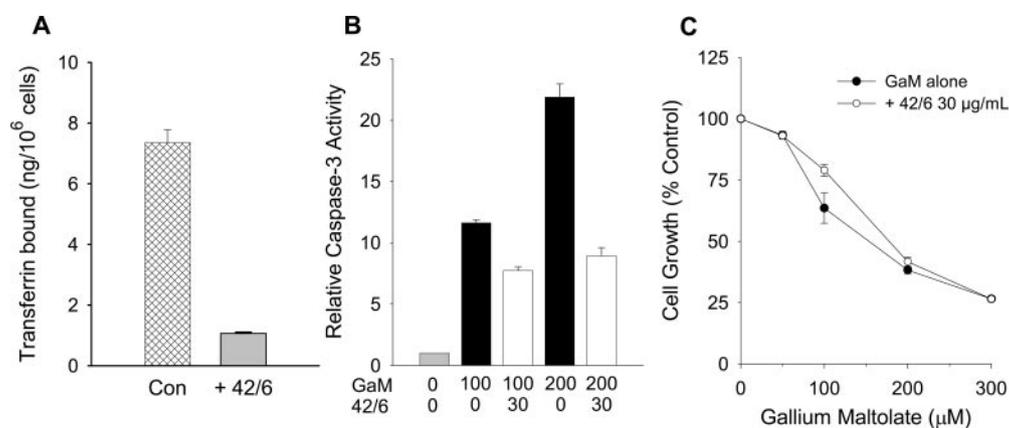


Fig. 4. Blockade of the TfR by MoAb 42/6 decreases caspase-3 activation but not the growth-inhibitory effects of gallium maltolate. **A**, Tf binding. Intact CCRF-CEM cells were assayed for Tf binding in the absence (Con) or presence of 10 $\mu\text{g}/\text{ml}$ MoAb 42/6, as described under *Materials and Methods*. **B**, caspase-3 activity. CCRF-CEM cells were assayed for caspase-3 activity after 24 h of incubation with gallium maltolate (GaM) and MoAb 42/6 at the concentrations shown in the figure. Values shown represent the means \pm S.E. ($n = 3$). **C**, cell proliferation. CCRF-CEM cells were incubated with increasing concentrations of gallium maltolate in the absence or presence of 30 $\mu\text{g}/\text{ml}$ MoAb 42/6. Cell growth was measured after a 24-h incubation. Values shown represent the means \pm S.E. of three experiments, each performed in quadruplicate.

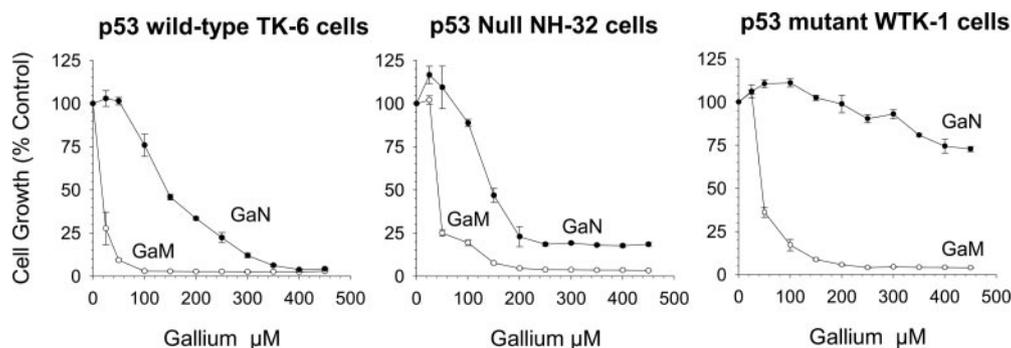


Fig. 5. Effects of gallium maltolate and gallium nitrate on the proliferation of p53 variant lymphoma cell lines. Cell growth was measured by MTT assay after 72-h incubation of cells with increasing concentrations of gallium maltolate (open circles) or gallium nitrate (closed circles). Values shown represent the means \pm S.E. ($n = 3$).

tracer for either gallium compound. As shown in Fig. 6, E and F, over a 24-h incubation, both gallium-sensitive and -resistant cells incorporated greater amounts of Ga-67 as gallium maltolate than as gallium nitrate, thus suggesting that the uptake of gallium is more efficient when gallium is presented to cells as gallium maltolate.

Discussion

The activity of gallium nitrate in non-Hodgkin's lymphoma and urothelial malignancies demonstrated in numerous clinical trials indicates that gallium-based drugs have significant potential for further advancement as chemotherapeutic agents in specific diseases. Hence, the development of compounds in which gallium is bound to ligands that may enhance its transport into malignant cells and facilitate its delivery to critical intracellular targets is of major interest. Prior studies have shown that a lipophilic iron chelator pyridoxal isonicotinyl hydrazone is capable of binding gallium and enhancing its transport into cells independent of Tf, thereby increasing the growth-inhibitory effects of this metal beyond that of gallium nitrate alone (Richardson et al., 1995; Chitambar et al., 1996). In the present investigation, we report on a novel compound, gallium maltolate, and show that it displays significantly greater cytotoxicity than gallium nitrate, and, most importantly, it induces apoptosis in cells that are resistant to gallium nitrate. Compared with gallium nitrate, gallium maltolate-induced apoptosis occurs not only at lower gallium concentrations but also at signifi-

cantly earlier incubation time-points. As demonstrated in this study and in previous investigations (Chitambar et al., 2006), the triggering of apoptosis by gallium nitrate in gallium-sensitive CCRF-CEM cells occurs after 24 to 30 h of incubation. In contrast, gallium maltolate induces apoptosis after 6 h of incubation.

Although gallium maltolate induces apoptosis more efficiently than gallium nitrate, both gallium compounds appear to eventually activate effector caspase-3 through a similar apoptotic pathway. Recently, we showed that gallium nitrate activates proapoptotic Bax, leading to the mitochondrial release of cytochrome *c*, downstream activation of caspase-3, and apoptosis (Chitambar et al., 2006). In the present study, using JC-1 dye, we show that the exposure of cells to gallium maltolate results in a loss of the mitochondrial membrane potential and an increase in mitochondrial permeability transition. These changes are known to herald the release of cytochrome *c* from the mitochondria and are consistent with the involvement of the mitochondrial pathway in apoptosis induced by gallium maltolate.

Having demonstrated an action of gallium maltolate on mitochondria, we examined whether its cytotoxicity was associated with the generation of intracellular ROS. These experiments revealed two important novel findings. First, an increase in intracellular ROS occurred as an early event (within 2 h) following the incubation of cells with gallium maltolate and preceded caspase-3 activation. Second, this increase in gallium-induced ROS production was completely

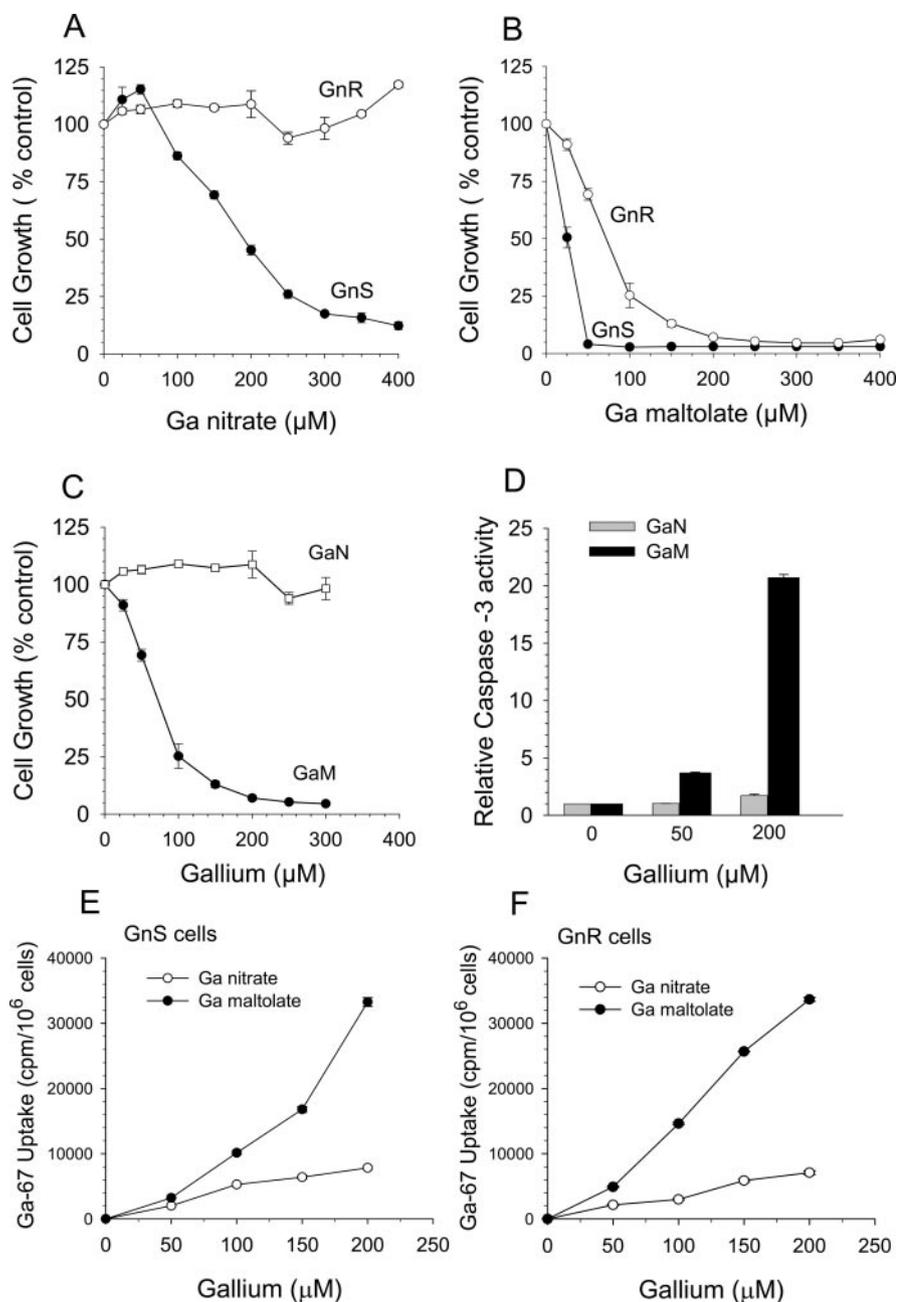


Fig. 6. Gallium maltolate induces apoptosis in CCRF-CEM cells resistant to gallium nitrate and is incorporated into cells to a greater extent than gallium nitrate. A, effect of gallium nitrate on the growth of gallium nitrate-resistant (GnR) and -sensitive (GnS) cells. Cellular proliferation was measured by MTT assay after 72-h incubation with gallium nitrate. Values shown are means \pm S.E. ($n = 4$). B, effect of gallium maltolate on the growth of gallium nitrate-resistant (GnR) and -sensitive (GnS) cells. Cellular proliferation was measured by MTT assay after 72-h incubation with gallium maltolate. Values shown are means \pm S.E. ($n = 4$). C, comparison of the effects of gallium maltolate and gallium nitrate on GnR cells. Cell growth was measured by MTT assay after 72-h incubation. D, gallium maltolate but not gallium nitrate activates caspase-3 in GnR cells. Caspase-3 activity was measured after a 24-h incubation of cells with gallium maltolate or gallium nitrate at the concentrations shown. E and F, cellular gallium uptake. Cells were incubated with increasing concentrations of gallium nitrate or gallium maltolate containing Ga-67 as a tracer. Ga-67 incorporated into cells was measured after a 24-h incubation. Values shown represent the means \pm S.E. ($n = 5$).

blocked by the mitochondria-targeted antioxidant mito-Q, suggesting that ROS generated in the presence of gallium maltolate originated from the mitochondrion. Not surprisingly, even in the absence of gallium maltolate, CCRF-CEM cells had a basal level of endogenous ROS, a finding that is consistent with other reports that a small amount of ROS is physiologically present in cells as a byproduct of mitochondrial metabolism (Raha and Robinson, 2000). The source of this ROS appears to be complexes I and III of the mitochondrial electron transport chain that produce superoxide, which is dismutated to form hydrogen peroxide. The latter may, via the Fenton reaction, lead to the formation of the highly damaging hydroxyl radical (Lubec, 1996). How gallium maltolate increases ROS production is not clear. Although gallium does share certain properties with iron, it is not redox active; therefore, its ability to generate ROS may result from a direct action on steps in mitochondrial oxidative phosphor-

ylation or the displacement of iron from intracellular proteins, thereby making iron available to catalyze ROS production. These potential mechanisms are currently being investigated.

For gallium to induce apoptosis, it must first be incorporated into cells. Prior studies have shown a role for Tf and the TfR in gallium uptake (Larson et al., 1980; Harris and Pecoraro, 1983; Chitambar and Zivkovic, 1987); however, cellular gallium uptake may also occur by a Tf-independent pathway (Chitambar and Zivkovic, 1987). Hence, to determine which uptake pathway is relevant to the mechanism of action of gallium maltolate, we examined the effects of gallium maltolate under conditions of TfR blockade. It should be noted that a limitation to the use of MoAb 42/6 for such studies is that TfR blockade may itself inhibit cellular iron uptake and reduce cell proliferation (Trowbridge and Lopez, 1982; Taetle et al., 1986). We

therefore considered these independent effects of MoAb 42/6 and noted that the concentration of this antibody used in our experiments did not significantly activate caspase-3 or inhibit cell proliferation over a 24-h incubation. Our studies showed that although MoAb 42/6 blocked TfR function and reduced the level of caspase-3 activity induced by gallium maltolate, it did not abrogate caspase-3 activity, nor did it reverse the growth-inhibitory effects of gallium maltolate. Interestingly, MoAb 42/6 did provide partial protection (~15%) against growth inhibition by lower concentrations of gallium maltolate, suggesting that when gallium concentrations are limited, TfR-mediated gallium uptake (as Tf-Ga) does contribute gallium maltolate's cytotoxicity. In the latter situation, it is likely that Tf-Ga is formed by a transfer of Ga from maltol to Tf present in the culture medium. Overall, however, we conclude that although caspase-3 activation by gallium maltolate may result from gallium uptake by TfR-dependent and -independent pathways, the amount of caspase-3 activation by the latter pathway is sufficient to inhibit cell proliferation. Thus, the growth-inhibitory effects of gallium maltolate cannot be reversed by blocking TfR function. Gallium maltolate appears, therefore, to exert its apoptotic action by bypassing the TfR and delivering gallium to intracellular targets.

An important finding in our studies was that cells resistant to growth inhibition by gallium nitrate, including WTK-1 p53 mutant cells, were sensitive to gallium maltolate. Gallium maltolate inhibited cell growth and activated caspase-3 in gallium nitrate-resistant cells, whereas gallium nitrate failed to do so. Prior studies examining the mechanisms of tumor cell resistance to gallium nitrate have suggested that the development of such resistance includes a decrease in TfR-mediated gallium uptake and changes in the intracellular trafficking of gallium and iron (Chitambar and Wereley, 1997; Davies et al., 2006). The cytotoxicity of gallium maltolate in gallium nitrate-resistant cells may, therefore, be due, at least in part, to the ability of this compound to circumvent the TfR pathway and transport greater amounts of gallium into cells. Indeed, our studies demonstrating that cellular radiogallium uptake is greater with gallium maltolate than with gallium nitrate support this conclusion.

The development of metal-based drugs as therapeutic agents for cancer treatment is an important area for research. Recent studies have shown that metal complexes of tridentate chelators display antiproliferative activity against malignant cells in vitro (Bernhardt et al., 2006). The ability of gallium maltolate to induce apoptosis more efficiently than gallium nitrate and its activity against cells resistant to gallium nitrate makes it an attractive agent for further investigation. Gallium maltolate is presently in development in phase I clinical trials; studies are in progress to understand its mechanisms of action and to identify intracellular targets and molecular markers in lymphomas and other malignancies that may be of value in predicting tumor sensitivity to this compound.

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