Motexafin Gadolinium: A Redox Active Drug That Enhances the Efficacy of Bleomycin and Doxorubicin

Richard A. Miller,1 Kathryn W. Woodburn, Qing Fan, Intae Lee, Dale Miles, George Duran, Branimir Sikic, and Darren Magda

ABSTRACT
The effect of motexafin gadolinium (MGd), a redox mediator, on tumor response to doxorubicin (Dox) and bleomycin (Bleo) was investigated in vitro and in vivo. MES-SA human uterine sarcoma cells were studied in vitro using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay. Rif-1, a murine fibrosarcoma cell line, was studied using a clonogenic survival assay. Tumor growth delay assays were performed using the EMT-6 murine mammary sarcoma cell line in BALB/c mice. MGd (25–100 μM) produced dose-dependent enhancement of Bleo cytotoxicity to MES-SA cells. The IC50 for Bleo was reduced by ~10-fold using 100 μM MGd. In clonogenic assays using Rif-1 cells, MGd enhanced the activity of Bleo ~1000-fold. This effect was shown to be mediated, in part, by MGd inhibition of potentially lethal damage repair. MGd enhanced the tumor response to bleomycin and Dox in vivo. MGd had no significant effect on the systemic exposure to Dox (expressed in terms of the plasma area under the curve, 0–24 h) and did not increase Dox myelosuppression. MGd enhanced the effectiveness of the redox active drugs, Bleo and Dox.

INTRODUCTION
The development of agents that improve or enhance the efficacy of cancer chemotherapy is an important area of research in medical oncology. Some of the approaches have involved the use of drugs that modify the metabolism or inhibit the efflux of cancer drugs from cells. Despite intense efforts in this area, chemosensitization of tumors has met with limited clinical success (1–3).

The texaphyrins are metal cation-binding macrocycles that bear strong resemblance to naturally occurring porphyrins. For instance, it has now been demonstrated in both animal tumor models and human clinical trials that texaphyrins accumulate selectively in cancers, similar to certain porphyrins (4–7). However, texaphyrins are far easier to reduce (more electron affinity) than typical metalloporphyrins with a half-wave potential of approximately −50 mV (versus normal hydrogen electrode; Ref. 8). These properties have led to the consideration that metallotexaphyrins might function as radiation-enhancing agents. Animal tumor model studies have demonstrated that the texaphyrin, MGd,2 selectively accumulates in cancers and renders tumors more responsive to ionizing radiation (4, 7, 9). Promising Phase II clinical results also have been reported in patients receiving radiation therapy for brain metastases (6, 10). In human Phase I and II studies, selective tumor localization of MGd has been confirmed using magnetic resonance imaging, which can detect the drug based on its paramagnetic properties (5, 6, 10).

Biological redox reactions are central to metabolism and cellular energy production. Recent studies with MGd have indicated that it catalytically reacts with various intracellular reducing metabolites (antioxidants), such as ascorbate and NADPH, to produce hydrogen peroxide and other reactive oxygen species (11). We hypothesize that the depletion of intracellular reducing metabolites and resulting bioenergetic disruption because of futile redox cycling would lead to increased tumor response to both radiation therapy and chemotherapy. This could explain the results of Bernhard et al. (12), who failed to observe radiation enhancement with MGd. In this report, we describe the effects of MGd on tumor response to two chemotherapy drugs, Bleo and Dox. These chemotherapeutic agents were selected because they are redox active and known to generate reactive oxygen species within cells (13, 14).

MATERIALS AND METHODS
Chemicals and Cell Lines. The synthesis and chemical characterization of MGd has been described previously (8). Dox and Bleo were obtained from Sigma Chemical Co. (St. Louis, MO).

The development and characterization of the human cell line, MES-SA, derived from sarcoma elements of a uterine mixed Mullerian tumor, have been described previously (15). Monolayer cultures of MES-SA cells were grown in McCoy’s 5A medium supplemented with 10% FCS, 25 mM HEPES (Life Technologies, Inc., Grand Island, NY), 2 mM l-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Sigma). Murine EMT-6 mammary sarcoma and Rif-1 radiation-induced fibrosarcoma cell lines were maintained through established in vivoin vitro propagation procedures (16). Rif-1 cells were selected because they are redox active and known to generate reactive oxygen species within cells (13, 14).

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1 To whom requests for reprints should be addressed, at Pharmacyclics, Inc., 995 East Arques Avenue, Sunnyvale, CA 94085. Phone: (408) 774-0330; Fax: (408) 774-0340.

2 The abbreviations used are: MGd, motexafin gadolinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bleo, bleomycin; Dox, doxorubicin; PLDR, potentially lethal damage repair.
were grown in RPMI 1640 supplemented with 15% FCS and penicillin/streptomycin. EMT-6 cells were grown in Waymouth’s medium (MB752/1; Life Technologies, Inc.) supplemented with 15% FCS and penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

**In Vitro MTT Cytotoxicity Assays.** MES-SA cells were allowed to adhere to 96-well microtiter plates (4000 cells/well) overnight in 180 µl of McCoy’s 5A medium. A freshly prepared stock solution of Bleo in medium (60 µl) was serially diluted (1:4) down the plate, discarding the final 60 µl. Stock solutions of MGd (1 mM, 750 µM, 500 µM, or 250 µM diluted in medium and 5% mannitol) were added to the plates to give a final volume of 200 µl. Final mannitol concentration was 0.25% in all wells. The plates were incubated at 37°C in a 5% CO2 atmosphere. Medium was exchanged after 24 h, and the plates were incubated an additional 48 h prior to analysis for viability using the tetrazolium dye MTT (17). In brief, a 5-mg/ml solution of dye in PBS (20 µl/well) was added, and after 2 h the medium was removed and replaced with 100 µl of isopropanol to dissolve the formazan crystals formed by the cells. The plate was read at a test wavelength of 570 nm and a reference wavelength of 650 nm on a multiwell spectrophotometer (ThermoMax; Molecular Devices, Sunnyvale, CA). Each concentration of drug was tested in quadruplicate. The percentage of drug-treated cells to that of the control. Data were adjusted to normalize for effect of MGd treatment alone, which produced 15–45% cytotoxicity in the range of MGd concentrations tested. Experiments with Dox were carried out by serial dilution as above, an exchange of medium after 8 h, followed by addition of MGd for 13 h. Medium was exchanged, and cells were incubated for an additional 2 days as above.

**In Vitro Clonogenic and PLDR Assays.** Rif-1 cells (200 to 105) were plated into 25-cm2 plastic tissue culture flasks and allowed to adhere for 24 h. Bleo and MGd were added to final concentrations of 0.88–4.4 and 50 µM, respectively. After a 4- or 24-h drug exposure, the cells were washed twice with Hank’s solution, replenished with complete medium, and returned to the incubator for an additional 6–10 days. The colonies were fixed with 10% buffered formalin and stained with crystal violet for quantitation (plating efficiencies were 43–50%). To evaluate the effects on PLDR, cultures of Rif-1 (4 × 105 cells/flask) were treated with Bleo and MGd for 18 h, washed with fresh medium, and then treated with trypsin either immediately or after incubation in Hank’s solution at room temperature for a period of 4 h. Cells were counted and replated, and colonies were determined after further incubation as above (plating efficiencies were 34–46%). To study the effect of drug sequence, Rif-1 cells in medium were exposed under ambient conditions to the following conditions: (a) Bleo for 4 h, rinsed, and then treated with MGd for 4 h; (b) MGd for 4 h, rinsed, and then treated with Bleo for 4 h; (c) both drugs together for 4 h; (d) Bleo alone in medium containing 0.125% mannitol (drug vehicle) for 4 h; (e) Bleo alone for 4 h, rinsed, and then treated with medium containing 0.125% mannitol for 4 h. Colonies were determined after further incubation as above (plating efficiencies were 32–44%). MGd alone had no effect on plating efficiency.

**In Vivo Tumor Models.** All animals received care in compliance with Guide for the Care and Use of Laboratory Animals (NIH Publication, 1996). Female BALB/c mice, 7–8 weeks of age, were obtained from Simonsen Laboratories (Gilroy, CA). EMT-6 cells (5 × 105) were injected s.c. into the right hind flanks. Animals were studied 10–14 days after inoculation with tumor cells when the tumors had reached 5–7 mm diameter and a depth of 2.5–3.5 mm. Tumor volume, measured three times/week using a caliper, was calculated assuming the conformation of a hemiellipsoid (16).

The dose of Dox was selected to produce an ∼50% reduction in tumor growth compared with untreated controls. Dox (7.5 mg/kg), Bleo (10 or 20 units/kg), and/or MGd (0.5–40 µmol/kg) were administered i.v. on days 0, 7, and 14. Control animals received either no treatment, Dox, Bleo, or MGd. Kaplan-Meier analyses were performed by scoring events as the time for the tumor to reach four times the pretreatment volume. A log-rank test was used to determine statistical significance.

**Pharmacokinetic Measurements.** The plasma pharmacokinetics of MGd and Dox was evaluated in 11-week-old male Sprague Dawley rats (Simonsen Laboratories) weighing 295–358 g. Each rat received injections i.v. via the tail vein with either a single administration of 20 µmol/kg MGd, a single administration of 1.3 mg/kg Dox, or an administration of 1.3 mg/kg Dox, followed by an administration of 20 µmol/kg MGd 1 h later. The Dox and MGd infusions were delivered over 30 s and 1 min, respectively. Blood was drawn into EDTA collection tubes by retroorbital bleeding under methoxyflurane anesthesia (Schering-Plough, Kenilworth, NJ). For each drug or drug combination, the combined data from three subgroups of rats (4 rats/subgroup) was used to characterize the 48-h postdosing period.

MGd and Dox concentrations in plasma were measured by high-performance liquid chromatography. MGd was analyzed using a modified technique based on the method of Parise et al. (18). Dox was assayed using a modified procedure based on the method of Robert (19), Camaggi et al. (20), and Pfeiffer et al. (21). Standards for both assays were prepared by adding known amounts of analyte to blank rat plasma. For both MGd and Dox, plasma concentrations at 48 h were below the lower limit of quantitation.

**Hematological Toxicity.** BALB/c mice receiving Dox (7.5 mg/kg) and MGd (40 µmol/kg) administered on days 0, 7, and 14 were compared with untreated animals and animals receiving Dox alone. There were 8 animals in each group, and complete blood counts were measured on days 14 and 28 by the Cell-dyn system (Abbott Laboratories).

**RESULTS**

**In Vitro Studies with Bleomycin.** MES-SA cells were treated in vitro with a range of concentrations of Bleo. The cytotoxicity of Bleo to MES-SA cells was enhanced by concomitant treatment with 25–100 µM MGd as shown in Fig. 1 using an MTT assay. The IC50 (50% inhibitory concentration) for Bleo was reduced by ~1 log using 100 µM MGd. These results demonstrate dose-dependent MGd enhancement of the in
vitro cytotoxicity of Bleo to MES-SA cells. Similar results were obtained using Rif-1 cells (data not shown).

Enhancement of the cytotoxic activity of Bleo also was studied using the Rif-1 tumor cell line in a clonogenic survival assay (Fig. 2). Plated cells were incubated with MGd for 4 or 24 h with increasing concentrations of Bleo. These data indicated that MGd enhanced the activity of Bleo ~1000-fold in this assay. In another experiment, cultures were incubated with MGd and Bleo and then treated with trypsin and plated, either immediately or after a 4-h “recovery” period. Improved survival was observed in control cultures allowed to recover for 4 h but not in cultures treated with MGd (Fig. 3). This suggests that PLDR was inhibited by MGd. In other experiments, drug treatment sequence was studied by treating Rif-1 cultures with Bleo and MGd for 4 h together or sequentially, after medium exchange (Fig. 4). No change in survival was observed when MGd was added after Bleo treatment. Conversely, the surviving fraction decreased considerably (~2 logs) when MGd was added 4 h before Bleo, although not to as great a degree as when the two drugs were added simultaneously (~3 logs).

Various in vitro studies with combinations of Dox and MGd were performed. These studies failed to demonstrate an enhancement of Dox activity. Instead, MGd displayed a dose-dependent cytoprotective effect on MES-SA cells when added before or simultaneously with Dox (Fig. 5). This effect was not seen when MGd was added after Dox. Similar results were found using Rif-1 cells (data not shown). The origins of this in vitro antagonism are unknown but could arise from interactions involving Dox and MGd directly or through the intermediacy of reducing metabolites present in the tissue culture medium (thiols, ascorbate, NADPH, and others). An analogous dependence on the scheduling of drug administration was not observed in vivo (see below).
Chemosensitization with Motexafin Gadolinium

MGd Enhances in Vivo Tumor Response to Bleo. MGd was found to enhance the effect of Bleo in vivo using a tumor growth assay. In these studies, the two drugs were administered within 5 min of each other. As shown in Fig. 6, animals receiving 20 μmol/kg of MGd with each of three weekly doses of Bleo showed significant delay in EMT-6 tumor growth compared with animals receiving Bleo alone. The median time required for tumors to grow to four times their original volume was increased to 17.7 days in animals receiving MGd in combination with a Bleo dose of 10 units/kg compared with 12.3 days for animals receiving Bleo alone \((P = 0.008)\). MGd combined with 20 units/kg Bleo delayed tumor growth to 20.8 days \((P = \text{0.015})\,\text{compared with 20 units/kg Bleo alone)}\).

MGd Enhances in Vivo Tumor Response to Dox. Animals bearing established EMT-6 tumors were treated with Dox on days 0, 7, and 14. Each Dox injection was followed by administration of MGd within 10 min. At MGd doses of 0.5 and 2.5 μmol/kg, some enhancement of tumor response to Dox was seen compared with animals receiving Dox alone. At doses of MGd of 5 μmol/kg or more, considerable enhancement of tumor response to Dox was observed (Table 1). No toxicity was observed with the MGd doses used.

We studied the effect of drug treatment sequence on the enhancement of tumor response to Dox. As shown in Table 2, a dose of 5 μmol/kg of MGd enhanced the tumor response to Dox when given either before or after Dox.

### Table 1: Dose-response effect on enhancement of tumor response to Dox by MGd

<table>
<thead>
<tr>
<th>MGd (\mu\text{mol/kg)}</th>
<th>\text{MRT (days)}^b</th>
<th>\text{No. of animals}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.8 ± 1.6</td>
<td>24</td>
</tr>
<tr>
<td>0.5</td>
<td>23.5 ± 9.0^b</td>
<td>7</td>
</tr>
<tr>
<td>2.5</td>
<td>21.9 ± 5.7</td>
<td>7</td>
</tr>
<tr>
<td>5.0</td>
<td>30.2 ± 5.4^b</td>
<td>19</td>
</tr>
<tr>
<td>20</td>
<td>32.2 ± 7.1^b</td>
<td>6</td>
</tr>
<tr>
<td>40</td>
<td>33.4 ± 7.9^b</td>
<td>18</td>
</tr>
</tbody>
</table>

^a Animals with established EMT6 tumors were given 7.5 mg/kg Dox and various doses of MGd. Treatment was once a week for 3 weeks. MGd was given 5–10 min after administration of Dox.

^b The mean regrowth time (MRT) is the number of days, ± SD, for the tumor to reach four times the pretreatment volume.

^c \(P < 0.05\) compared with Dox alone.

### Table 2: Effect of schedule on enhancement of tumor response to Dox by MGd

<table>
<thead>
<tr>
<th>Schedule^a</th>
<th>\text{MRT (days)}^b</th>
<th>\text{No. of animals}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox + MGd, 10 min</td>
<td>30.2 ± 5.5^c</td>
<td>19</td>
</tr>
<tr>
<td>Dox + MGd, 5 h</td>
<td>33.2 ± 6.9^c</td>
<td>6</td>
</tr>
<tr>
<td>MGd + Dox, 5 h</td>
<td>27.9 ± 10.8^c</td>
<td>8</td>
</tr>
<tr>
<td>MGd alone</td>
<td>9.6 ± 1.4</td>
<td>8</td>
</tr>
<tr>
<td>Dox alone</td>
<td>18.4 ± 7.9</td>
<td>26</td>
</tr>
<tr>
<td>No treatment</td>
<td>10.8 ± 3.5</td>
<td>6</td>
</tr>
</tbody>
</table>

^a Animals with established EMT6 tumors were given 7.5 mg/kg Dox and/or 5 μmol/kg of MGd. Treatment was given once a week for 3 weeks.

^b The mean regrowth time (MRT) is the number of days, ± SD, for the tumor to reach four times the pretreatment volume.

^c \(P < 0.05\) for the MGd treatment groups plus Dox compared with Dox alone.
calculated from the log-transformed data. Fraction of 20 profiles for Sprague Dawley rats receiving either a single i.v. administration of 20 μmol/kg i.v. dose of MGd.

The studies presented here have important implications regarding the mechanism of action of MGd. These findings also suggest potential novel uses for this drug in cancer treatment as an enhancer of chemotherapy or chemosensitizer. MGd high electron affinity and has been shown to react with various species have been shown to enhance radiation response and to be proapoptotic through release of cytochrome c from mitochondria (22–26). Cellular reducing metabolites, or antioxidants, such as glutathione, ascorbate, and NADPH protect the cell from the damaging effects of reactive oxygen species (27–29).

MGd enters tumor cells and enhances tumor response to ionizing radiation (4, 7, 9). Ionizing radiation induces the formation of hydroxyl radicals, placing the cells under oxidative stress (30). These radicals damage DNA, which is believed to be the culprit cytotoxic event. The depletion of reducing metabolites and production of reactive oxygen species caused by MGd may render the cells more susceptible to oxidative damage and lower the threshold for apoptosis.

Cytotoxic chemotherapy agents may lead to the generation of reactive oxygen species within cells and cause oxidative stress. Bleo and Dox are two agents known to generate reactive oxygen species (13, 14). Bleo is a glycopeptide that chelates iron inside the cell and binds to DNA. In reactions involving Fe(II) and oxygen, an “activated” Bleo species is formed that damages DNA through free radical intermediates (31). Superoxide and hydrogen peroxide can also react with Fe(II) or Fe(III) bleomycin, respectively, to produce the activated form of the drug. DNA damage from Bleo and ionizing radiation is similar both in induction and repair (32). Dox has several modes of action, including DNA double- and single-strand breakage associated with DNA intercalation and inhibition of topoisomerase II (33). Dox can also form complexes with iron and copper and produce cytotoxic reactive oxygen species (14). In the presence of reducing metabolites, Dox can undergo a one-electron reduction to the semiquinone radical. This radical can rapidly react with oxygen to form superoxide and, in the presence of Fe(II), highly reactive hydroxyl radicals (34). The generation of free radicals is also associated with mitochondrial membrane damage (35).

A dose-response effect was observed for MGd enhancement of tumor responsiveness to Bleo and Dox. The dose-response effect could be readily measured in vitro with Bleo (Figs. 1–4) assayed by either MTT viability or clonogenic survival using MES-SA or Rif-1 cells, respectively. The observed enhancement of Bleo activity may partly be attributable to inhibition of PLDR in cells oxidatively stressed by MGd. This assertion is supported by the data presented in Fig. 3, which show that the survival benefit attributable to delayed trypsin treatment was abrogated in MGd-treated cultures. However, the magnitude of the Bleo enhancement suggests that additional mechanisms must be operative that are unique to this agent. One possibility is that reactive oxygen species generated in the presence of MGd contribute to the formation of activated Bleo within cells, either by electron transfer or via the release of Fe(II) ions from intracellular storage sites (31). Another possible mechanism is increased membrane permeabilization by MGd. Electroporation of cells in culture has been reported to enhance Bleo activity by 3 orders of magnitude (36).

Interestingly, activity was enhanced when MGd was added before but not after exposure of cultures to Bleo (Fig. 4). This finding suggests that it may be possible to use the selective treatment was abrogated in MGd-treated cultures. However, the magnitude of the Bleo enhancement suggests that additional mechanisms must be operative that are unique to this agent. One possibility is that reactive oxygen species generated in the presence of MGd contribute to the formation of activated Bleo within cells, either by electron transfer or via the release of Fe(II) ions from intracellular storage sites (31). Another possible mechanism is increased membrane permeabilization by MGd. Electroporation of cells in culture has been reported to enhance Bleo activity by 3 orders of magnitude (36).

Interestingly, activity was enhanced when MGd was added before but not after exposure of cultures to Bleo (Fig. 4). This finding suggests that it may be possible to use the selective
tumor localization of MGd, obtained by its prior administration, to improve the therapeutic index of Bleo. It is important to note that differences in PLDR would not be detected in this experiment, because cells remain in growth phase. Consistent with the above results, administration of MGd and Bleo (MGd within 5 min of Bleo) led to increased tumor responsiveness in vivo (Fig. 6).

Dox activity was antagonized when MGd was added to cells before or simultaneously with Dox (Fig. 5). Addition of MGd after Dox had no effect on activity in vitro (also observed with Bleo). The source of antagonism is currently unclear. These findings could be explained based on a direct interaction between MGd and Dox or interference with cellular uptake in vitro. Alternatively, cells grown in tissue culture may not accurately represent tumor cell concentrations of redox active molecules such as thiols and ascorbate. These differences may mask the effects of redox active drugs such as MGd.

Indeed, as is shown in Table 2, MGd could be given before or after Dox administration in vivo. This suggests that the metabolic perturbations induced by MGd do not need to precede or follow Dox administration in vivo to reduce Dox activity. MGd synergizes with nonoverlapping toxicities will be best suited for use in combination.

These findings suggest important potential clinical uses for MGd as a chemotherapeutic enhancer. MGd selectively accumulates in tumor cells, suggesting the possibility that chemotherapy activity could be enhanced at the tumor but not in normal tissue, thereby increasing the therapeutic margin. Preclinical studies have shown that MGd is excreted primarily by the liver (4). In human clinical trials conducted to date, MGd has shown dose-limiting renal toxicity at 26 μmol/kg in a single dose escalation Phase I trial (5). In a Phase Ib/II trial in patients receiving whole brain radiation therapy for brain metastases, dose-limiting hematological toxicity has been seen at a dose of 8.4 mg/kg given daily for 10 days (6, 10). It is likely that chemotherapy drugs with nonoverlapping toxicities will be best suited for use in combination with MGd.

MGd is a novel redox active drug that may have diverse applications in cancer therapy. It enhances tumor responsiveness to radiation and certain chemotherapy drugs such as Bleo and Dox. Other drugs are currently being tested for use in combination with MGd, and the potential toxicity of combined use will need to be examined for each agent. The studies reported here support redox modulation and inhibition of PLDR as important mechanisms of action for MGd as an enhancer of radiation and chemotherapy. Moreover, these studies and the putative mechanism provide potential new directions for discovering other novel cancer drugs with unique mechanisms of action involving redox reactions in biological systems.

REFERENCES


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