Evaluation of Transferrin and Gallium-Pyridoxal Isonicotinoyl Hydrazone as Potential Therapeutic Agents to Overcome Lymphoid Leukemic Cell Resistance to Gallium Nitrate

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ABSTRACT
Gallium nitrate is active against lymphoma and bladder cancer; however, little is understood about tumor resistance to this drug. Transferrin, the iron transport protein, increases gallium uptake by cells, whereas pyridoxal isonicotinoyl hydrazone (PIH), an iron chelator, transports iron into cells. Therefore, we examined whether these metal transporters would increase the cytotoxicity of gallium in gallium nitrate-resistant CCRF-CEM cells. Transferrin, in increasing concentrations, enhanced the cytotoxicity of gallium nitrate. One mg/ml transferrin decreased the 50% inhibitory concentration of gallium nitrate from 1650 to 75 μM in gallium-resistant cells and from 190 to 150 μM in gallium-sensitive cells. Transferrin also enhanced the cytotoxicity of gallium even at drug concentrations that were not growth inhibitory. The gallium chelate Ga-PIH inhibited the growth of both gallium nitrate-resistant and sensitive cells. Fifty μM Ga-PIH inhibited cellular proliferation by 50%, whereas similar concentrations of PIH or gallium nitrate were not growth inhibitory. However, because higher concentrations of PIH also inhibited cell growth, the cytotoxicity of Ga-PIH was greater than PIH only at concentrations of <100 μM. Cross-titration experiments demonstrated that the cytotoxicity of PIH was partially reversed by gallium nitrate, whereas the cytotoxicity of gallium nitrate was enhanced by PIH. Our studies suggest that Ga-PIH warrants further evaluation as a potential antineoplastic agent. Because transferrin increases the cytotoxicity of gallium nitrate in transferrin receptor-bearing, gallium nitrate-resistant cells, future clinical trials of this drug should incorporate the development of strategies to increase plasma transferrin levels.

INTRODUCTION
Gallium nitrate (NSC 15200) is a group IIIa metal salt that more than 2 decades ago was found to possess significant antineoplastic activity against malignant cells in vitro and in tumor-bearing animals (1). Detailed studies over the last several years have shown that the cellular uptake of gallium involves the binding of gallium to transferrin (the physiological transport protein for iron) and subsequent endocytosis of transferrin-gallium complexes by cell surface transferrin receptors (2–4). In addition, gallium and iron can be incorporated into HL60 cells through a transferrin-independent transport system (5); however, gallium uptake by this process appears to be relatively minor when compared with the transferrin receptor-mediated mechanism (3). The cytotoxicity of gallium is, in part, due to inhibition of transferrin receptor-mediated iron uptake by cells and perturbation of cellular iron-dependent processes, including deoxyribonucleotide synthesis by ribonucleotide reductase (6, 7).

Whereas preclinical studies have shown that gallium inhibits the growth of a variety of malignant cells, clinical trials have shown that gallium nitrate is active primarily against lymphoma and bladder cancer (8–13). The reason for the sensitivity of these cancers to gallium nitrate is not known; however, information gained from earlier studies suggests that the antineoplastic activity of gallium in lymphoma and bladder cancer may relate to the presence of transferrin receptors on these cells in vivo (14, 15). Accordingly, transferrin-gallium complexes, formed in the circulation following the administration of gallium nitrate, would preferentially deliver gallium to these transferrin receptor-bearing tumors.

The resistance of neoplastic cells to cancer chemotherapeutic agents remains a major obstacle to the successful treatment of cancer. Therefore, studies are warranted to understand the mechanisms of drug resistance and to develop strategies to overcome such resistance. Previously, we reported that a clone of human leukemic HL60 cells made resistant to the growth-inhibitory effects of gallium nitrate differed from the parent wild-type cells with respect to iron uptake and transferrin receptor and ferritin expression (16), thus suggesting a role for altered iron metabolism in the development of drug resistance to gallium. Interestingly, gallium-resistant HL60 cells are not cross-resistant to the majority of standard chemotherapeutic drugs, including the metal indium (16), indicating that the mechanism of drug resistance to gallium nitrate is unique.

The mechanism of drug resistance to gallium is being investigated in our laboratory. In the present study, we have tested the hypothesis that the sensitivity of gallium-resistant cells to gallium can be enhanced by increasing the transport of gallium into gallium nitrate-resistant CCRF-CEM cells by the use of transferrin or PIH, a metal chelating agent (17). In prior

Received 12/29/95; revised 2/28/96; accepted 3/8/96.
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3 The abbreviations used are: PIH, pyridoxal isonicotinoyl hydrazone; IC50, concentration of drug producing a 50% inhibition of cellular proliferation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
studies, we had shown that the cytotoxicity of gallium nitrate could be potentiated by the lipophilic iron chelator N,N'-bis(o-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid, suggesting a potential role for chelators in enhancing the delivery of gallium into cells (18). We have now examined the potential of PIH to transport gallium into cells, because, unlike most chelators, which sequester iron and render it unavailable for use by cells, PIH is able to deliver iron to cells independent of transferrin to support iron-dependent processes, including cellular proliferation and hemoglobin synthesis (19, 20). Our studies show that both transferrin and PIH can potentiate the cytotoxicity of gallium in gallium-resistant cells; however, the ability of PIH to deliver gallium to cells to inhibit their proliferation is positively or negatively influenced by the relative concentrations of gallium and PIH.

MATERIALS AND METHODS

Gallium nitrate was obtained from Alpha Products, (Danvers, MA). A stock solution (10 mg/ml) was prepared in water or sodium citrate buffer (pH 6.8). Human transferrin (substantially iron free), pyridoxal hydrochloride, isonicotinic acid, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]Thymidine was purchased from Amersham (Arlington Heights, IL).

PIH was prepared from pyridoxal hydrochloride and isonicotinic acid by Schiff base condensation, as described previously (17, 21). Ga-PIH was prepared by combining PIH with a solution of gallium nitrate in citrate buffer (pH 6.8) at a ratio of 2 mol PIH:1 mol Ga3+ at a ratio of 2 mol PIH:1 mol Ga3+. After mixing overnight at room temperature, the insoluble Ga-PIH complex was filtered and dried. A 4 mM stock solution of Ga-PIH was prepared by dissolving the Ga-PIH in a few drops of 1 N NaOH and then diluting the sample with water (final NaOH concentration, 10 mM). The UV visual wavelength spectrum (250−500 nm) of Ga-PIH thus formed was compared with that of PIH using a DU-40 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA).

Tissue Culture and Incubation Conditions. Human T-lymphoblastic leukemic CCRF-CEM cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 medium supplemented with 10% FCS. Cells were incubated in an atmosphere of 6% CO2 at 37°C and were allowed to grow to confluency before their use in cell growth studies. Gallium nitrate-resistant CCRF-CEM cells were
developed in our laboratory through a process of continuous exposure of these cells to stepwise increments of gallium nitrate over the course of several months. Gallium nitrate-resistant cells were routinely cultured in medium containing 150 μM gallium nitrate. Prior to their use in experiments, cells were washed by centrifugation to remove gallium nitrate and were then plated in fresh medium as described below.

**Cell Growth Experiments.** Unless otherwise stated, all cell growth and viability experiments were performed in 96-multiwell plates using the MTT assay as described by Mossman (22). The effects of gallium nitrate, Ga-PIH, PIH, and transferrin on cell growth either as single agents or in combination were examined using the concentrations shown in the figures. Gallium nitrate-sensitive or -resistant cells were plated at an initial density of 2 × 10^5 cells/ml and were incubated for 72 h in the presence of various additives. At the end of the incubation, 10 μl MTT (5 mg/ml stock solution) were added to each well, and the cells were incubated at 37°C for an additional 4 h. One hundred μl 0.04 N HCl in isopropanol were then added to each well, and the absorbance of each well was determined spectrophotometrically at dual-wavelength 570/630 nm using an EL 310 microplate autoreader (Biotec Instruments, Winooski, VT). The absorbance of the wells containing different additives was compared with that of control wells.

**[^3H]Thymidine Uptake.** Gallium nitrate-resistant cells were plated in 96-well plates with varying concentrations of gallium nitrate, PIH, or Ga-PIH for 72 h. [^3H]Thymidine (1 μCi/well) was added to wells during the final 4 h of incubation, and cells were harvested onto a glass fiber filter using a Mini-Mash harvester (M. A. Bioproducts, Walkersville, MD). Disks corresponding to individual wells were cut out of the filter, and the radioactivity in each disk was counted in an LKB scintillation counter.

**RESULTS**

**Effect of Transferrin on the Cytotoxicity of Gallium Nitrate.** Because gallium nitrate has clinical activity in lymphoid cancers, a line of human lymphoid leukemic CCRF-CEM cells resistant to the growth-inhibitory effects of this drug was developed for the study. The dose-response curves of gallium nitrate-sensitive and -resistant cells shown in Fig. 1A demonstrate that the IC50 of gallium nitrate for sensitive cells was approximately 190 μM, whereas for resistant cells, it was greater than 1000 μM. By extrapolation, the IC50 of gallium nitrate was determined to be approximately 1650 μM for resistant cells, indicating that these cells had an 8.8-fold increase in resistance to gallium nitrate. To determine whether transferrin could potentiate the cytotoxicity of gallium in these cells, the effects of gallium nitrate on cell growth were examined in the absence or presence of increasing concentrations of transferrin. As shown in Fig. 1C, transferrin markedly potentiated the antiproliferative effect of gallium nitrate in resistant cells, resulting in a decrease in the IC50 from 1650 to 5 μM gallium nitrate in the presence of 1000 μg/ml transferrin. In gallium nitrate-sensitive cells, the same concentration of transferrin produced a decrease in the IC50 from 190 to 150 μM gallium nitrate (Fig. 1B). It should be appreciated, however, that although transferrin produced only a minor decrease in the IC50 of gallium nitrate in gallium nitrate-sensitive cells, it did enhance the cytotoxicity of gallium nitrate in these cells at lower drug concentrations. The addition of 1000 μg/ml transferrin to cells exposed to 100 μM gallium nitrate resulted in decreases in cellular proliferation by 30% in gallium nitrate-sensitive cells and 55% in gallium nitrate-resistant cells (Fig. 1, B and C).

**Comparison of the Effects of Ga-PIH, PIH, and Gallium Nitrate on the Proliferation of Gallium Nitrate-resistant and -sensitive Cells.** Spectroscopic analysis was done to confirm that the Ga-PIH prepared actually contained gallium complexed to PIH. Consistent with that reported by others (23), the Ga-PIH used in our experiments displayed a shift in the spectrum from that seen with PIH alone, indicating that Ga was indeed complexed to PIH (Fig. 2). In contrast to gallium nitrate, Ga-PIH produced a marked inhibition of cellular proliferation and DNA synthesis ([^3H]thymidine incorporation) in gallium nitrate-resistant cells (Fig. 3). Consistent with the known antiproliferative effects of iron chelation (23–27), PIH alone also inhibited cell growth, but to a lesser degree than that seen with Ga-PIH (Fig. 3). Comparison of Ga-PIH and PIH in gallium nitrate-sensitive cells also showed that Ga-PIH was more effective than PIH in inhibiting cellular proliferation (Fig. 4). However, because PIH alone inhibited cell growth, Ga-PIH could be demonstrated to be superior to PIH only at concentrations of less that 100 μM. However, this differential is significant, because 50 μM Ga-PIH inhibited cellular proliferation by approximately 50%, whereas similar concentrations of PIH or gallium nitrate alone were not growth inhibitory.

**Interaction of Transferrin and Ga-PIH.** Although transferrin enhanced the cytotoxicity of gallium nitrate, it did not significantly increase the cytotoxicity of Ga-PIH in gallium nitrate-resistant cells (Fig. 5). In gallium nitrate-sensitive cells, however, a small increase in growth inhibition was seen when cells were coincubated with transferrin and increasing concentrations of Ga-PIH (Fig. 5).
Transferrin and Ga-PIH Overcome Drug Resistance to Ga

Cross-Titration Experiments Examining the Dose Relationship of Gallium Nitrate and PIH. Ga-PIH used in the above studies is a complex of gallium and PIH in a 1:2-mol ratio. Because PIH is a strong chelator, we sought to determine whether cell growth could be effectively inhibited by adding gallium nitrate and PIH as single agents in various concentrations directly to the incubation medium. When cells were incubated with a constant growth-inhibitory concentration of PIH, the addition of increasing concentrations of gallium nitrate resulted in an initial partial restoration of cell growth, followed by a return to baseline (Fig. 6A). In contrast, when cells were incubated with a constant growth-inhibitory concentration of gallium nitrate, the addition of increasing concentrations of PIH resulted in a further decrease in cellular proliferation (Fig. 6B).

DISCUSSION

The present study was performed to gain an insight into strategies that may be potentially applicable to enhancing the cytotoxicity of gallium nitrate in cells that have acquired or inherent resistance to growth inhibition by this drug. We show that, transferrin, the physiological transporter for iron in the circulation, and PIH, a compound with strong metal-chelating properties, can increase the antiproliferative action of gallium nitrate and can significantly overcome drug resistance to this agent.

Evidence that the effect of transferrin is specifically relevant to gallium resistance is the finding that transferrin produced a marked reduction in the IC50 of gallium nitrate for resistant cells but produced only a minor reduction in the IC50 of gallium nitrate for sensitive cells. This finding is particularly intriguing, because it suggests that gallium nitrate-resistant cells differ from sensitive cells in their handling of transferrin and, therefore, transferrin-dependent delivery of gallium to intracellular targets. It is possible that the interaction of transferrin with its receptor or the intracellular trafficking of transferrin and transferrin receptors in resistant cells is altered such that gallium uptake requires higher concentrations of exogenous transferrin, and that once within the cell, gallium is preferentially shunted to inhibit DNA synthesis. Previously, gallium nitrate-resistant HL60 cells were shown to have an up-regulation in transferrin receptors without a change in the affinity of the receptors for transferrin (16). However, in gallium nitrate-resistant CCRF-CEM cells, we have been unable to find consistent changes in the binding of transferrin to its receptor.4 Nevertheless, changes in the trans-
Fig. 5 Effect of Ga-PIH and transferrin in combination on cellular proliferation. CCRF-CEM cells were incubated with increasing concentrations of Ga-PIH in the absence or presence of transferrin. Cell growth was determined after 72 h of incubation. △, Ga-PIH alone; ✦, transferrin, 250 μg/ml; ○, transferrin, 500 μg/ml. A, gallium nitrate-sensitive cells. At 50 and 100 μM Ga-PIH, P < 0.01 for comparison between Ga-PIH alone and Ga-PIH plus 500 μg/ml. B, gallium nitrate-resistant cells.

Fig. 6 Cross-titration experiments (gallium nitrate-sensitive cells). A, cell growth in the presence of a constant concentration of PIH and increasing concentrations of gallium nitrate. Control, growth in the presence of gallium nitrate alone. B, cell growth in the presence of a constant concentration of gallium nitrate and increasing concentrations of PIH. Control, growth in the presence of PIH alone. Values represent means of two experiments, each performed in quadruplicate. Bars, SE. Cell growth was determined after a 72-h incubation.

The transferrin receptor may prove to be more subtle. In lymphoma, for example, transformation from an indolent to a more aggressive biological behavior is accompanied by the appearance of new antigenic sites on cell surface transferrin receptors (28). Further analyses of the transferrin receptor in gallium nitrate-resistant cells are in progress, which, along with other studies, may elucidate mechanisms responsible for drug resistance to gallium.

In addition to using transferrin to enhance the cytotoxicity of gallium, we examined whether a similar result could be obtained with Ga-PIH, a gallium chelate. The rationale for this approach came from previous studies that showed that Fe-PIH complexes could deliver iron to HL60 and other cells to support their proliferation in serum-free, transferrin-free medium (19, 29), and that the cytotoxicity of gallium nitrate in HL60 cells could be potentiated by the lipophilic iron chelator N,N’-bis(o-hydroxybenzyl)ethylenediamine-N,N’-diacetic acid but not by the hydrophilic iron chelator deferoxamine (18). Additional preliminary experiments suggested that PIH could also increase the cytotoxicity of gallium in HL60 cells. Because gallium, like iron, readily forms a complex with PIH, it appears highly likely that PIH is capable of facilitating the transport of gallium into cells. Our present study shows that Ga-PIH complexes inhibit the proliferation of both gallium nitrate-sensitive and -resistant cells to a greater extent than either PIH or gallium nitrate alone. However, in contrast to transferrin, which had no growth-inhibitory effect, PIH alone inhibited cellular proliferation. This latter finding is not unexpected, because iron chelators are known to have antiproliferative effects (23–27). Based on these results,
we suggest that the cytotoxicity of Ga-PIH may result from action at two levels: (a) delivery of gallium into cells, with subsequent targeting of gallium to intracellular molecules; and (b) chelation of intracellular iron by PIH after dissociation of gallium from PIH (thereby limiting the amount of available iron necessary for cellular proliferation).

Because PIH readily chelates gallium, we examined whether the addition of both of these agents individually to the medium would inhibit cellular proliferation (presumably through the formation of Ga-PIH complexes in the medium). Potentiation of gallium cytotoxicity by PIH was recently demonstrated in neuroblastoma cells; however, only a single concentration of PIH and gallium nitrate was examined (23). Our studies clearly show that when gallium and PIH are added individually, their combined growth-inhibitory effects are variable and are positively or negatively influenced by the relative concentrations of both agents. When the concentration of PIH is limited, and the concentration of gallium nitrate is increased, the antiproliferative effect of PIH produced through iron chelation is partially neutralized by competitive binding of gallium to PIH. For PIH to significantly enhance the cytotoxicity of gallium, it must be present in sufficient concentrations to effectively transport gallium into cells (Fig. 6B). Because of the variable interaction of gallium nitrate and PIH, we conclude that preformed Ga-PIH is more reliable and preferable as a cytotoxic agent than the combination of gallium nitrate and PIH.

In comparing the potential of transferrin and Ga-PIH as agents to enhance the cytotoxicity of gallium, certain advantages and disadvantages become apparent. Ga-PIH appears to inhibit cellular proliferation through mechanisms involving enhanced transport of gallium into cells and chelation of intracellular iron. Excess iron stores could, therefore, neutralize the cytotoxicity produced by the chelator component of the complex, whereas its lipophilic nature could lead to nonspecific uptake of gallium by nonmalignant tissues. Clearly, further studies of Ga-PIH will need to focus on the evaluation of this agent in vivo to address these issues. In contrast to Ga-PIH, transferrin-gallium complexes specifically target transferrin receptor-bearing cells. The binding of gallium to transferrin is obviously an advantage when malignant cells express transferrin receptors; however, it could be a disadvantage when tumors inherently display few or no transferrin receptors. In the latter situation, the binding of gallium to transferrin may limit the amount of free gallium available for cellular uptake through transferrin-independent mechanisms and thus actually diminish its cytotoxicity.

The ability of transferrin to increase the cytotoxicity of gallium nitrate in transferrin receptor-bearing, gallium nitrate-resistant cells clearly has clinical implications. Under physiological conditions in adults, the level of transferrin in the circulation ranges from 1.76 to 3.9 mg/ml, with approximately one-third of it being saturated with iron (30). This leaves a considerable amount of transferrin free to bind gallium. In disease states, however, transferrin levels may vary significantly. Cancer, infection, inflammation, and liver dysfunction tend to reduce serum transferrin levels. A decrease in the availability of transferrin, coupled with variable tumor cell transferrin receptor expression (or abnormal transferrin receptors), could adversely affect the efficacy of gallium nitrate in the treatment of cancer. Unfortunately, clinical trials evaluating gallium nitrate as a chemotherapeutic agent have not taken into account transferrin levels or tumor transferrin receptor expression as a variable that could influence the tumor response rate. Based on the above, it seems reasonable to consider the development of therapeutic approaches that involve the concomitant administration of transferrin with gallium nitrate or the infusion of transferrin-gallium complexes. This, along with further investigations of the potential of Ga-PIH as an antineoplastic agent, may result in increased efficacy of gallium in the treatment of cancer.

REFERENCES


Evaluation of transferrin and gallium-pyridoxal isonicotinoyl hydrazone as potential therapeutic agents to overcome lymphoid leukemic cell resistance to gallium nitrate.

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