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Report

Microtubule-disrupting effects of gallium chloride *in vitro*

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Gallium chloride ($GaCl_3$), an antitumor agent with antagonistic action on iron, magnesium and calcium, was tested for its ability to alter the polymerization of purified tubulin (2.2 mg/ml) in a cell-free system *in vitro*. $GaCl_3$ (250 μM) does not mimic the effect of 10 μM paclitaxel and, therefore, is not a microtubule (MT)-stabilizing agent that can promote tubulin polymerization in the absence of glycerol and block MT disassembly. In contrast, $GaCl_3$ mimics the effect of 1 μM vincristine (VCR) and inhibits glycerol-induced tubulin polymerization in a concentration-dependent manner (IC_{50}: 125 μM), indicating that $GaCl_3$ is a MT de-stabilizing agent that prevents MT assembly. However, 150 μM $GaCl_3$ must be used to match or surpass the inhibitions of tubulin polymerization caused by 0.25 μM of known MT de-stabilizing agents, such as colchicine (CLC), nocodazole, podophyllotoxin, tubulozole-C and VCR. The inhibitory effect of 250 μM $GaCl_3$ persists in the presence of up to 9 mM $MgCl_2$, suggesting that the exogenous Mg^{2+} cations absolutely required for the binding of GTP to tubulin and MT assembly cannot overcome the antitubulin action of Ga^{3+} ions of a higher valence. The binding of [^{3}H]vinblastine (VBL) to tubulin (0.5 mg/ml) is inhibited by unlabeled VBL but enhanced by concentrations of $GaCl_3 > 200$ μM. However, increasing concentrations of $GaCl_3$ mimic the ability of cold CLC to reduce the amount of [^{3}H]CLC bound to tubulin, suggesting that $GaCl_3$ may interact with the CLC binding site to inhibit tubulin polymerization. The binding of [^{3}H]GTP to tubulin is decreased by unlabeled GTP but markedly enhanced by $GaCl_3$, especially when concentrations of this metal salt of 32 μM or higher are added to the reaction mixture before rather than after the radiolabeled nucleotide. These data suggest that changes in protein conformation following $GaCl_3$ binding might increase the interactions of tubulin with nucleotides and Vinca alkaloids. After a 24 h delay, the viability of $GaCl_3$-treated L1210 leukemic cells is reduced in a concentration-dependent manner at days 2 (IC_{50}: 175 μM), 3 (IC_{50}: 35 μM) and 4 (IC_{50}: 16 μM). Since $GaCl_3$ (100–625 μM) increases the percentage of mitotic cells at 2–4 days, it might arrest tumor cell progression in M phase, but its antimitotic activity is much weaker than that of 0.25 μM VCR. Because the concentrations of $GaCl_3$ that inhibit tubulin polymerization also increase the mitotic index and decrease the viability of L1210 cells *in vitro*, the antitubulin and antimitotic effects of $GaCl_3$ might contribute, at least in part, to its antitumor activity. [© 1999 Lippincott Williams & Wilkins.]

Key words: Gallium, L1210 cell viability, mitotic index, tubulin binding site and polymerization.

Introduction

Gallium (Ga), a trivalent metal ion, may inhibit tumor cell proliferation and viability, and reduce cancer-related hypercalcemia because of its ability to decrease the cellular concentrations of magnesium (Mg), calcium (Ca), zinc (Zn) and, especially, iron (Fe), which may be the reason why Ga-treated human leukemic cells undergo apoptosis.[1–3] Ga decreases cell membrane permeability and the ionic transport of Na^+/K^+.[4–6] Ga induces protonation of biomolecules and conformational changes in DNA.[7,8] Ga inhibits ATPase and protein tyrosine phosphatase activities and arrests cell cycle progression in S phase.[9–11] Ga, which competes with binding sites of Ca and Mg,[12] may prevent cancer-related hypercalcemia by blocking the release of Ca from bone.[13,14] Ga competes with Fe for the binding to the serum Fe-binding transport protein transferrin and the tissue Fe storage protein ferritin.[15–19] Since Ga and Fe appear to compete for cellular uptake through the transferrin receptor pathway,[20,21] Ga may reduce the uptake and limit the utilization of Fe by the cells, and may interact with Fe-containing

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cellular targets to disrupt their bioactivity.[19,22] Indeed, the cytotoxicity of Ga is enhanced by transferrin and antagonized by Fe.[22,23] Moreover, resistance to Ga results primarily from the ability of tumor cells to overcome the Ga-induced block in transferrin receptor-mediated Fe uptake into cells.[24]

Prior work has focused on gallium nitrate [$Ga(NO_3)_3$].[25] The antitumor activity of $Ga(NO_3)_3$ has been demonstrated in various animal tumor models,[25-27] and this agent is also effective in phase II clinical trials against lymphoma and bladder cancer,[28-32] doses being limited by renal toxicity.[33,34] Renal toxicity may be avoided by prolonged oral administration of gallium chloride ($GaCl_3$), which enhances the bioavailability of Ga and favors the selective uptake of Ga by the tumor.[35] $GaCl_3$ has been successfully included in combination chemotherapy for lung cancer.[36]

$Ga(NO_3)_3$ can inhibit DNA and RNA polymerases,[26,27,37] and decrease replicative DNA synthesis rather than DNA repair.[38] By perturbing the utilization of Fe by cells, $Ga(NO_3)_3$ may block DNA synthesis and the proliferation of tumor cells in S phase because it inhibits the Fe-containing R2 subunit of ribonucleotide reductase, a key rate-limiting enzyme in deoxyribonucleotide synthesis.[22,39,40] Moreover, $Ga(NO_3)_3$ acts synergistically with the ribonucleotide reductase inhibitors fludarabine, gemcitabine and hydroxyurea to inhibit tumor cell growth.[41-43]

Because Ga salts exhibiting antitumor activity *in vitro* and *in vivo* might be useful in combination chemotherapy, further studies are required to elucidate their molecular mechanism of action. Therefore, the present study was undertaken to determine whether $GaCl_3$ would (i) interact with purified tubulin and disrupt microtubule (MT) dynamics in cell-free binding and turbidity assays, and (ii) increase the mitotic index and decrease the viability of L1210 leukemic cells *in vitro*. The antitubulin effects of $GaCl_3$ were compared to those of antimitotic drugs known to block MT assembly or disassembly.[44]

Materials and methods

Cell culture and drug treatments

Solutions of $GaCl_3$ were prepared and diluted in double-distilled water (DDW), whereas colchicine (CLC), nocodazole, podophyllotoxin, paclitaxel, tubulozole-C, vinblastine (VBL) (all from Sigma, St Louis, MO) and vincristine (VCR; a gift from Lilly Research, Indianapolis, IN) were all dissolved in dimethyl sulfoxide (DMSO). Murine L1210 lymphoblastic leukemia cells (ATCC, Rockville, MD) were maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 7.5% fortified bovine calf serum (HyClone, Logan, UT) and penicillin (100 IU/ml)-streptomycin (100 μg/ml). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Drugs were added to the culture medium in 1 or 2 μl aliquots and the concentrations of vehicle in the final incubation volume (0.5 ml) never exceeded 0.2% for DMSO and 0.4% for DDW. Such low concentration of DMSO did not affect the rates of macromolecule synthesis and growth in L1210 cells.[45] Control cells incubated in the absence of drugs were similarly treated with vehicle and, in each experiment, all incubates received the same volume of solvent.

Tubulin polymerization and binding assays

The polymerization of purified tubulin protein from bovine brain in the presence or absence of glycerol was analyzed using the Tubulin/Microtubule Biochem kit purchased from Cytoskeleton (Denver, CO).[46] The polymerization reactions contained, in a final volume of 0.2 ml, either tubulin minus glycerol or tubulin plus 10% glycerol (2.2 mg/ml) in 80 mM PIPES buffer, pH 6.8, supplemented with 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP and either 0 or 10% glycerol. $GaCl_3$ and known MT-disrupting agents were respectively added to the assay mixture in 2 μl aliquots of DDW or 1 μl aliquots of DMSO:tubulin buffer (40:60) to obtain the final concentrations of drugs tested. These vehicles did not affect the kinetics of tubulin polymerization in drug-untreated control reactions. Samples were immediately incubated at 35°C in quartz microcells, and the rate and plateau of tubulin polymerization were followed over 20-30 min by recording the increased absorbance (ΔA) of the solution at 340 nm, using a Shimadzu UV-160 spectrophotometer equipped with dual-beam optics and a thermostatically controlled cell holder.[47]

Tubulin binding assays were performed using the DEAE-cellulose filter method.[48,49] Increasing concentrations of unlabeled $GaCl_3$, VBL, CLC and GTP were compared for their ability to alter the binding of [^{3}H(G)]VBL sulfate (5.3 Ci/mmol; Moravek Biochemicals, Brea, CA), [ring C, methoxy-^{3}H]CLC (61.4 Ci/mmol; NEN Life Science Products, Boston, MA) and [8-^{3}H]GTP tetrasodium salt (10 Ci/mmol; ICN Pharmaceuticals, Irvine, CA) to purified tubulin. Tubulin was diluted to a final concentration of 0.5 mg/ml in 80 mM PIPES, pH 6.8, containing 1 mM EGTA and 1 mM $MgCl_2$ (PEM buffer). Glycerol must be absent since CLC binds to tubulin dimers but not to polymerized MTs.[44] Mixtures (0.1 ml) were supplemented with increasing

concentrations of unlabeled drugs and then incubated with [^{3}H]VBL (0.5 μCi; 10 μM) or [^{3}H]CLC (0.5 μCi; 1 μM) for 90 min at 37°C, or with [^{3}H]GTP (1 μCi; 5 μM) for only 10 min at 4°C.[50] After dilution with 5 ml of ice-cold 0.1 × PEM buffer, the CLC and VBL reaction mixtures were filtered through stacks of three Whatman DE81 ion exchange paper disks and the drug-protein complexes retained on the filters were washed with 3 × 10 ml of 0.1 × PEM buffer to eliminate residual levels of free radiolabeled drugs. However, the GTP binding reactions were diluted with 2.5 ml of ice-cold 0.1 × PEM buffer and the filters were washed only once with 2.5 ml of this buffer to avoid gradual dissociation of the GTP-tubulin complex on the filters during chronic washing.[50] After drying the filters, the radioactivity bound to tubulin was estimated by liquid scintillation counting. Control VBL, CLC and GTP binding assays were incubated in the absence of unlabeled drugs and blank values for free [^{3}H]VBL, [^{3}H]CLC or [^{3}H]GTP absorbed on filters in the absence of tubulin were subtracted from the results.

Cell viability and mitotic index

Decreasing concentrations of cells were initially plated at time 0 in order to collect samples with approximately equal cell densities at days 1, 2, 3 and 4. The viability of $GaCl_3$-treated L1210 cells was assessed from their ability to bioreduce the 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm.[51] After 1–4 days of growth in the presence or absence (control) of various concentrations of $GaCl_3$, samples with equal cell densities (about 10^6 cells/0.5 ml/well) were incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) reagent and the relative cell viability was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were substracted from the results.

For the mitotic index, 10^6 cells/0.5 ml/well grown for 1–4 days in the presence or absence (control) of various concentrations of $GaCl_3$ or known antimitotic drug were collected by centrifugation for 10 min at 100 *g*. For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After addition of 1 ml of methanol:glacial acetic acid (3:1), the cells stood for 10 min at room temperature and were centrifuged. Cell pellets were fixed for another 1 h in 0.5 ml of methanol:acetic acid (3:1) on ice. Final cell pellets were collected by centrifugation, resuspended in 100 μl of methanol:acetic acid (3:1), dispensed onto glass slides and air-dried. Samples were stained by spreading 50 μl of 0.1% crystal violet under a coverslip. The percentage of cells in mitosis was determined microscopically by counting 500 cells/slide. The mitotic index was calculated as the percentage of mitotic cells in drug-treated cultures divided by the percentage of mitotic cells in non-treated controls.[47,52,53] Data of all *in vitro* experiments were analyzed using Student's *t*-test with the level of significance set at $p < 0.05$.

Results

$GaCl_3$ and MT assembly

The classic turbidity assay was used to study the effects of $GaCl_3$ on tubulin polymerization in the presence or absence of glycerol. Normally, glycerol and paclitaxel stabilize tubulin and lower the critical cencentration (CC) of protein required to initiate MT assembly.[46] As shown by the control curve in Figure 1(A), therefore, a concentration of purified tubulin below 10 mg/ml cannot polymerize in the absence of 10% glycerol. However, the MT-stabilizing drug paclitaxel can easily induce the polymerization of such low concentration of tubulin (2.2 mg/ml) in the absence of 10% glycerol (Figure 1A). In contrast to 10 μM paclitaxel, 250 μM $GaCl_3$ cannot promote tubulin polymerization in the absence of glycerol and, thus, is not a MT-stabilizing agent that blocks MT disassembly like paclitaxel (Figure 1A). The control curve in Figure 1(B) shows the three typical phases of MT assembly taking place when purified tubulin (2.2 mg/ml) undergoes polymerization in the presence of 10% glycerol: a short lag phase, an exponential growth phase almost linear between 200 and 600 s, and a steady phase reaching a plateau after 15 min.[46] At 250 μM, $GaCl_3$ inhibits the control rate and plateau of glycerol-induced tubulin polymerization by 87 and 75%, respectively, and mimics the inhibitory effect of 1 μM VCR, indicating that $GaCl_3$ is a MT de-stabilizing agent that prevents MT assembly (Figure 1B).

The control rate of glycerol-induced tubulin polymerization between 200 and 600 s is represented by the striped area at 100% in Figure 2. $GaCl_3$ inhibits this control rate of glycerol-induced tubulin polymerization in a concentration-dependent manner (IC_{50}: 125 μM) but is about 833 times less potent than VCR (IC_{50}: 0.15 μM) (Figure 2). Tubulozole-C is the most effective inhibitor of glycerol-induced tubulin polymerization

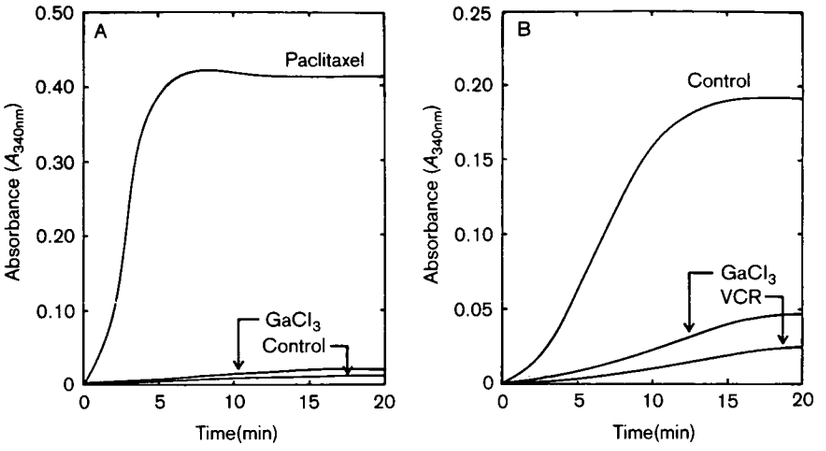

Figure 1. Comparison of the abilities of $GaCl_3$ and known MT-stabilizing (A) or MT de-stabilizing (B) anticancer drugs to respectively alter the kinetics of tubulin polymerization in the absence (A) or presence (B) of glycerol *in vitro*. (A) Purified tubulin was diluted to a final concentration of 2.2 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 1 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP. The polymerization reactions were placed in quartz microcells and incubated at 35°C in the presence or absence (control) of 250 μM $GaCl_3$ or 10 μM paclitaxel. (B) The turbidity assay mixtures were identical to those of (A) but contained 10% glycerol. The polymerization reactions were similarly incubated in the presence or absence (control) of 250 μM $GaCl_3$ or 1 μM VCR. The rate of MT assembly was continuously monitored by scanning over 20 min the increase in turbidity at $A_{340\ nm}$. Assays were performed in duplicate.

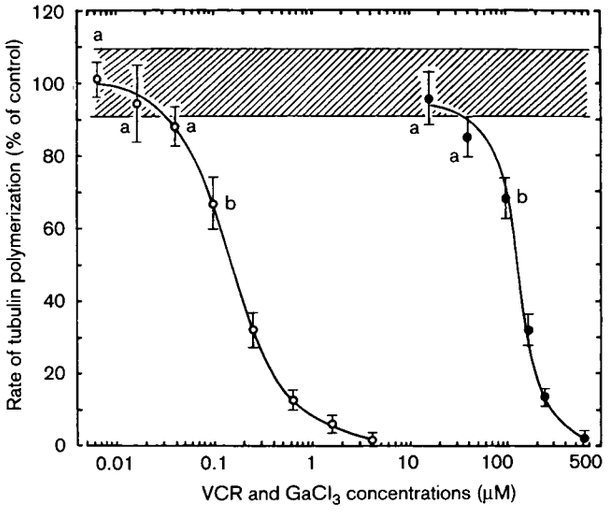

Figure 2. Comparison of the concentration-dependent inhibitions of glycerol-induced tubulin polymerization by $GaCl_3$ (●) and the known MT de-stabilizing anticancer drug VCR (○). The conditions of the turbidity assays were identical to those of Figure 1(B). The polymerization reactions were incubated in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale. Results are expressed as percentage of the rate of glycerol-induced tubulin polymerization between 200 and 600 s in vehicle-treated control assays ($\Delta A_{340\ nm}=0.115\pm0.010$; $100\pm9\%$; striped area). Bars: means $\pm$ SD ($n=2$). [a]Not significantly different from control; [b]$p<0.05$, significantly smaller than control.

among a spectrum of five known MT de-stabilizing agents tested at 0.25 μM in the turbidity assay (Figure 3). Since 16–40 μM $GaCl_3$ cannot inhibit the rate of tubulin polymerization (Figure 2), a greater concentration of $GaCl_3$ (150 μM) must be used to approximately match or surpass the inhibitions of MT assembly caused by 0.25 μM CLC, nocodazole, podophyllotoxin and VCR (Figure 3).

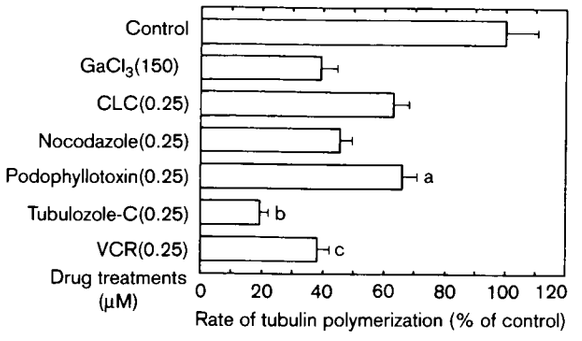

Figure 3. Comparison of the abilities of $GaCl_3$ and a spectrum of known MT de-stabilizing agents to alter the kinetics of glycerol-induced tubulin polymerization *in vitro*. The conditions of the assays were identical to those of Figure 1(B). The polymerization reactions were incubated in the presence or absence (control) of 150 μM $GaCl_3$ or 0.25 μM CLC, nocodazole, podophyllotoxin, tubulozole-C or VCR. Results are expressed as percentage of the rate of glycerol-induced tubulin polymerization between 200 and 600 s in vehicle-treated control assays (ΔA_{340nm}= 0.126±0.013; 100±10%). Bars: means±SD (n=2). [a]$p<0.05$, smaller than control but not different from CLC; [b]$p<0.05$, smaller than VCR; [c]$p<0.05$, smaller than CLC but not different from nocodazole or $GaCl_3$.

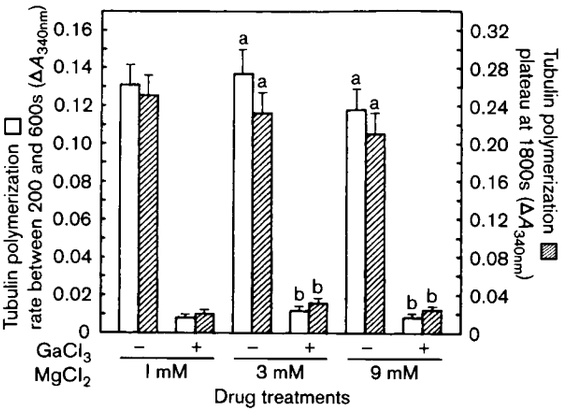

Figure 4. Effect of increasing concentrations of $MgCl_2$ on the inhibition of glycerol-induced tubulin polymerization by $GaCl_3$. The assay mixtures were identical to those of Figure 1(B) but contained either 1, 3 or 9 mM $MgCl_2$. The polymerization reactions were incubated at 35°C for 30 min in the presence (+) or absence (−) of 250 μM $GaCl_3$. The rate (open) and plateau (striped) of MT assembly respectively represent the increases in turbidity ($\Delta A_{340\ nm}$) between 200 and 600 s or at 1800 s. Bars: means±SD (n=2). [a]Not different from respective control values at 1 mM $MgCl_2$; [b]Not different from respective $GaCl_3$ values at 1 mM $MgCl_2$.

MTs require hydrolysis of GTP for their assembly and the tubulin dimer is a GTPase protein containing 2 mol of GTP.[54] Only one of them is loosely bound, complexed to Mg^{2+}, and can be exchanged within 5 s to 5 min with the free GTP or GDP present in the medium.[44,50] However, GTP has a higher affinity for the exchangeable site than GDP. Since exogenous Mg^{2+} is required for the binding of GTP to the exchangeable site on tubulin and for MT assembly,[44,46,55] it is of interest to determine if the antitubulin activity of $GaCl_3$ might be linked to its antagonistic action with Mg^{2+} ions and could be modulated by exogenous Mg^{2+}. However, the ability of 250 μM $GaCl_3$ to inhibit the control rate and plateau of glycerol-induced tubulin polymerization persists in the presence of 1-9 mM $MgCl_2$ (Figure 4). This failure of increasing concentrations of $MgCl_2$ to prevent or reverse the inhibition of tubulin polymerization caused by $GaCl_3$ suggests that competition with Mg^{2+} is unlikely to be involved in the antitubulin action of Ga^{3+}.

$GaCl_3$ and tubulin binding sites

MT de-stabilizing agents interact with tubulin either on the CLC or vinca alkaloid binding sites.[44] The striped areas at 100% in Figure 5 represent the control bindings of radiolabeled VBL, CLC or GTP to purified tubulin (0.5 mg/ml). The binding of [^{3}H]VBL (0.5 μCi; 10 μM) to tubulin is obviously inhibited by increasing concentrations of unlabeled VBL (1.28-125 μM) but not by $GaCl_3$?, which above 200 μM enhances such binding (Figure 5A). Indeed, the binding of [^{3}H]VBL to tubulin is more than doubled in the presence of 1250-3125 μM $GaCl_3$ (Figure 5A). In contrast, increasing concentrations of $GaCl_3$ (32-3125 μM) mimic the ability of increasing concentrations of cold CLC (0.51-125 μM) to reduce the amount of [^{3}H]CLC (0.5 μCi; 1 μM) bound to tubulin, suggesting that $GaCl_3$ may interact with tubulin at the CLC binding site to block MT assembly (Figure 5B). As expected, the binding of [^{3}H]GTP (1 μCi; 5 μM) to tubulin is inhibited by increasing concentrations of unlabeled GTP (1.28-125 μM) but markedly enhanced by $GaCl_3$, especially when concentrations of this metal salt above 32 μM are added to the reaction mixture before rather than after the radiolabeled nucleotide (Figure 5C). For instance, pre- or post-treatments with 500 μM $GaCl_3$, respectively, induce 2.5- and 9.7-fold increases in the level of [^{3}H]GTP bound to tubulin (Figure 5C). These data suggest that changes in protein conformation following $GaCl_3$ binding might increase

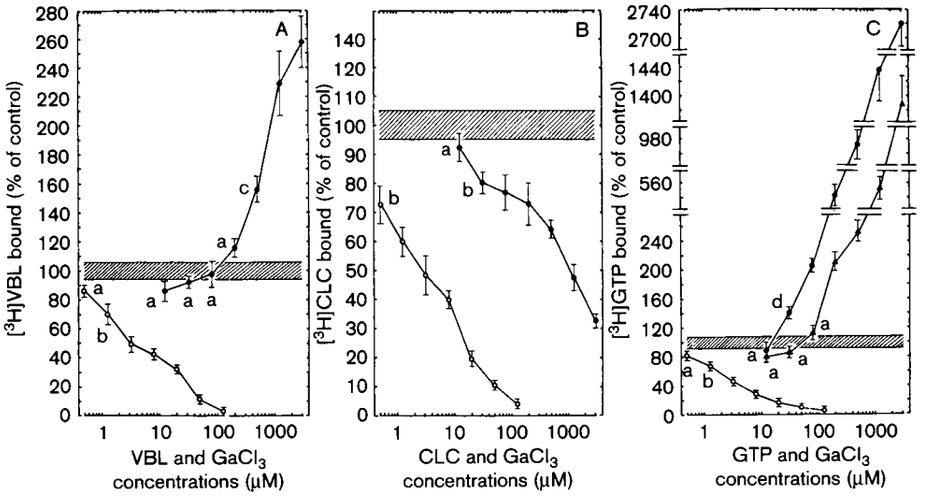

Figure 5. Comparison of the effects of $GaCl_3$ (●), VBL (○), CLC (○) and GTP (○) on the binding of [^{3}H]VBL (A), [^{3}H]CLC (B) and [^{3}H]GTP (C) to purified tubulin. Tubulin was diluted to a final concentration of 0.5 mg/ml in 80 mM PIPES buffer, pH 6.8, containing 1 mM $MgCl_2$ and 1 mM EGTA. The assay mixtures (0.1 ml) were supplemented with the indicated concentrations of unlabeled drugs, which are plotted on a logarithmic scale, and were incubated with [^{3}H]VBL (0.5 μCi; 10 μM) or [^{3}H]CLC (0.5 μCi; 1 μM) for 90 min at 37°C (A and B) or with [^{3}3H]GTP (1 μCi; 5 μM) for 10 min at 4°C (C). In (C), $GaCl_3$ was added to the assay mixture 5 min before (●) or after (▲) [^{3}H]GTP. The radioactivity bound to tubulin was determined by the DEAE-cellulose filter method. Results are expressed as percentage of [^{3}H]VBL (33 954 ± 2139 c.p.m.; 100 ± 6%; striped area in A), [^{3}H]CLC (95 761 ± 6224 c.p.m.; 100 ± 5%; striped area in B) or [^{3}H]GTP (25 563 ± 2096 c.p.m.; 100 ± 8%; striped area in C) bound to tubulin in control assays incubated in the absence of unlabeled drugs. Blank values for free [^{3}H]VBL (18 157 ± 853 c.p.m.), [^{3}H]CLC (223 ± 7 c.p.m.) and [^{3}H]GTP (19 514 ± 706 c.p.m.) retained on filter stacks in the absence of tubulin have been respectively substracted from the results in (A), (B) and (C). Bars: means ± SD (n=2). [a]Not different from control; [b]$p<0.05$, smaller than control; [c]$p<0.025$ and [d]$p<0.05$, greater than control.

the interactions of tubulin with nucleotides and vinca alkaloids.

Cytotoxicity of $GaCl_3$

The striped area at 100% in Figure 6 represents the control viability of L1210 cells growing in culture at days 1, 2, 3 and 4. The ability of $GaCl_3$ to decrease the viability of leukemic cells over a 4-day period is clearly concentration dependent between 16 and 625 μM, and the effectiveness of each of these cytotoxic concentrations of $GaCl_3$ clearly increases over time (Figure 6). For example, the antileukemic activities of 40 and 16 μM concentrations of $GaCl_3$ become only apparent after 3 and 4 days in culture, respectively (Figure 6). Moreover, the 100 μM concentration of $GaCl_3$ is ineffective at day 1 but reduces the viability of L1210 cells at days 2, 3 and 4 by 24, 82 and 94%, respectively (Figure 6). These results, therefore, suggest that the effectiveness of $GaCl_3$ as an inhibitor of tumor cell viability *in vitro* is a combination of drug concentration and duration of action. After a 24-h delay, the concentrations of $GaCl_3$ that reduce by 50% (IC_{50}) the viability of untreated leukemic cells in control wells at 2, 3 and 4 days are about 175, 35 and 16 μM, respectively (Figure 6). Under similar conditions, however, 0.64 and 0.04 μM concentrations of VCR already decrease L1210 cell viability at day 2 by 89 and 73%, respectively, and the cytotoxicity of VCR at day 4 is characterized by an IC_{50} value around 5 nM (data not shown), suggesting that $GaCl_3$ is an antitumor agent about 3200 times less potent than VCR.

Antimitotic activity of $GaCl_3$

In relation with their ability to block MT assembly (Figure 2) and slowly decrease tumor cell viability (Figure 6), concentrations of $GaCl_3$ of 100 μM or higher consistently increase the percentage of mitotic cells after 2–4 days of culture *in vitro* (Table 1). After a 24 h delay, 625 μM $GaCl_3$, which inhibits maximally tubulin polymerization in the turbidity assay (Figure 2), produces 1.8-, 4.0- and 10.5-fold increases in the mitotic index of L1210 cells at 2, 3 and 4 days, whereas 40 μM $GaCl_3$, a concentration ineffective against tubulin polymerization in a cell-free assay (Figure 2),

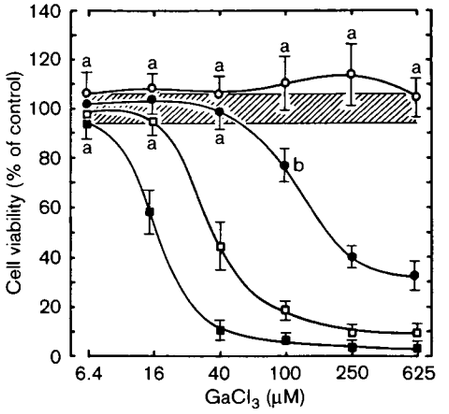

Figure 6. Concentration–response curves for the cytotoxic effect of $GaCl_3$ on L1210 cells at days 1 (○), 2 (●), 3 (□) and 4 (■). Cells were seeded in triplicate at an initial density of 300 000, 75 000, 18 750 or 4687 cells/0.5 ml/well in RPMI 1640 medium, containing 7.5% fortified bovine calf serum and penicillin (100 IU/ml)–streptomycin (100 μg/ml), and respectively grown for 1, 2, 3 or 4 days in the presence or absence (control) of $GaCl_3$ in a humidified incubator at 37°C with 5% CO_2 in air. The ability of viable cells/0.5 ml/well to bioreduce 0.1 ml of MTS:PMS (20:1) reagent over a 3 h incubation period at 37°C was assessed by measuring the absorbance of the water-soluble formazan products at $A_{490\ nm}$. Cell viability results are expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells (100±6%; striped area) at days 1 (1.022±0.061), 2 (1.308±0.081), 3 (1.451±0.084) and 4 (1.179±0.067). Blank values ($A_{490\ nm}$=0.195, 0.194, 0.210 and 0.150 at days 1, 2, 3 and 4) for culture medium supplemented with MTS:PMS reagent have been substracted from the results. The concentrations of $GaCl_3$ are plotted on a logarithmic scale. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.01$, smaller than control.

is largely unable to raise the mitotic index of cells in culture (Table 1). In contrast, the antitubulin (Figure 2) and cytotoxic concentration of 0.25 μM VCR produces a rapid 23.6-fold increase in the percentage of mitotic cells at 24 h and the mitotic index of these VCR-treated leukemic cells remains elevated at 2–4 days (Table 1). Because it blocks tubulin polymerization and increases the mitotic index, the antitubulin agent $GaCl_3$ may also arrest cell cycle progression in M phase but its antimitotic and cytotoxic activities are much slower and weaker than those of VCR.

Discussion

Decreased cell viability after several days of drug treatment may be a better predictor of anticancer activity than antiproliferation, since growth delay may allow survivors to resume dividing to form tumors once the drug is metabolized and its effect is waning.[56] However, delayed cytotoxic/cytostatic effects may develop in $GaCl_3$-treated tumor cells several days after cessation of treatment.[57] Since the concentrations of $GaCl_3$ of 100 μM or higher that inhibit tubulin polymerization and the binding of CLC to tubulin in cell-free assays also increase the percentage of mitotic cells and decrease the viability of leukemic cells after a 24 h delay *in vitro*, the weak antitubulin and slow antimitotic effects of $GaCl_3$ might contribute, at least in part, to its antiproliferative and antitumor activities. The cytotoxicity of $GaCl_3$ in L1210 cell culture suggests that the inability of $Ga(NO_3)_3$ injected i.p. to decrease the growth of L1210 and other leukemias

Table 1. Comparison of the abilities of $GaCl_3$ and the known MT de-stabilizing anticancer drug VCR to increase the mitotic index of L1210 cells *in vivo*

Drug treatment[a] (μM)	24 h		48 h		72 h		96 h	
	Mitotic cells[b] (%)	Mitotic index[c]	Mitotic cells[b] (%)	Mitotic index[c]	Mitotic cells[b] (%)	Mitotic index[c]	Mitotic cells[b] (%)	Mitotic index[c]
Control	1.28±0.16		1.49±0.09		2.35±0.21		1.19±0.13	
VCR (0.25)	30.20±1.96	23.59	19.85±1.63	13.32	30.62±6.71	13.03	11.11±1.87	9.34
$GaCl_3$ (40)	1.44±0.08[d]	1.13	1.56±0.08[d]	1.05	2.57±0.86[d]	1.09	1.69±0.29[e]	1.42
$GaCl_3$ (100)	1.49±0.32[d]	1.16	2.17±0.43[e]	1.46	3.29±0.57[e]	1.40	2.38±0.43[f]	2.00
$GaCl_3$ (250)	1.66±0.51[d]	1.30	2.43±0.59[e]	1.63	5.59±1.72[e]	2.38	3.78±0.94[g]	3.18
$GaCl_3$ (625)	2.13±0.76[d]	1.66	2.71±0.52[f]	1.82	9.36±2.94[f]	3.98	12.44±4.76[f]	10.45
$GaCl_3$ (1562.5)	2.98±1.04[e]	2.33	6.10±1.18[h]	4.09	27.56±12.40[f]	11.73	15.57±2.80[h]	13.08

[a]L1210 cells were seeded in triplicate at an initial density of 1 000 000, 250 000, 62 500 or 15 625 cells/0.5 ml/well and respectively incubated for 1, 2, 3 or 4 days at 37 C in the presence or absence (control) of the indicated concentrations of drugs.
[b]Results are expressed as percentage of a total of 500 cells/slide scored for mitotic figures (means±SD, n=3).
[c]Percentage of mitotic cells in drug-treated cultures divided by the percentage of mitotic cells in vehicle-treated controls.
[d]Not different from control.
[e]$p<0.05$, [f]$p<0.025$, [g]$p<0.01$ and [h]$p<0.005$, greater than control.

in mice *in vivo* might be due to the failure of these protocols to deliver sustained therapeutic doses of Ga.[25] The concentration and time dependency for the antitubulin, antimitotic and cytotoxic effects of $GaCl_3$ reported in the present study substantiate and extend previous findings concerning the bioactivity of Ga compounds *in vitro*. The growth inhibition by graded concentrations of Ga depends on the time of exposure.[38] A 24 h delay is also required for Ga to block DNA synthesis, arrest cells in S phase and inhibit tumor cell growth. In agreement with the antitubulin (IC_{50}: 125 μM) and cytotoxic (IC_{50}: 175, 35 and 16 μM at 2, 3 and 4 days) effects of $GaCl_3$ observed in our study, IC_{50} values of 21–200 μM have been reported for the antiproliferative activity of Ga after 2–3 days, which is maximal at 480 μM.[38,39,43,57] Interestingly, Ga inhibits DNA synthesis,[38] whereas few of the known antimitotic drugs tested at 10 μM in mammary tumor cell culture can do so.[47] Even though it is a much weaker MT-disrupting agent than other antimitotic drugs, Ga might be a more versatile anticancer compound able to target a wider range of molecular events and affect several phases of the cell cycle because of its ability to inhibit ribonucleotide reductase activity and DNA synthesis, with a consequent accumulation of cells in S phase.[38] Ga is perhaps a bifunctional anticancer drug with a self-limiting mechanism of action: by arresting in S phase the tumor cells of an unsynchronized population, Ga might limit the fraction of tumor cells entering M phase and susceptible to MT disruption.

The Vinca alkaloid VCR is a spindle poison which binds to tubulin, prevents MT assembly, causes metaphase arrest and kills cells attempting mitosis.[44,58] The IC_{50} values for the cytostatic/cytotoxic effects of VCR and VBL in different cell lines are in the 1.1–14 nM range.[44,47] A 4 day treatment with 16 μM $GaCl_3$ can reduce L1210 cell viability by 50% but is still about 3200 times less potent than VCR on an equal molecular basis. Although the polymerization of tubulin is assayed in a cell-free turbidity assay, VCR reduces L1210 cell viability with an IC_{50}=5 nM, which is 30 times smaller than the IC_{50}=0.15 μM required for this antimitotic drug to block MT assembly. Similarly, $GaCl_3$ reduces L1210 cell viability at 3–4 days with IC_{50} values of 35–16 μM, which are 3.6–7.8 times smaller than the IC_{50}=125 μM required for this metal salt to inhibit tubulin polymerization. The fact that the concentrations of antimitotic agents effective in the tubulin polymerization assay are consistently higher than those with cytostatic/cytotoxic activities has been noticed before.[44,47,49] Antimitotic drugs interacting with a few essential sites in the MTs might disrupt the mitotic spindle and be cytostatic/cytotoxic over a 3–4 day period at concentrations much lower than those required to directly block the rate of glycerol/Mg^{2+}-induced tubulin polymerization in a cell-free turbidity assay.[49] Indeed, mitotic arrest occurs when less than 5% of the cellular tubulin is complexed by CLC.[44] Moreover, other molecular alterations besides MT disruption might contribute to the overall cytostatic/cytotoxic actions of these antimitotic drugs.

Tubulin is a labile protein, which is unstable below 80 mM PIPES, should not be exposed to pH<6.8 or pH>7.0 and will not polymerize in the presence of Ca^{2+}.[46] The propensity of tubulin subunits to assemble into MTs is dependent upon their affinity for MT ends. In order to achieve MT assembly, the value of this affinity (called CC) has to be less than the total concentration of free tubulin subunits.[46] GTP and Mg^{2+} are necessary for tubulin nativity and glycerol stabilizes tubulin, and lowers the CC required to initiate polymerization.[46] Paclitaxel, which also lowers the CC and eliminates the requirement for GTP, promotes tubulin polymerization in the absence of glycerol and stabilizes MTs by inhibiting their depolarization.[44,59] The effect of paclitaxel on tubulin minus glycerol and the effect of glycerol on tubulin, therefore, can be used to screen for MT-stabilizing and destabilizing drugs.[44] A short lag phase is necessary to create nucleation sites, which are small tubulin oligomers from which larger MT polymers can form. Because MT polymerization is readily reversible, a given population of MTs is continually growing and shortening, a phenomenon called dynamic instability.[46] Thus, the control growth phase observed between 200 and 600 s in Figure 1(B) reflects the rapid increase in the ratio of MT assembly:disassembly in the presence of glycerol. Finally, a steady phase is established when the residual concentration of free tubulin heterodimer becomes equal to the CC required to initiate polymerization.[46] Of course, the rate and plateau of paclitaxel-induced tubulin polymerization are faster and higher in Figure 1(A) since this drug blocks MT disassembly. The kinetics of paclitaxel- or glycerol-induced MT assembly shown in Figure 1 appear consistent with the initial concentration of 2.2 mg tubulin/ml used in our reactions.

In contrast to the MT-stabilizing drug paclitaxel,[44,59] an effective cytotoxic concentration of $GaCl_3$ (250 μM) cannot induce the polymerization of tubulin minus glycerol, suggesting that this antimitotic agent neither promotes MT assembly nor blocks tubulin depolymerization and MT disassembly. However, cytotoxic concentrations of $GaCl_3$ prevent the binding of [^{3}H]CLC to tubulin, and inhibit the rate and plateau of glycerol/Mg^{2+}-induced tubulin polymerization, de-

monstrating that this metal salt blocks MT assembly like the known MT de-stabilizing drugs that interact with the CLC binding site of tubulin.[44] However, at 125 μM the IC_{50} value for the antitubulin effect of $GaCl_3$ is well above the following ranges of IC_{50} values reported for known MT de-stabilizing drugs using the turbidity assay: CLC, 0.2–20 μM; nocodazole, 1–5 μM; podophyllotoxin, 0.3–3 μM; tubulozole-C, 0.3–0.5 μM; and VCR, 0.1–2 μM.[44,47,49,60,61] The superior inhibition of tubulin polymerization caused by tubulozole-C in our study confirms the reports that tubulozole-C is more effective against MT assembly, DNA synthesis and tumor cell growth than CLC, nocodazole and VCR.[44,47,60–63] The weak antitubulin effect of Ga is probably not limited by the 1 mM concentration of EGTA used in the turbidity assay since chelating agents structurally similar to EGTA, such as Na_2Ca-ethylenediaminetetraacetate and Na_3Ca-diethylenetriaminepentaacetate, are ineffective antidotes against acute Ga^{3+} and Al^{3+} intoxication.[64]

Ga^{3+} is unlikely to displace Mg^{2+} to inhibit glycerol/Mg^{2+}-induced tubulin polymerization since the antitubulin effect of $GaCl_2$ persists in the presence of 36 times more $MgCl_2$, suggesting that the exogenous Mg^{2+} cations absolutely required for the binding of GTP to tubulin and MT assembly cannot overcome the antitubulin action of Ga^{3+} ions of a higher valence. $GaCl_3$ might inhibit tubulin polymerization as a consequence of its interaction with the CLC binding site but its ability to dramatically enhance the binding interactions of VBL and GTP with tubulin suggests that $GaCl_3$ also induces conformational changes at other sites in the α/β tubulin dimer. Although they do not affect the binding of GTP to tubulin, most CLC site binding agents enhance tubulin-dependent GTP hydrolysis.[44] CLC binds tightly to a site on the tubulin dimer, which is not exposed when the dimer is assembled into MTs *in vitro*.[44,46] CLC site binding agents induce conformational changes which promote the fluorescence of tubulin.[65] Free sulfhydryl (SH) groups are essential for MT assembly and may be used as potential probes for characterizing drug binding sites, based on their reactivity with the alkylating agent iodo[^{14}C]acetamide.[66,67] CLC-induced conformational changes of tubulin block SH groups and prevent cysteine cross-linking.[66] The hypothesis that conformational changes caused by the interaction of $GaCl_3$ with tubulin might alter the fluorescence of this protein, the alkylation of SH groups by iodo[^{14}C]acetamide and the hydrolysis of exchangeable GTP[54] remains to be investigated. When MT assembly is prevented in CLC- or nocodazole-treated cells, the level of unpolymerized tubulin is increased and this, in turn, inhibits the formation of new tubulin mRNA while the pre-existing message decays rapidly.[68] Since Ga inhibits DNA and RNA polymerases,[26,27,37] its ability to affect the level of translatable tubulin mRNA and tubulin synthesis should be studied.

The complex interactions of tubulin with several, mostly divalent, cations has been reviewed.[44] Tubulin is able to bind them at both high- and low-affinity sites, with as many as 60 reported.[44] Perhaps Ga^{3+} inhibits polymerization by saturating the regulatory C-terminal domains of each α and β subunits containing high-affinity Ca^{2+} binding sites, which normally block polymerization when bound to Ca^{2+} in the absence of EGTA.[44,46] However, the high-affinity Ca^{2+} sites located between amino acids 418 and the carboxy-terminals are distinct from the CLC, VBL and exchangeable nucleotide sites. High concentrations of Hg^{2+}, Cu^{2+} and Cd^{2+} inhibit MT assembly and substantially reduce the free SH content of tubulin.[47,69] $CdCl_2$, a SH-binding compound, partially inhibits tubulin polymerization at 1000 μM. High concentrations of Sr^{2+} and Ba^{2+}, like Ca^{2+}, also inhibit MT assembly and cause disassembly of preformed MTs.[44] In contrast, Al^{3+}, Zn^{2+}, Mn^{2+} and Co^{2+} all induce tubulin polymerization, some of these cations even promoting lateral tubulin–tubulin interactions between protofilaments to form extensive sheet polymers that are wider and shorter than MTs.[44]

Based on its ability to disrupt MT dynamics, $GaCl_3$ would be expected to arrest cells in G_2/M phase. The mitotic index can differentiate between the antimitotic drugs that cause G_2 or M phase arrest. Agents that arrest cells in M phase, such as VCR, increase the mitotic index but agents that cause G_2 arrest, such as etoposide (VP-16), decrease it.[53] Since $GaCl_3$ increases the percentage of mitotic figures and the mitotic index after 24 h, it may be capable of causing metaphase arrest and blocking the progression of L1210 cells in the M phase of their cycle, although to a much slower pace and lesser degree than VCR. The fact that Ga increases the proportion of murine[11] and human leukemic cells[38] in the G_0/G_1 and S phases of their cell cycle at 24 h might explain why, in contrast to the large and early increase in mitotic index caused by VCR at 24 h, only a much smaller fraction of Ga-treated L1210 cells displaying mitotic figures can slowly accumulate after 48–96 h in Table 1. Since the CLC site binding agent $GaCl_3$ also enhances the interaction of VBL with tubulin, it would be of interest to determine if the combination of Ga and VCR has more antitumor activity than either drug alone.

Conclusion

Ga may be a bifunctional antitumor agent, which not only interferes with Fe-containing enzymes to inhibit

DNA synthesis in S phase but also interacts with tubulin subunits to block MT assembly in M phase and slowly exert its antiproliferative and cytotoxic activities. The antitubulin action of $GaCl_3$ might differ from that of standard CLC site binding agents and be valuable to boost the effectiveness of combination chemotherapy in multidrug-resistant cells.[43]

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