Gallium-containing anticancer compounds

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Abstract

There is an ever pressing need to develop new drugs for the treatment of cancer. Gallium nitrate, a group IIIa metal salt, inhibits the proliferation of tumor cells *in vitro* and *in vivo* and has shown activity against non-Hodgkin's lymphoma and bladder cancer in clinical trials. Gallium can function as an iron mimetic and perturb iron-dependent proliferation and other iron-related processes in tumor cells. Gallium nitrate lacks cross resistance with conventional chemotherapeutic drugs and is not myelosuppressive; it can be used when other drugs have failed or when the blood count is low. Given the therapeutic potential of gallium, newer generations of gallium compounds are now in various phases of preclinical and clinical development. These compounds hold the promise of greater anti-tumor activity against a broader spectrum of cancers. The development of gallium compounds for cancer treatment and their mechanisms of action will be discussed.

Research into the potential anticancer activity of gallium was stimulated by the discovery that ^{67}Ga, when injected into rodents bearing implanted tumors, localized in high concentration within these tumors. This discovery led the development of the ^{67}Ga scan as a method for detecting tumors in patients and prompted further evaluation of the potential antineoplastic activity of stable gallium salts. In studies conducted at the National Cancer Institute (USA), the antineoplastic activities of gallium, thallium, indium and aluminum salts were investigated in tumor-bearing rodents [1]. Of these metals, gallium nitrate was found to display the greatest *in vivo* anti-tumor activity and, as a result, it was further evaluated for toxicity in animals and then for both toxicity and antineoplastic activity in humans in Phase I and II **clinical trials** [2–4]. These studies showed gallium nitrate to have appreciable antineoplastic activity in certain malignancies, as well as to display additional interesting effects in other non-neoplastic conditions [5]. The results of these early studies have subsequently stimulated further research in the field, which has led to the development of newer gallium-based compounds that have the promise of greater antineoplastic activity than the parent compound gallium nitrate. Beginning with gallium nitrate, we will review the different gallium-based **metallodrugs** that have emerged as cancer therapeutic agents in the preclinical and clinical arenas. Since these gallium compounds have mechanisms of action that are similar in some ways, but different in others, the known mechanisms of antineoplastic activity of these agents will be discussed collectively in the latter part of this

review. Important gallium compounds in cancer treatment and the status of their advancement from the laboratory to the clinic are summarized in Table 1.

Similarities between gallium(III) & iron(III)

Gallium(III) shares certain properties with iron(III) with respect to its ionic radius. The octahedral ionic radius is 0.620 Å for Ga^{3+} and 0.645 Å for high spin Fe^{3+}, respectively; while the tetrahedral ionic radius is 0.47 Å for Ga^{3+} and 0.49 Å for Fe^{3+}, respectively [6]. The ionization potential and electron affinity values for Ga^{3+} are 64 eV and 30.71 eV, respectively, while those for high spin Fe^{3+} are 54.8 eV and 30.65 eV, respectively [6]. As a result of these properties, gallium is able to form complexes with proteins and ligands that bind iron, such as transferrin, the iron-transport protein in blood, and certain organic compounds. The aqueous chemistry of gallium has been summarized by Bernstein [6]. Ga^{3+} has been shown to bind to the two metal-binding domains of transferrin with binding constants $\log K_1 = 20.3$ and $\log K_2 = 19.3$ at normal plasma bicarbonate concentrations; these binding constants are comparable to binding constants of $\log K_1 = 22.8$ and $\log K_2 = 21.7$ reported for the binding of Fe^{3+} to transferrin [7,8]. These metal–protein interactions are relevant to the development of gallium compounds as therapeutic agents in cancer [9]. At concentrations of up to 50 μM in the blood, gallium binds almost entirely to transferrin while at higher gallium concentrations that exceed transferrin saturation, the percentage of gallium bound to transferrin diminishes and gallium circulates as gallate, $[Ga(OH)_4^-]$[6]. Despite gallium's similarities with iron, it differs from iron in that it does not transition from a trivalent to a divalent state and cannot participate in redox reactions.

Gallium nitrate

Gallium nitrate, a simple gallium salt, shown in Figure 1A, can be considered a 'first-generation' gallium compound that was investigated for its anticancer activity in humans [2]. This drug is, therefore, the standard against which newer gallium compounds should be compared. In Phase II clinical trials conducted to evaluate the spectrum of its antineoplastic activity, gallium nitrate was administered to patients with a variety of different cancers, including breast, prostate, lung, ovarian, cervical, bladder, renal, melanoma, sarcoma, chronic lymphocytic leukemia, Hodgkin's lymphoma and non-Hodgkin's lymphoma. Of these malignancies, gallium nitrate was found to have antineoplastic activity primarily against advanced bladder cancer and non-Hodgkin's lymphoma [10–12]. In these malignancies, significant responses to gallium nitrate were seen in patients whose tumors had relapsed or failed to respond to conventional chemotherapy. Encouraged by these findings, a limited number of clinical trials ensued in which gallium nitrate was administered in combination with other chemotherapeutic agents (vinblastine, ifosfamide, 5-fluorouracil, mitoguazone, etoposide and hydroxyurea) to patients with bladder cancer or non-Hodgkin's lymphoma. These trials demonstrated that gallium nitrate could be safely combined with other drugs with a good clinical outcome [10–12]. An important consideration in gallium nitrate-based studies is that, unlike most chemotherapeutic drugs, gallium nitrate does not suppress the white blood cells or platelets and it can, therefore, be used to treat patients with low blood counts or can be combined with other antineoplastic agents without exacerbating their myelosuppressive effects.

Gallium nitrate has been administered to patients by two different treatment schedules. In the early clinical trials, patients received this drug at a dose of 700 mg/m^2 by rapid intravenous infusion over 15–30 min. Although responses to treatment were noted, this schedule resulted in high peak plasma levels of gallium and toxicity to the kidneys that proved to be dose limiting. Under these conditions, 69 and 91% of the gallium administered were excreted in the urine by 24 and 48 h, respectively [13]. In a second treatment schedule,

patients received gallium nitrate by continuous intravenous infusion at a dose of 200–400 mg/m^2/day over 5–7 days. Steady-state gallium levels of 0.9–1.9 µg/ml were achieved during the infusion; these levels decreased to 0.45–0.7 µg/ml 4 days after gallium was discontinued. The continuous infusion schedule also allowed patients to receive a greater amount of gallium over time when compared with the brief infusion schedule [14]. Moreover, continuous intravenous infusion gallium nitrate for 5–7 days was better tolerated by patients and it is now the recommended mode of administration for this drug. Alternative treatment schedules for gallium nitrate that are less cumbersome warrant further investigation. It should be noted that, while the anticancer activity of gallium nitrate has been examined in the aforementioned malignancies, its action against a number of other malignancies remains to be investigated. However, while efficacious, the continuous intravenous treatment schedule for gallium nitrate is inconvenient since it requires that patients receive this drug intravenously in the hospital or as an outpatient via a pump device. Unfortunately, the bioavailability of oral gallium nitrate is poor, thus limiting the use of gallium nitrate to the intravenous route.

Gallium nitrate has important effects that are independent of its anti-tumor activity. In the course of its development as an antineoplastic agent, gallium nitrate was found to impact on bone metabolism and to reduce calcium levels in the blood [15]. These observations led to additional trials in patients with cancer and metabolic bone disease that confirmed its ability to inhibit bone resorption and lower pathologically elevated blood calcium levels [15]. As a result, gallium nitrate was approved by the US FDA as a prescription drug for the treatment of malignancy-associated hypercalcemia.

Gallium chloride

Early investigations into the anti-tumor activity of gallium salts demonstrated that gallium chloride, like gallium nitrate, inhibited the proliferation of malignant cell lines *in vitro* at concentrations that were within the range seen with gallium nitrate. The antineoplastic activity of gallium chloride has been reviewed by Collery *et al.* [3]. Intraperitoneal administration of gallium chloride to mice bearing adenocarcinoma CA755 produced a decrease in tumor growth [16]. In contrast, daily oral administration of gallium chloride to C3H/HeJ mice bearing C3HBA mammar y adenocarcinoma resulted in significant intratumor concentrations of gallium but cytotoxicity was less than that seen with the parenteral route [17]. Similar to gallium nitrate, the bioavailability of gallium is low following oral administration of gallium chloride. Nonetheless, with prolonged oral administration of gallium chloride to rats, gallium was shown to accumulate in tissues, with the greatest gallium concentrations seen in bone and lung [18]. Lower accumulation of gallium occurred in the spleen, kidneys, liver, adrenals and ovaries, while even lower concentrations of gallium were present in muscle and the brain [18]. In clinical trials, oral gallium chloride at doses of 100–1400 mg/24 h produced partial responses in ovarian cancer but not in lung cancer [3,19]. While the low bioavailability of oral gallium chloride appears to have dampened its further development as an antineoplastic agent, it has stimulated the development of newer gallium compounds with much greater oral bioavailability [3].

G4544

G4544 is an oral tablet formulation of gallium nitrate manufactured using a proprietary drug-delivery technology developed by Emisphere Technologies. The drug is presently in Phase I clinical trials [20]. **Preclinical studies** in dogs showed that the drug was rapidly absorbed and achieved a mean plasma level of 1800 ng/ml, which was fourfold greater than that achieved with gallium nitrate. Long elimination half-lives of 101 and 338 h were seen in rat and dog, respectively. In a Phase I clinical trial, 30 normal volunteers received a single

oral dose of G4544 at doses ranging from 30–150 mg [20]. At the highest dose level, a peak gallium plasma concentration of 485 ng/ml was achieved, which is similar to levels seen with continuous intravenous gallium nitrate. G4544 was tolerated without adverse side effects. These initial Phase I results are encouraging and further trials to examine the efficacy of this agent in skeletal diseases and cancer are planned [20].

Gallium maltolate

Gallium maltolate, *tris*(3-hydroxy-2-methyl-4*H*-pyran-4-onato)gallium (Figure 1B) is a gallium compound that has been developed for oral use. It consists of three maltolate ligands bidentately bound to a central gallium atom in a propeller-like arrangement, with one of the ligands disordered in two possible orientations [21]. Maltol, 3-hydroxy-2-methyl-4-pyrone, is a hydroxypyrone with high affinity for a range of metal ions [22]; it has been shown to enhance the gastrointestinal absorption of orally administered iron in iron-deficient and normal subjects [22,23]. Bernstein *et al.* compared the bioavailability of orally administered or intraduodenal administered (via a feeding tube) gallium maltolate solution with that of oral gallium nitrate solution in male beagle dogs [21]. They showed that the maximum serum concentration of gallium achieved with the administration of 1.5 mg/kg of gallium was 2.2 μg/ml and 1.47 μg/ml for oral and intraduodenal administered gallium maltolate, respectively, and was 0.3 μg/ml for intraduodenal administered gallium nitrate solution. The same group examined the bioavailability of gallium in human subjects following a single oral dose of 100–500 mg of gallium maltolate (equivalent to 15.7–78.4 mg of gallium). The maximum serum concentrations of gallium achieved were 0.115 μg/ml and 0.569 μg/ml with the 100- and 500-mg doses, respectively [21]. Allamneni *et al.* reported that following oral administration of gallium maltolate to normal subjects, gallium in the circulation is found bound to transferrin, the iron transport protein, with very little of it unbound [24]. These findings are consistent with the earlier studies of Vallahajosula *et al.*, which showed that ^{67}Ga injected intravenously in rabbits binds exclusively to transferrin in circulation [25].

Preclinical investigations of the cytotoxicity of gallium maltolate in various cell lines *in vitro* have shown that the IC_{50} value of gallium maltolate (concentration required to inhibit cell growth by 50%) is significantly lower than that of gallium nitrate. In four hepatocellular carcinoma cell lines, the IC_{50} values of gallium maltolate ranged from 25 to 35 μM while the IC_{50} value of gallium nitrate ranged from 60 to 250 μM [26]. In another study utilizing a panel of lymphoma cell lines, gallium maltolate was shown to induce apoptosis at earlier time points and at significantly lower concentrations than gallium nitrate [27]. The basis for the enhanced sensitivity of certain cancer cells to gallium maltolate over gallium nitrate remains to be determined; however, it may, in part, be related to more efficient entry of the former compound into cells [27].

In addition to displaying greater cytotoxicity when compared with gallium nitrate, gallium maltolate was shown to block the proliferation of lymphoma cells resistant to growth inhibition by gallium nitrate. In studies utilizing human lymphoma/leukemic CCRF-CEM cells resistant to gallium nitrate, the IC_{50} of gallium maltolate was 72 μM, whereas the IC_{50} for gallium nitrate was >400 μM [27].

It is known that mutations of p53 may impact the antineoplastic activity of certain chemotherapeutic drugs. However, the cytotoxicity of gallium maltolate does not appear to be influenced by p53 mutations in B-lymphoma cells. Similar growth–inhibitory activity of gallium maltolate *in vitro* was seen in p53 wild-type TK-6 cells, p53-null NH-32 cells and p53-mutant WTK-1 cells [27]. In contrast, gallium nitrate did not significantly inhibit the growth of WTK-1 cells [27]. The observation that cells with acquired or inherent resistance to gallium nitrate remain sensitive to the cytotoxicity of gallium maltolate indicates that

these gallium compounds do not share cross resistance; it also suggests that tumors that fail to respond to gallium nitrate may respond to gallium maltolate *in vivo*.

Evidence that gallium maltolate has antineoplastic activit y in humans was recently provided by Bernstein *et al.* [28]. These investigators reported a patient with advanced hepatocellular carcinoma that had failed to respond to treatment with the drug sorafenib. Avid uptake of ^{67}Ga by the tumor on radiogallium scanning suggested that the hepatic mass would incorporate nonradioactive gallium. Treatment of this patient with 1500 mg/day of oral gallium maltolate resulted in marked clinical improvement of the patient's symptoms and a significant reduction in the size of the hepatic mass, as measured by imaging studies of the liver [28]. Further studies to evaluate the antineoplastic activity of oral gallium maltolate in hepatoma, lymphoma, bladder cancer and other malignancies are warranted.

Tris(8-quinolonato)gallium(III) (KP46)

Tris(8-quinolonato)gallium(III) (KP46), a gallium complex with an organic ligand 8-quinolinol is currently in clinical trials as an oral gallium compound (Figure 1C). KP46 is reported to have an *n*-octanol: water partition coefficient, log P value, of 0.88, which represents a hydrophilic/lipophilic balance that should favor adequate membrane permeability with oral administration [29]. The high thermodynamic stability of KP46 (log $\beta_3 = 40.7$), kinetic stability and the observation that this complex retains chemical stability in solution for hours suggests that, unlike gallium maltolate, orally absorbed KP46 may not rapidly release gallium to transferrin in the circulation, but may do so within an acidic tumor environment [30].

Comparative studies by Collery *et al.* showed that *tris*(8-quinolinolato)gallium(III) possesses a tenfold greater growth-inhibitory effect on malignant cells when compared with gallium chloride [31]. The IC$_{50}$ values of KP46 in a variety of melanoma, ovary, breast, colon and lung cancer cell lines *in vitro* ranged from 0.85 to 10.4 μM [32], which is much lower than that reported for gallium nitrate by others. In addition, the combination of KP46 and platinum drugs acts synergistically to inhibit the proliferation of ovarian and colon cancer cell lines *in vitro* [33].

Studies examining the preclinical toxicology and tissue distribution of oral KP46 in mice defined the LD$_{50}$ dose to be 2870 mg/kg (410 mg Ga^{3+}/kg) and 2370 mg/kg (339 mg Ga^{3+}/kg) for males and females, respectively; a dose of 62.5 mg/kg/day for 14 days was well-tolerated and showed the highest accumulation of gallium in bone, followed by liver, spleen and kidney [34]. Additional studies conducted in animal tumor models confirmed that KP46 produced a reduction in tumor mass *in vivo* and a lowering of serum calcium levels. The latter finding indicates that in addition to its antineoplastic activity KP46 (like gallium nitrate) may also be beneficial in the treatment of hypercalcemia.

In a Phase I/II clinical trial of oral KP46 in patients with solid tumors (renal, ovarian, stomach and parotid gland), the drug was found to be well-tolerated over a dose range of 30 to 480 mg/m^2 daily for 14 days; no significant dose-limiting toxicities were observed [35,36]. Responses to treatment were seen in three out of four patients with renal cancer [35,36]. The treatment of renal cancer is challenging since this disease does not respond to conventional chemotherapy or to gallium nitrate; hence, the initial activity of KP64 in this malignancy is noteworthy and should be further explored.

Gallium thiosemicarbazones

Early studies demonstrated that many α-*(N)*-heterocyclic carboxaldehyde thiosemicarbazones possessed significant anti-tumor activity and were capable of chelating

metals [37]. Saryan *et al.* showed that the iron complexes of these thiosemicarbazones were three- to six-fold more potent than the free ligand in inhibiting **ribonucleotide reductase**, the enzyme responsible for the synthesis of deoxyribonucleotides [38]. Kratz *et al.* reported on the synthesis of gallium(III) complexes of different 2-acet ylpyridine thiosemicarba zones [39]; while Arion *et al.* later demonstrated the anti-tumor action of gallium complexes with 2-acetylpyridine [4]*N*-dimethylthiosemicarbazone in SW480 (colon adenocarcinoma), SK-B-3 (breast adenocarcinoma) and 41-M (ovarian cancer) cell lines *in vitro*, thus setting the stage for further development of this gallium complex [40]. Kowol *et al.* reported on the synthesis and cytotoxicity of gallium complexes with five different [4]N-substituted α-*N*-heterocyclic thiosemicarbazones. One such complex is shown in Figure 1D [41]. Others showed that the coordination of 2-pyridineformamidethiosemicarbazones to gallium greatly increased their ability to induce apoptosis in glioblastoma cell lines *in vitro* [42]. In these studies, the coordination of these thiosemicarbazones to gallium increased their cytotoxicity by 15–37-fold in RT2 glioblastoma cells with wild-type p53 and by 7–36-fold in T98 glioblastoma cells with mutant p53. It is known that mutations in p53 may affect pathways that determine the sensitivity of cancer cells to chemotherapeutic drugs [43]. Therefore, it is notable that these thiosemicarbazone–gallium complexes were cytotoxic to both wild-type p53- and mutant p53-containing glioblastoma cells. Both RT2 and T98 cells were resistant to the cytotoxicity of gallium nitrate alone. These data indicate that it is the thiosemicarbazone–gallium complex *per se* and not the individual actions of thiosemicarbazone or gallium, which induces apoptosis in these cells.

Although several thiosemicarbazones have been examined in preclinical investigations, at this time only 3-aminopyridine-2-carboxyaldehyde thiosemicarbazone (3-AP, Triapine[®]) was advanced to Phase I and II clinical trials several years ago [44], and was recently shown to enhance the response to radiation therapy in cervical cancer [45]. The ability of gallium to potentiate the antiproliferative action of 3-AP was demonstrated in studies that compared the effects of iron and gallium complexes of 3-AP on ribonucleotide reductase and tumor cell proliferation *in vitro*; these studies showed that the cytotoxicity of this thiosemicarbazone was enhanced by gallium, but weakened by iron [41]. Further preclinical and clinical investigation to explore the antineoplastic activity of the gallium-3AP *in vivo* would be of interest.

Pyridine & phenolate ligand complexes of gallium

Shakya *et al.* reported the synthesis of five novel gallium(III) complexes described as $[Ga^{III}(L^X)_2]ClO_4$, where L^X is a negatively charged ligand containing 2-methylpyridine and 2-methylphenolate groups attached to a secondary amine (Figure 1E) [46]. The phenol moiety has substituents (X) that encompass the electron-withdrawing and electron-donating methoxy (complex **1**), nitro (complex **2**), chloro (complex **3**), bromo (complex **4**) and iodo groups (complex **5**) [46]. Complex **5** is shown in the right panel of Figure 1E. Complexes **2**–**5** displayed the greatest apoptosis-inducing activity in BE(2)-C neuroblastoma cells *in vitro* with the IC_{50} levels for these complexes ranging from 13.3–23.8 μM. In contrast, the IC_{50} value for complex **1** was 245.4 μM [46]. Chen *et al.* demonstrated that of these complexes, complex **5** (Figure 1E, right-hand panel) displayed the greatest induction of apoptosis in prostate cancer cell lines *in vitro* and inhibited the growth of PC-3 prostate cancer cell xenografts in nude mice by 66% [47]. Tumor extracts from mice treated with complex **5** showed an increase in ubiquitinated proteins, an accumulation of p27 (a proteasomal target protein), a decrease in proteasomal chymotrypsin activity, and an induction of apoptosis [47]. The interesting preclinical antineoplastic activity of these gallium complexes, especially complex **5**, suggests that they have potential for further development as antineoplastic agents.

Other gallium complexes

Several additional gallium complexes that display antiproliferative activity against malignant cell lines *in vitro* have been reported. Many of these gallium complexes are in very early stages of their preclinical development and are yet to be advanced to human studies. Some examples of these complexes follow.

Gallium-pyridoxal isonicotinyl hydrazone

Pyridoxal isonicotinyl hydrazone (PIH) and its analogues belong to a class of lipophilic iron chelators that has been shown to inhibit the proliferation of a variety of malignant cells *in vitro* [48,49]. An interesting property of PIH is that it can also deliver iron to cells to support cell growth and function [50]. Consistent with its ability to facilitate metal uptake, PIH in a complex with gallium (Ga-PIH, Figure 1F) displayed greater cytotoxicity than PIH alone [48,51]. Ga-PIH was also shown to inhibit the growth of CCRF-CEM cells that were resistant to growth inhibition by gallium nitrate [52]. These gallium-resistant cells have a downregulation of transferrin receptor-mediated gallium uptake [53]. The ability of Ga-PIH to inhibit the growth of these gallium-resistant cells suggests that gallium, when coupled with PIH, is able to enter cells by a transferrin-independent route and, thus, induce cell death [52].

Gallium *tris*(salicylate) ethanol

Sodium salicylate has been shown to inhibit the proliferation of malignant cells *in vitro* [54]. Ismail *et al.* reported the synthesis of gallium *tris*(salicylate)-ethanol and showed that it inhibited the proliferation of Erlich's ascites carcinoma cells in a dose-dependent fashion [55]. The antineoplastic activity of this gallium complex was examined by measuring the survival of Swiss mice inoculated intraperitoneally with Erlich ascitic carcinoma cells. At 30 days following inoculation of tumor cells, 30% of the mice treated with gallium *tris*(salicylate) ethanol had survived compared with 0% survivors in the untreated group of mice. However, although treatment with this gallium complex prolonged survival in tumor-bearing mice, the cancer cells were not completely eradicated [55].

Gallium complexes with azole ligands

The synthesis of gallium complexes of 2,1,3-benzothiadiazole, 1,2,3-benzotriazole, and 1-methyl-4,5-diphenylimidazole has been described by Zanias *et al.* [56]. Compound **1**, a Ga(III) complex with 1,2,3-benzotriazole, is shown in Figure 1G. These compounds inhibit the proliferation of breast, ovarian, cervical and colon tumor cell lines *in vitro* [56].

Bi- & tetranucleargallium(III) complexes with heterocyclic thiolato ligands & dinuclear gallium carboxylate complexes

A variety of gallium complexes with these ligands (structure shown in Figure 1H) have been characterized and reported to display cytotoxicity against solid tumor cell lines *in vitro* [57,58]. The gallium(III)complex containing the thiolato ligand was shown to bind to fish sperm DNA and display greater cytotoxicity in malignant cell lines than in fibroblast cells [58–60]. The latter property is relevant to their further development as anticancer drugs.

Mechanisms of anti-tumor activity by gallium compounds

Action on cellular iron homeostasis

A brief discussion of cellular iron metabolism is relevant to understanding some of gallium's mechanisms of action. Some of the steps in the interaction of gallium with cellular iron uptake and trafficking are summarized in Figure 2. Iron plays an important role in the

function of numerous proteins critical for cell viability and proliferation. It is an essential component of numerous iron–sulfur-containing enzymes of the citric acid cycle and the mitochondrial electron-transport chain. Iron is required for the activity of the R2 subunit of ribonucleotide reductase, the enzyme responsible for deoxyribonucleotide synthesis, a rate-limiting step in DNA synthesis [61]. In the absence of iron, this subunit exists as a non-functional apoR2 protein leading to a block in the synthesis of deoxyribonucleotides [62]. More recent studies have revealed that iron is also needed for the transition of cells through the G1 phase of the cell cycle [63]. While iron deprivation may lead to loss of cell proliferation and viability, excess iron may be equally deleterious as it can serve as a catalyst for the generation of cytotoxic hydroxyl radicals via the Fenton reaction [64]. Hence, iron homeostasis is tightly regulated in the cell through a complex interplay of various proteins responsible for regulating its transport, cellular uptake, intracellular trafficking, storage and efflux from cells [65]. Several studies have demonstrated that cancer cells have a significantly higher requirement for iron than normal cells. Iron is transported in the circulation bound to transferrin and its uptake by cells occurs via transferrin receptor-mediated endocytosis of transferrin–iron complexes. An increase in the number of cell surface transferrin receptors is seen in lymphoma, bladder cancer and breast cancer *in vivo* [66–68]. In fact, an increased level of transferrin receptor expression has been correlated with an aggressive clinical behavior and adverse outcome in lymphoma, urothelial cancer and other malignancies [66,67,69]. The level of intracellular ferritin, the iron storage protein, is known to be altered in certain malignant cells [68,70]. Recently, it was shown that cancer cells in patients with breast cancer display a decrease in their expression of the iron efflux protein ferroportin. In breast cancer patients, the combined effect of decreased tumor ferroportin and elevated serum hepcidin (a protein that degrades hepcidin) was associated with a poorer clinical outcome than patients who did not display this ferroportin/hepcidin profile [71]. An interpretation of this finding is that a decrease in ferroportin and an increase in transferrin receptor would be expected to elevate intracellular iron status to support aggressive tumor cell growth. Hence, the above clinical findings provide support to the notion that certain tumor cells have a greater requirement for iron than normal cells. It also indicates that treatment strategies directed at iron-dependent tumor growth have potential in cancer therapeutics.

Several studies have suggested that gallium acts as an iron mimetic that can perturb cell proliferation by interposing itself into iron-dependent processes. The mechanisms of action of gallium nitrate and transferrin–gallium can be viewed as occurring in two steps (Figure 2). The first involves the preferential targeting of transferrin–gallium to transferrin receptor 1-expressing tumor cells followed by its uptake via transferrin receptor-mediated endocytosis. Under physiological conditions, approximately one-third of transferrin in the circulation is occupied by iron thus leaving the remainder of transferrin available to bind gallium and to deliver it to cells. The second step involves the action of intracellular gallium on iron-dependent processes resulting in disruption of iron homeostasis and cell death. Gallium, as transferrin–gallium, blocks cellular iron uptake by competitively inhibiting the binding of transferrin–iron to cell surface transferrin receptors and by interfering with the dissociation of iron from transferrin in the endosome [72]. In the latter situation, gallium interferes with endosomal acidification, a step necessary for the release of iron from transferrin [72]. The net result of gallium's action at this level is cellular iron deprivation [72]; this, in turn, can impact on downstream iron-dependent processes. Iron deprivation has been shown to produce apoptosis [73,74]. Evidence that gallium actually produces cellular iron deficiency *in vivo* was presented in clinical studies that showed that red blood cells from patients receiving gallium nitrate contained increased levels of zinc protoporhyrin, a marker of iron deficiency [75], and that anemia in patients being treated with gallium chloride may be due to gallium-induced iron deficiency [76].

The effect of gallium on cellular iron homeostasis is, however, not limited to the production of cellular iron deprivation through blockade of iron uptake. Within cells, gallium inhibits DNA synthesis through action on the iron-containing R2 subunit of ribonucleotide reductase [77–79]. Cytoplasmic extracts from cells incubated with gallium contain apoR2 protein that lacks an EPR-detectable tyrosyl free radical signal; this signal can be restored within minutes by the addition of iron salt, thus indicating that gallium blocks the interaction of iron with R2 without decreasing the level of R2 protein [79]. Consistent with inhibition of ribonucleotide reductase activity, cells exposed to gallium display decreased levels of deoxyribonucleotides [77]. Although it could be surmised that the inhibition of R2 subunit activity by gallium results from gallium-induced cellular iron deprivation, other studies show that gallium directly inhibits CDP and ADP reductase activity in a cell-free enzyme assay [78]. Hence, it is likely that ribonucleotide reductase inhibition by gallium occurs through both indirect (cellular iron deprivation) and direct mechanisms.

Actions of gallium unrelated to iron homeostasis

Whereas perturbation of cellular iron homeostasis and inhibition of ribonucleotide reductase are sufficient to inhibit cell proliferation, many of the upstream and downstream events involved in gallium-induced cell death remain to be elucidated. Moreover, it is possible (and likely) that steps independent of iron and iron proteins also contribute to the cytotoxicity of gallium. Early studies into the mechanism of action of gallium showed that it could partially inhibit DNA polymerases, however, the concentration of gallium required to produce this effect could not be correlated with its anti-tumor activity [80]. Inhibition of tyrosine phosphatase activity by gallium nitrate in Jurkat leukemia and HT-29 human colon cancer cell lines has been demonstrated, but since this effect could not be correlated with an inhibition of proliferation its contribution to the antineoplastic activity of gallium is unclear. Competitive inhibition of magnesium-dependent ATPase and inhibition of tubulin formation by gallium has been demonstrated [81–85]; these effects could impact tumor cell growth. Studies into the mechanisms of action of the pyridine and phenolate ligand complexes of gallium have revealed an important target for these compounds: the proteasome. Complexes **2–5** of these gallium compounds (Figure 1E) inhibited proteasome function and induced apoptosis in cells *in vitro* and in a prostate cancer xenograft in a rat model [47]. In other studies, gallium maltolate was found to inhibit proteasome activity and to synergistically enhance the cytotoxicity of the proteasome inhibitor bortezomib [86]. These mechanisms of action of gallium are important since in recent years the proteasome has emerged as an important target for cancer therapeutics in multiple myeloma and certain lymphomas; these malignancies have an increased sensitivity to proteasome inhibition [87,88]. A report by Niesvizky *et al.* suggested that patients with multiple myeloma treated with gallium nitrate for bone disease had a better survival than those who did not receive this drug [89]. The action of certain gallium compounds on proteasome function is relevant to the further development of these compounds for the treatment of tumors that are sensitive to proteasome inhibition.

Induction of apoptosis by gallium & cellular adaptation to gallium

Recent investigations conducted in human leukemic/lymphoma cells show that the pathways involved in gallium-induced cell death include the mitochondria [27,90]. Earlier studies had reported an effect of gallium on calcium retention in rat liver mitochondria [91]. Human leukemia/lymphoma cells exposed to gallium nitrate or gallium maltolate display an initial translocation of inositol phosphotidylserine to the cell surface, an early marker of apoptosis. This is followed by a loss of mitochondrial membrane potential leading to the release of cytochrome C from the mitochondria to the cytoplasm and the activation of caspase-3 and morphologic changes of apoptosis (both of which can be inhibited by z-VAD-fmk, a pancaspase inhibitor) [27,90]. In DoHH2 and CCRF-CEM cells exposed to gallium nitrate,

the loss of mitochondrial membrane potential is associated with activation of cytoplasmic Bax, a proapoptotic protein. It is known that activated Bax translocates to the mitochondria to induce the loss of mitochondrial membrane potential that precedes apoptosis [92]. Exposure of cells to gallium maltolate results in a loss of mitochondrial membrane potential much more rapidly than gallium nitrate [27]; this may occur without the involvement of Bax [Chitambar CR, Unpublished Data].

The cellular handling of gallium is only partly understood. Although gallium compounds can induce cell death, the initial response of cells to gallium appears to be to mount a cytoprotective response. A study of metal-related genes expressed in CCRF-CEM cells sensitive or resistant to growth inhibition by gallium nitrate revealed a marked upregulation of MT2A and zinc transporter-1 (a zinc efflux protein) genes in gallium-resistant cells [93]. These findings were entirely unexpected since MT2A is a low-molecular weight protein that binds divalent metals. MT is involved in the metabolism of zinc and the sequestration of cadmium (to decrease its toxicity); it is not involved in iron metabolism. An early study by Capel *et al.* reported a lowering of tissue zinc levels in C57/BL mice treated with gallium nitrate [94], while the more recent studies clearly demonstrate an impact of gallium on zinc metabolism [93]. The extent to which cellular MT levels influence gallium's cytotoxicity remains to be determined. Upregulation of MT in CCRF-CEM cells by pre-incubation of cells with endogenous zinc sulfate partially diminished the growth-inhibitory effect of gallium; this protection was lost when MT levels returned to baseline [93]. In a panel of lymphoma cell lines, the cytotoxicity of gallium nitrate was shown to correlate with their endogenous level of MT [93]. Although these studies lend support to a role for MT in modulating gallium's cytotoxicity, it remains to be determined whether this occurs through a direct effect (sequestration of gallium by MT) or an indirect effect mediated by another mechanism that also impacts on MT expression.

Other studies have demonstrated that gallium nitrate produces a shift in intracellular zinc and an increase in *MT2A* and *HO-1* gene expression [95]. The activation of HO-1 expression by gallium involves the p38 MAP kinase pathway and the activation of Nrf2, a zinc finger transcription factor. The increase in both MT2A and HO-1 is possibly explained by the fact that these proteins can be upregulated in response to increased levels of reactive oxygen species (ROS) in gallium-treated cells. In support of this cause-and-effect relationship is the observation that the gallium-induced increase in metallothionein and HO-1 can be abrogated by the antioxidant *N*-acetyl cysteine [95]. An important question centers on the significance of the gallium-induced increase in MT and HO-1 expression. It is known that both these genes are activated in response to oxidative stress [96,97], and an early event seen within 4 h of exposure of CCRF-CEM cells to gallium nitrate is a decrease in the cellular GSH/GSSG ratio and an increase in intracellular oxidative stress [95]. Hence, gallium-induced increase in cellular ROS precedes the increase in MT and HO-1. These new insights into the cellular response to gallium suggest a model (shown in Figure 3) in which the initial exposure of cells to gallium results in an elevation of intracellular ROS which, in turn, invokes a cytoprotective response (i.e., an increase in MT2A and HO-1). Thus, cell death occurs only when these cytoprotective responses are overwhelmed. How gallium exposure results in ROS production and whether the cytoprotective response to gallium varies among different cell types remains to be determined.

Future perspective

Advances in the field of gallium research, championed primarily by a relatively small number of investigators, have enhanced our understanding of this interesting metal. However, there remain many areas for future development that warrant additional study to

move gallium compounds from the laboratory to the clinic. Some of these areas are discussed below.

Gallium compounds that can target cells independently of transferrin

The binding of gallium to transferrin in the circulation can, theoretically, be a double-edged sword. On one hand, transferrin–gallium complexes have the advantage of directly targeting transferrin receptor-bearing malignant cells. On the other hand, the binding of gallium to transferrin may prevent it from reaching tumor cells that lack transferrin receptors or display low levels of these receptors. In the latter situation, the antineoplastic action of gallium on tumor cells lacking transferrin receptors may require plasma levels of gallium to exceed the metal-binding capacity of transferrin so that gallium's cytotoxicity can be exerted through transferrin–independent mechanisms. In consideration of these factors, gallium compounds designed to deliver gallium to tumor cells independent of transferrin and its receptor may prove to be more effective against tumors with a low number of transferrin receptors.

Combination therapy with gallium compounds & other antineoplastic agents

Successes in cancer treatment have often stemmed from the use of several drugs in combination. Selecting drugs with synergistic interactions against cancer cells but not against normal cells is key in developing a scientifically based therapeutic strategy. In this regard, gallium compounds can synergistically increase the cytotoxicity of hydroxyurea, fludarabine, IFN-α, gemcitabine, bortezomib, paclitaxel and cisplatin; these drugs are currently in use for the treatment of various malignancies [33,77,86,98–101]. The results of these preclinical studies form a scientific rationale for further studies of gallium-based drug combinations and their evaluation in the clinic.

Biologic markers that predict tumor responsiveness to gallium-based therapy

Cancers are molecularly heterogeneous disorders; tumors that appear to be similar in morphologic appearance can display diverse clinical behavior and response to treatment. Tumor cell resistance to chemotherapeutic agents remains a significant obstacle to success in the treatment of cancer. Hence, the identification of markers (proteins, pathways, or processes) in cancer cells that predict tumor sensitivity to gallium compounds would be a significant advance since it would allow for the selection of patients most likely to benefit treatment with gallium metallodrugs. While reliable markers for tumor sensitivity to gallium treatment have not been established, preclinical studies have provided clues that are worthy of further rigorous investigation. For example, the intensity of cellular ^{67}Ga uptake and cell surface transferrin receptor density would appear to be of value in determining whether gallium might target to a specific tumor. However, a caveat here is that the homing of gallium to a malignant cell does not necessarily mean that the cell will be killed since the mechanisms of gallium-induced cell death depends primarily on its action on intracellular targets. The discovery of elevated MT2A levels in lymphoma CCRF-CEM cells resistant to gallium nitrate begs the question as to whether measurement of metallothionein might have value in identifying gallium-responsive tumors in patients. Clearly, further research is needed to understand the intracellular targets of gallium in malignant cells. It is envisioned that such research will advance our ability to use gallium compounds optimally in the treatment of cancer.

Key Terms

Clinical trials	Drug studies conducted in humans. Phase II clinical trials evaluate the efficacy of a new anticancer drug in different malignancies to establish where it may be most effective

Metallodrugs	Drugs that contain metals
Preclinical studies	Studies of a drug conducted *in vitro* and in animals prior to its use in humans
Ribonucleotide reductase	An iron-containing enzyme responsible for the synthesis of deoxyribonucleoside diphosphates. This enzyme is an important target for gallium

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Executive summary

Gallium compounds in cancer treatment

- Gallium compounds are unique metallodrugs with therapeutic potential in cancer and other diseases.

- The first-generation gallium nitrate has, in several clinical trials, consistently demonstrated efficacy against bladder cancer and non-Hodgkin's lymphoma while displaying an acceptable toxicity profile to normal cells.

- In preclinical studies, the newer generation gallium compounds hold promise as agents with greater antineoplastic activity against a wider spectrum of cancers than gallium nitrate.

Similarities between iron & gallium chemistry, & gallium's mechanisms of anti-tumor activity

- The unique mechanism of antineoplastic action of gallium that involves its ability to target and disrupt tumor cell iron homeostasis sets it apart from other chemotherapeutic drugs. Gallium shares certain similarities with iron that allow it to function as an iron mimetic, bind to iron transport proteins, and thus inhibit iron-dependent cell function.

- At the cell surface, transferrin–gallium complexes interfere with transferrin receptor-mediated uptake of transferrin–iron. Within the cell, gallium can inhibit ribonucleotide reductase and iron-dependent mitochondrial function and induce apoptosis.

- Malignant cells have an unusually high requirement for iron and may thus alter their expression of proteins responsible for iron influx, storage and efflux. Hence, targeting tumor iron homeostasis is an attractive strategy for cancer treatment.

- Gallium compounds may also inhibit tumor growth through mechanisms unrelated to iron; more recent studies suggest an interaction of gallium with proteins involved in zinc metabolism. In addition, some of the newer gallium compounds have important mechanisms of cytotoxic action such as inhibition of proteasome function in cancer cells.

Advancing the field

- Numerous clinical trials have confirmed the clinical efficacy of gallium nitrate in the treatment of lymphoma and bladder cancer. Newer gallium compounds may prove to be more efficacious in other malignancies.

- Their development should be vigorously pursued with the goal of advancing them to clinical trials.
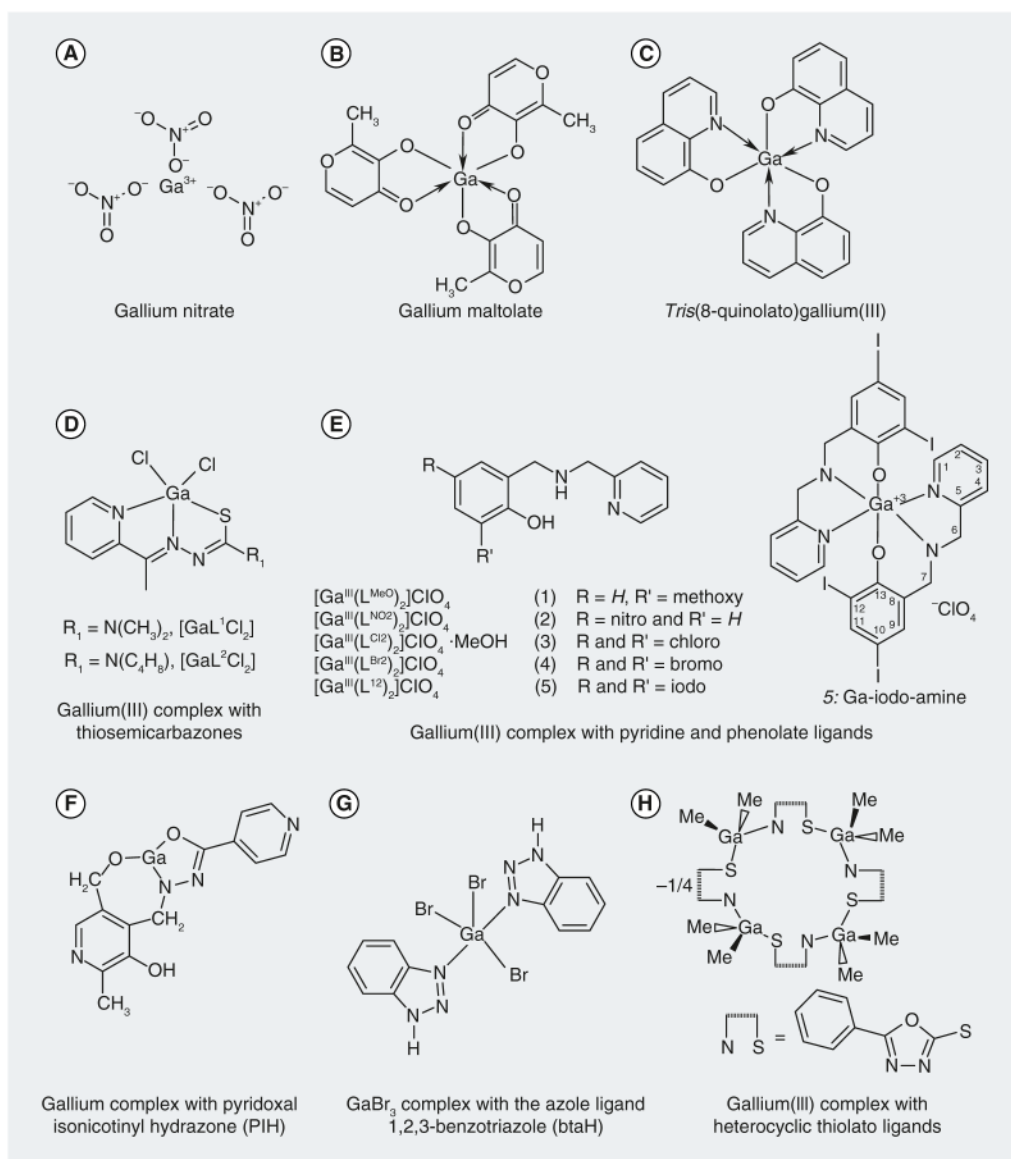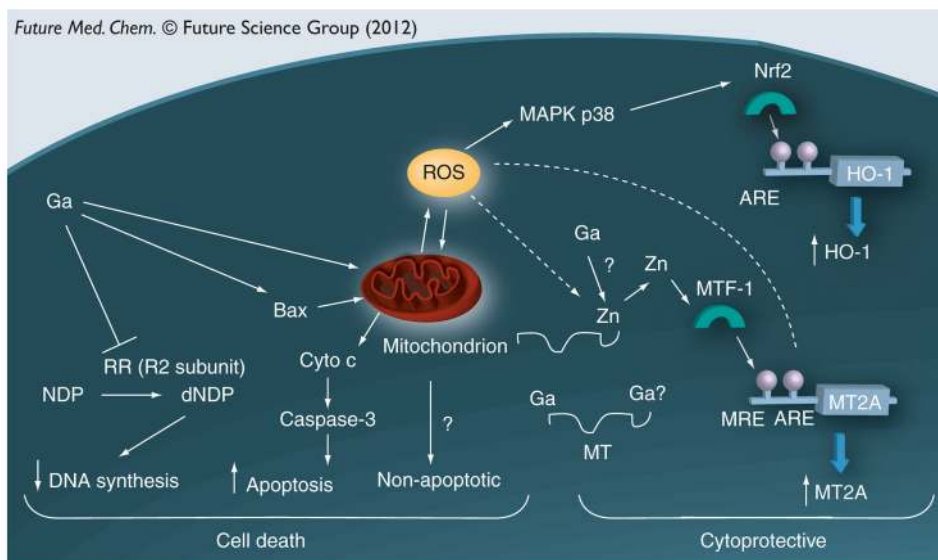
Figure 1.
Gallium complexes (A–H).
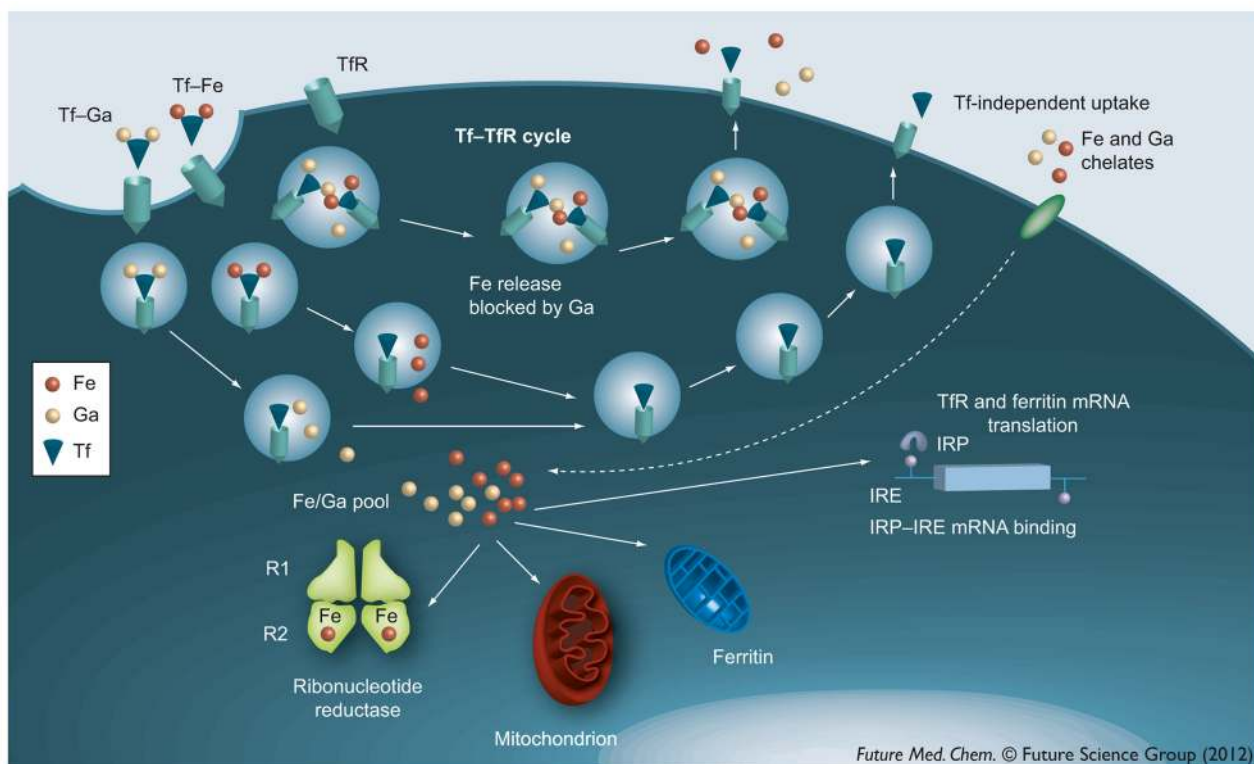
Figure 2. Cellular uptake and interaction of iron and gallium
Iron(III) and gallium(III) are taken up by cells via transferrin receptor-mediated endocytosis of transferrin–iron (Tf–Fe^{3+}) and transferrin–gallium (Tf–Ga^{3+}). These complexes compete with each other for binding to the cell surface TfR. Theoretically, the endosome can contain Tf-Fe^{3+} or Tf-Ga^{3+}, or both Tf-Fe^{3+} and Tf-Ga^{3+} in variable proportions. Fe^{3+} and Ga^{3+} are unloaded from transferrin in an acidic endosome and transferred to an intracellular labile pool containing both iron and gallium. However, gallium may also block the unloading of iron from the endosome, thus resulting in recycling of both metals back to the cell surface to be released to the exterior. In some cells, iron and gallium may enter cells through a transferrin-independent route. Under physiological conditions, the iron in the pool is utilized for ribonucleotide reductase activity, mitochondrial function and other iron-dependent processes. Excess iron(III) is stored in ferritin. Transferrin receptor and ferritin syntheses are regulated by cellular iron status via the interaction of IRP with IREs present on the 5′ or 3′ untranslated regions of the ferritin or transferrin receptor mRNAs, respectively. Gallium can disrupt cellular iron homeostasis and inhibit cell proliferation through action on iron-dependent processes at different levels.
IRE: Iron-response element; IRP: Iron regulatory protein; TfR: Transferrin receptor.

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Figure 3. Proposed model for the cytotoxic and cytoprotective events triggered by gallium nitrate
An early event in the cellular response to gallium(III) is an increase in intracellular ROS of
mitochondrial origin. Changes in zinc homeostasis along with ROS generation induce the
expression of MT2A. *HO-1* gene expression is triggered by ROS via the p38 MAP kinase
pathway and activation of transcription factor Nrf2. MT2A and HO-1 are cytoprotective.
The iron-dependent R2 subunit of ribonucleotide reductase is inhibited by gallium(III).
Apoptosis is triggered through Bax activation and the mitochondrial release of Cyto c
leading to caspase-3 activation. Direct action of gallium(III) on the mitochondria is also
likely. Cell death ensues when cytoprotective responses are overwhelmed.
ARE: Antioxidant response element; Cyto c: Cytochrome c; MRE: Metal-responsive
elements; ROS: Reactive oxygen species; RR: Ribonucleotide reductase.

Table 1

Gallium compounds in cancer treatment: advancement from the laboratory to the clinic.

Gallium complex	*In vitro* studies	Animal studies	Human studies
Gallium nitrate	Cytotoxic against a spectrum of malignant cell lines, especially lymphoma and leukemia cells	Antineoplastic activity demonstrated in rodent tumor models. Animal toxicity profile established	Human-toxicity profile established in clinical Phase I studies. Antineoplastic activity against bladder cancer and non-Hodgkin's lymphoma demonstrated in numerous Phase II clinical studies. Combination chemotherapy trials conducted
Gallium chloride	Cytotoxic against a variety of malignant cell lines at concentrations similar to gallium nitrate	Intraperitoneal administration inhibited the growth of adenocarcinoma CA755 in mice. Low bioavailability with oral administration. Tissue-distribution studies conducted	Clinical pharmacology studies with oral GaCl₃ conducted in lung cancer patients. Partial responses to treatment were seen in patients with ovarian cancer
G4544	Not reported	Pharmacokinetic studies conducted	Clinical pharmacology studies conducted
Gallium maltolate	IC₅₀ values lower than gallium nitrate. Inhibits the growth of lymphoma cells resistant to gallium nitrate	Pharmacokinetic studies conducted	Clinical pharmacology studies conducted. Anti-tumor activity demonstrated in a patient with hepatocellular carcinoma
Tris(8-quinolonato)gallium(III) (KP46)	Cytotoxic in melanoma, ovarian, breast, colon and lung cancer cell lines. IC₅₀ values lower than that reported for gallium nitrate	Tissue distribution studies conducted in mice. Anti-tumor activity demonstrated *in vivo*	Clinical pharmacology and toxicity studies conducted in clinical Phase I/II trials. Anti-tumor activity noted in renal cancer
Pyridine and phenolate ligand complexes of gallium	Cytotoxic to cisplatin-resistant neuroblastoma cells and prostate cancer cell lines. Inhibitors of proteasome activity	Anticancer activity demonstrated against PC-3 prostate cancer xenografts in a nude mouse model	Not reported
Gallium *tris*(salicylate) ethanol	Inhibits the growth of Erlich carcinoma cell lines	Inhibits the growth of Erlich carcinoma cells in ascites in mice	Not reported
Gallium thiosemicarbazones	Complexes of gallium with thiosemicarbazones display greater cytotoxicity than either component alone in colon, breast, ovarian and brain tumor cell lines. Cytotoxicity not influenced by p53 mutations	Not reported	Not reported
Gallium-pyridoxal isonicotinyl hydrazone	Cytotoxic to solid tumor and lymphoma cell lines, including those resistant to gallium nitrate	Not reported	Not reported
Gallium complexes with azole ligands	Cytotoxic to breast, ovarian, cervical and colon cancer cell lines	Not reported	Not reported
Bi- and tetra-nuclear gallium(III) complexes with heterocyclic thiolato ligands	Cytotoxic against human and murine solid tumor cell lines, including breast and cervical cancer	Not reported	Not reported