Motexafin Gadolinium, a Tumor-selective Drug Targeting Thioredoxin Reductase and Ribonucleotide Reductase*

Seyed Isaac Hashemy1, Johanna S. Ungerstedt1, Farnaz Zahedi Avval, and Arne Holmgren2

From the Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, SE-171 77 Stockholm, Sweden

Motexafin gadolinium (MGd) is a chemotherapeutic drug that selectively targets tumor cells and mediates redox reactions generating reactive oxygen species. Thioredoxin (Trx), NADPH, and thioredoxin reductase (TrxR) of the cytosol/nucleus or mitochondria are major thiol-dependent reductases with many functions in cell growth, defense against oxidative stress, and apoptosis. Mammalian TrxRs are selenocysteine-containing flavoenzymes; MGd was an NADPH-oxidizing substrate for human or rat TrxR1 with a $K_m$ value of 8.65 μM ($k_{cat}/K_m$ of 4.86 x $10^4$ M$^{-1}$ s$^{-1}$). The reaction involved redox cycling of MGd by oxygen producing superoxide and hydrogen peroxide. MGd acted as a non-competitive inhibitor (IC50 of 6 μM) for rat TrxR. In contrast, direct reaction between MGd and reduced human Trx was negligible. The corresponding reaction with reduced Escherichia coli Trx was also negligible, but MGd was a better substrate ($k_{cat}/K_m$ of 2.23 x $10^5$ M$^{-1}$ s$^{-1}$) for TrxR from E. coli and a strong inhibitor of TrxR-dependent protein disulfide reduction. Ribonucleotide reductase (RNR), a 1:1 complex of the non-identical R1- and R2-subunits, catalyzes the essential de novo synthesis of deoxyribonucleotides for DNA synthesis using electrons from Trx and TrxR. MGd inhibited recombinant mouse RNR activity with either 3 μM reduced human Trx (IC50 2 μM) or 4 mM dithiothreitol (IC50 6 μM) as electron donors. Our results demonstrate MGd-induced enzymatic generation of reactive oxygen species by TrxR plus a powerful inhibition of RNR. This may explain the effects of the drug on cancer cells, which often overproduce TrxR and have induced RNR for replication and repair.

Texaphyrins are porphyrin-like synthetic macrocycles that form highly stable complexes with large metal cations (1). One such drug is motexafin gadolinium (MGd,3 Xcytrin®), a chemotherapeutic agent, undergoing clinical trials including phase III for the treatment of brain metastasis of lung cancer (2, 3). MGd (see Fig. 1), similar to porphyrins (4), selectively targets tumor cells by an unknown mechanism. Drug localization in tumors has been confirmed in animal models and in human clinical trials using magnetic resonance imaging, which detects the paramagnetic gadolinium ion (5). MGd is easily reduced (half-wave potential 50 mV versus normal hydrogen electrode), and in vitro, it may catalyze slow oxidation of intracellular reducing metabolites such as ascorbate, GSH, and NADPH, generating superoxide and reactive oxygen species (ROS) in a process called futile redox cycling (6). The drug is known to enhance treatment of tumor cells by radiation and cytotoxic drugs (7–9). Generation of ROS in myeloma cells and induction of apoptosis has been demonstrated (10). Recently, MGd has been shown to disrupt zinc metabolism and induce oxidative stress in human cancer cell lines (11). These studies showed increased levels of intracellular free zinc, increased metallothionein transcripts, inhibition of thioredoxin reductase, and cell death following incubation of human lung carcinoma cells with MGd in the presence of exogenous zinc acetate (11). These findings could possibly result from oxidation of vicinal thiols or generation of ROS, but the in vivo mechanisms are still largely unknown.

We hypothesized that MGd may target ribonucleotide reductase, thioredoxin (Trx), or thioredoxin reductase (TrxR) (12–14). All these enzymes have redox-active Cys residues and are central to cell growth and survival. The thioredoxin system, comprising Trx, TrxR, and NADPH, acts as a general protein disulfide reductase, where reduced Trx (Trx-(SH)$_2$) is used to reduce protein disulfides to protein thiols (protein-(SH)$_2$) and Trx-S$_2$ is reduced by TrxR using electrons from NADPH (13, 14). Reactions 1 and 2 are as follows.

**REACTION 1**

$$\text{Trx-(SH)}_2 + \text{Protein-S}_2 \rightarrow \text{Trx-S}_2 + \text{Protein-(SH)}_2$$

**REACTION 2**

$$\text{TrxR} \rightarrow \text{Trx-S}_2 + \text{NADPH} + H^+ \rightarrow \text{Trx-(SH)}_2 + \text{NADP}^+$$

The Trx system participates in a large number of biological functions via its protein disulfide reductase activity, involving thioredoxin peroxidases (peroxiredoxins), methionine sulfoxide reductases, regulation of transcription factors, ribonucleotide reduction, and DNA synthesis or selenium metabolism (14–18). Mammalian TrxR enzymes in the cytosol/nucleus (TrxR1) or mitochondria (TrxR2) are selenoproteins with a selenocysteine (Sec) in the active site (-Gly-Cys-Sec-Gly) and have a broad substrate specificity (19, 20). Increased levels of Trx have been reported in many different tumors with a correlation to malignancy and poor prognosis (21, 22). Inhibition of apoptosis by the Trx system has been suggested to be involved in drug resistance of tumor cells (23–26).

Ribonucleotide reductase (RNR), another target for antitumor therapy and a key S-phase enzyme reducing all four ribonucleotides to deoxyribonucleotides with electrons from Trx-(SH)$_2$, is essential for DNA synthesis (27, 28). A compound that interferes with the activity of RNR leads to the inhibition of DNA synthesis and cell proliferation.

In this report, we show that MGd is a substrate for mammalian TrxR,
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generating reactive oxygen species. It also acts as an inhibitor of Trx-dependent disulfide reduction by blocking reduction of Trx-S2. No reaction was found between Trx-(SH)2 and MGd. RNR was also strongly inhibited by MGd. We believe that these results are important to explain the mechanism of action of MGd on tumor cells.

EXPERIMENTAL PROCEDURES

Materials—MGd was obtained from Pharmacia, Inc (Sunnyvale, CA) as a stock solution dissolved in 5% aqueous mannitol at a concentration of 2.3 mg/ml (2 mM). Bovine serum albumin, DTNB, DTT, insulin, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. [3H]CDP and PD-10 Sephadex G-25 columns were from Amersham Biosciences. Amplex® Red hydrogen peroxide/peroxidase assay kit was from Molecular Probes. Escherichia coli Trx1 and TrxR (29), human Trx1, and the double mutant C61S/C72S were prepared as described (30). Calf thymus, human placenta TrxR1, as well as rat recombinant TrxR1 (31) with 50% specific activity due to a content of truncated enzyme were used. E. coli RNR (32) was a kind gift of Prof. Britt-Marie Sjöberg, and mouse R1 and R2 proteins of RNR were expressed and purified and the R2 protein activated as described by Thelander and co-workers (33, 34). The E. coli strains expressing the subunits were kind gifts from Prof. Lars Thelander, Umeå University.

Spectrophotometric Measurements—The spectra of 20 μM MGd, with and without 1 mM DTT, were recorded at 25 °C in 50 mM Tris-Cl, 1 mM EDTA, pH 7.5 (TE buffer), in 1-cm semimicro quartz cuvettes, using a Shimadzu UV2100 spectrophotometer. Other measurements were made with a recording Zeiss PMQ III spectrophotometer.

Oxidation of NADPH by MGd—Assays were carried out in a total volume of 0.50 ml, containing 100 μM NADPH, 50 mM Tris-Cl, 1 mM EDTA, pH 7.5, and different concentrations of MGd. MGd was excluded from the reference cuvette. The reaction was initiated by adding 0.2 μM rat TrxR or 0.015 μM E. coli TrxR to the reference and reaction cuvettes, after which NADPH oxidation was followed at 340 nm. A molar extinction coefficient of 6200 M−1 cm−1 was used in calculations.

Production of H2O2 by Redox Cycling of MGd—This assay was performed in TE buffer with 200 μM NADPH, 20 μM MGd, and 0.2 μM rat TrxR, under aerobic or anaerobic conditions. Anaerobic conditions were generated by using degassed and nitrogen-equilibrated buffers and special cuvettes covered by rubber septa. After following the consumption of NADPH at A340 nm, the reactions were terminated, and the concentration of H2O2 was measured using Amplex® Red hydrogen peroxide/peroxidase assay kit, according to the protocol from Molecular Probes.

Oxidation of Trx-(SH)2 by MGd—E. coli or human Trx (0.5 mM) was reduced with 5 mM DTT for 30 min under nitrogen, after which DTT was removed by running the sample through a Sephadex G-25 column, with nitrogen-equilibrated TE buffer. After mixing 20 μM Trx-(SH)2 and 20 μM MGd in potassium phosphate buffer, pH 7.5, or in 50 mM Tris-Cl, pH 7.5, with or without 1 mM EDTA, oxidation of reduced Trx was measured as the decrease of SH groups over time, after stopping the reaction with 6 M guanidine hydrochloride in TE buffer containing 1 mM DTNB. We used a molar extinction coefficient at 412 nm of 13.600 M−1 cm−1 for each SH group.

Binding between MGd and TrxR—MGd bound to Sephadex and Sepharose resins in a saturable way. A PD-10 Sephadex G-25 desalting column was equilibrated with 40 μM MGd in TE buffer until the absorbance at 470 nm was constant in the eluate. Then, a mixture of 40 μM MGd and 4 μM rat TrxR or 40 μM MGd and 0.4 μM E. coli TrxR in a final volume of 0.50 ml of TE buffer was incubated for 15 min at 37 °C and applied to the column. Fractions of 0.50 ml were collected by eluting the column by TE buffer containing 40 μM MGd, and the absorbance at 280 and 470 nm was recorded.

Protein Disulfide Reduction Using Insulin—This assay was performed to measure the activity of Trx or TrxR (Reactions 1 and 2) as described (29). A total volume of 120 μl was generated, containing 100 mM Tris-Cl, pH 7.5, 2 mM EDTA, 0.1 mM NADPH, 0.3 mM insulin, 1.8 μM Trx, and 0–140 μM MGd. The reaction was initiated by adding 5 nM rat TrxR to all tubes. After 20 min, the reaction was terminated by adding 500 μl of 1 mM DTNB in 6 mM guanidine hydrochloride in TE buffer (pH 8.0); thereafter, the amount of generated insulin SH groups was measured at A412 nm and quantified by the molar extinction coefficient of 13600 M−1 cm−1 for 2-nitro-5-thiobenzoic acid (29). All kinetic experiments were performed at 20 °C. Both reference and reaction cuvettes contained MGd.

Disulfide Reduction Assay with an NADPH Regenerating System—The catalytic activity of TrxR with Trx (Reactions 1 and 2) in the presence of MGd with varying NADPH was tested using glucose-6-phosphate to regenerate NADPH (35). This experiment was performed in 50 mM potassium phosphate, pH 7.6, 1.5 mM EDTA, 10 mM glucose-6-phosphate, 0.2 units/ml glucose-6-phosphate dehydrogenase, 0.2 mM insulin, 10 μM human Trx, and varying concentrations of NADPH (0.25–50 μM). After initiating the reactions by adding 10 μM TrxR, the samples were incubated at 37 °C for 15 min, and reactions were terminated by the addition of 6 mM guanidine hydrochloride, 1 mM DTNB in TE buffer to the samples, and the absorbance at A412 nm was determined to measure insulin SH groups.

TrxR Activity by DTNB Reduction—In a final volume of 0.50 ml, 100 mM Tris-Cl, pH 7.5, 1 mM EDTA, 5 mM DTNB, 0.2 mM NADPH, and varying concentrations of MGd were mixed. The reaction was initiated by the addition of 15 nM rat TrxR to all cuvettes except the reference, and A412 nm was recorded for 5 min.

Ribonucleotide Reductase Assay—RNR activity was determined by following the conversion of [3H]CDP into [3H]dCDP with mouse RNR according to the methods of Thelander and co-workers (33, 34). Reducing equivalents were provided through either 4 mM DTT or 3 μM human Trx and 0.1 μM calf liver TrxR and 1 mM NADPH. The reaction was initiated by adding mouse RNR (5.5 μg of R1 and 2.1 μg of R2) (33, 34) to the reaction mixture containing 40 mM Tris-Cl buffer, pH 7.5, 2 mM ATP, 10 mM magnesium chloride, 200 mM potassium chloride, and 0.5 mM [3H]CDP (23,000 cpm/nmol) in a final volume of 50 μl. Samples were incubated at 37 °C, and the reaction was terminated after 30 min by the addition of 1 M HClO4 followed by acid hydrolysis. The amount of [3H]dCDP formed was calculated from the results of liquid scintillation counting after ion-exchange chromatography separation of the monophosphates on Dowex-50 columns.

Statistical Analysis—Nonlinear least-squares curve fitting and error calculations were done using the software package Grace.

RESULTS

The Ultraviolet and Visible Spectrum of MGd—MGd has a strong green color, and as seen from the spectrum (Fig. 1), there was a relatively strong absorbance at 340 and 412 nm, which are wavelengths for spectrophotometric assays of the oxidation of NADPH or sulphydryl groups by DTNB, respectively (29). Adding 1 mM DTT to a 20 μM MGd solution resulted in no change of the spectrum after 30 min. The dithiol DTT is known to be a slow reductant of MGd, but regeneration of the parent molecule by oxygen-dependent redox cycling obviously is quickly generating ROS (6). In our studies, we measured redox reactions
at 340 nm in the presence of MGd and the Trx system without any corrections due to spectral changes in MGd.

**MGd as a Substrate for Thioredoxin Reductase**—MGd is supplied as a solution in mannitol, and previous measurements of the redox properties of the molecule using an oxygen electrode have been made in 50 mM HEPES, pH 7.5, 100 mM NaCl (6). Initial experiments using this buffer with NADPH and 0.2 μM rat TrxR showed oxidation of NADPH at 340 nm. The addition of 1 mM EDTA, a standard condition for assays of Trx and TrxR (29), did not inhibit the reaction as if MGd was not complexed but rather slightly stimulated the reaction rate. This may be due to complexation of heavy metal ions in buffer salts, which may inhibit TrxR. Buffers used in the present study therefore all contained EDTA.

As seen in Fig. 2A, the addition of increasing amounts of MGd resulted in a saturable oxidation of NADPH. Examination of the data with Michaelis-Menten kinetics yielded an apparent 

$$K_m = 8.65 \text{ μM}$$

$$k_{cat} = 0.42 \times 10^3 \text{ s}^{-1}$$

and a $$k_{cat}/K_m$$ of 4.86 × 10^4 M$^{-1}$ s$^{-1}$ for the rat TrxR1. The addition of up to 10 μM human Trx1 did not stimulate the reaction. The pure human placenta TrxR (29) showed a similar activity as the rat recombinant enzyme (data not shown).

To test whether the smaller non-selenium-dependent prokaryotic TrxR from *E. coli* (14) used MGd as a substrate, the same experiments were performed. As shown in Fig. 2B, MGd acted faster with the *E. coli* enzyme, and a $$K_m$$ value of 22.1 μM, a $$k_{cat}$$ of 4.93 × 10^3 s$^{-1}$, and a $$k_{cat}/K_m$$ of 2.23 × 10^5 M$^{-1}$ s$^{-1}$ were calculated. As in the case of mammalian TrxR, the addition of *E. coli* Trx1 did not increase the reaction rate. In conclusion, the slow oxidation of NADPH by MGd described previously (6) is dramatically increased by the addition of catalytic amounts of TrxR. Surprisingly, *E. coli* TrxR, which usually is highly specific (14, 29) had a higher catalytic efficiency than the selenocysteine-containing mammalian TrxR.

**MGd Redox Cycling Leads to Production of Hydrogen Peroxide**—MGd takes electrons from NADPH, which was catalyzed by TrxR. Then, MGd reacts with oxygen to be regenerated by redox cycling (6). This phenomenon leads to the production of reactive oxygen species, including superoxide (Reaction 3) and hydrogen peroxide by spontaneous decay of superoxide (Reaction 4).

$$\text{NADPH} + \text{H}^+ + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{O}_2^- + 2\text{H}^+$$

**REACTION 3**

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

**REACTION 4**

Previous results showed that MGd leads to O$_2$ consumption in the presence of NADPH as detected with an oxygen electrode (6). Our results showed that the addition of TrxR dramatically accelerates NADPH oxidation (Reaction 3). This reaction was studied under aerobic and anaerobic conditions, using 200 μM NADPH, 20 μM MGd, and 0.2 μM rat-TrxR. Only in the presence of oxygen did significant NADPH oxidation occur (Fig. 3). The reaction was further analyzed after another 6 h, when more than 150 μM NADPH had been oxidized. The cuvette then contained 50 μM H$_2$O$_2$, which was determined using a hydrogen peroxide assay. This amount of reaction products is rather low, which can be explained by H$_2$O$_2$ being a direct substrate for mammalian TrxR, yielding H$_2$O, thus removing H$_2$O$_2$ (19).

**The Reaction between MGd and Thioredoxin Is Surprisingly Slow**—As mentioned above, the addition of 10 μM reduced Trx to the reaction with TrxR did not increase the rate of NADPH oxidation. This was unexpected since Trx is generally a fast disulfide reductant (13, 14). To study the reaction between Trx and MGd, we generated reduced Trx1 by incubating with excess DTT and removed the reductant by gel chromatography. Then, 20 μM Trx-(SH)$_2$ and 20 μM MGd were mixed in TE buffer followed by determination of free SH groups in Trx by using 1 mM DTNB in 6 mM guanidine hydrochloride. The result of this analysis showed that the half-life of the reaction was more than 20 min, indicat-
ing that the reaction is very slow. This experiment was repeated several times with E. coli as well as human Trx1 under different conditions, with and without EDTA, and at different temperatures. No reaction was observed. In addition, there was no evidence of complex formation between MGd and reduced or oxidized Trx using the gel chromatography method described later.

Characterization of the Reaction between MGd and Mammalian Thioredoxin Reductase—MGd induced an NADPH oxidase activity in TrxR similar to the effect of 1-chloro-2,4-dinitrobenzene (DNCB), which acts as an irreversible inhibitor of human TrxR with loss of Trx disulfide reductase activity but induction of a 34-fold higher NADPH oxidase activity (36). To investigate whether the reaction between TrxR and MGd required the active enzyme, 100 μM DNCB was added to TrxR and NADPH. The DNCB-modified enzyme without Trx disulfide reductase activity showed no reaction with MGd and NADPH at 340 nm above the

control. However, if fresh TrxR was added, a reaction with MGd was observed (data not shown). DNCB alkylates the active site selenocysteine and cysteine residues of the enzyme (37), and therefore, an interaction of MGd with the FAD or alternative redox active disulfide of the enzyme to oxidize NADPH-generating ROS is excluded. Further evidence that the reaction between TrxR and MGd required the fully active enzyme was that a mutant TrxR with the Sec-498 → Cys mutation (19), with a 100-fold lower $k_{cat}$ for Trx reduction than the wild type rat enzyme, did not show any reactivity with MGd (data not shown).

**Figure 3.** Oxygen-dependent redox cycling of MGd. 200 μM NADPH, 20 μM MGd, and 0.2 μM rat TrxR were mixed, and NADPH oxidation was recorded at 340 nm, under aerobic (continuous line) and anaerobic (dashed line) conditions, showing that NADPH can be oxidized by MGd in the presence of O₂, which was catalyzed by TrxR.

**Figure 2.** MGd is a direct substrate for both mammalian and E. coli TrxR. This experiment was performed using either 0.2 μM rat recombinant TrxR (A) or 0.015 μM E. coli TrxR (B) with 100 μM NADPH and 0–24 μM MGd. Insets show Lineweaver-Burk plots. The curves represent the non-linear curve fitting of the data points to the Michaelis-Menten equation (the correlation coefficient $R^2$ was 1.0 for both A and B, and the chi-square was $3.3 \times 10^{-3}$ and $2.4 \times 10^{-4}$ for panels A and B, respectively).

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- **Figure 4.** A: Enzyme from fresh TrxR (filled square) or the same amount of enzyme from TrxR that had been dialyzed against TE buffer for 24 h (open square). The concentration of insulin from 0.3 to 0.1 mM changed this $K_i$ to 70 μM. B: A similar assay system, indicating some binding to dialysis membranes or gel filtration columns used to separate excess drug. However, we found that MGd binds to dialysis membranes and plastic surfaces as well as Sephadex columns. To overcome this, we equilibrated an 8.3-ml PD-10 Sephadex G-25 column by running 40 μM MGd in TE buffer until a constant absorbance at 470 nm was reached. Then, enzyme incubated with MGd was added to the green-colored PD-10 column, and a chromatogram was generated. The peak at 470 nm of MGd co-eluted with protein at 280 nm (Fig. 4A), indicating that MGd bound to rat TrxR, and a corresponding trough was present at the low molecular weight eluted fractions. In fact, E. coli TrxR (Fig. 4B) bound more MGd, and a calculation showed that approximately up to six molecules of MGd were eluted per enzyme dimer. In contrast, the rat TrxR enzyme gave a ratio of 1:4.1, indicating a smaller amount of MGd bound.

**MGd Inhibits the Activity of the Thioredoxin System**—The Trx system is an NADPH-dependent general disulfide reductase (13, 14, 18). TrxR catalyzes the NADPH-dependent reduction of Trx–S₂ to Trx–(SH)₂ (Reaction 2). Through a combination of Reactions 1 and 2, the Trx system will catalyze NADPH-dependent disulfide reduction, and insulin disulfides are generally used to follow the reaction (13, 14, 18). To study whether MGd inhibited protein disulfide reduction, the mammalian Trx system was evaluated (Fig. 5). In this experiment, a low rate-limiting amount of TrxR (5 nM) was used, and the IC₅₀ was around 50 μM for MGd. In other experiments, we used varying concentrations of Trx (0–10 μM), with and without 16 μM MGd. Reactions were started by the addition of 10 nM rat TrxR. The change at 340 nm was measured, showing that MGd was a non-competitive inhibitor with a $K_i$ in the range of 180 μM. This is a very high concentration, and we suspected that this was due to the binding of MGd to insulin. Lowering the concentration of insulin from 0.3 to 0.1 mM changed this $K_i$ to 70 μM, and again, a non-competitive inhibition was observed. A similar magnitude of change was seen for the E. coli assay system, indicating some binding.
of MGd and insulin (data not shown). The reducing substrate for TrxR is NADPH. To vary NADPH and study the effect of MGd, we used a method to continuously regenerate NADPH by the presence of glucose-6-phosphate dehydrogenase and glucose-6-phosphate. As shown in Fig. 6, MGd acted as a non-competitive inhibitor of NADPH in this assay system (a $K_m$ value of 8.06 $\mu M$ and a $k_{cat}$ of 26.9 $s^{-1}$ without MGd and a $K_m$ value of 8.03 $\mu M$ and a $k_{cat}$ of 24.0 $s^{-1}$ with MGd). 

To study whether TrxR was inhibited by MGd independent of Trx, we used the artificial direct substrate DTNB (29). When varying MGd (0–16 $\mu M$) and NADPH with saturating DTNB (5 mM), an IC$_{50}$ of 6 $\mu M$ was obtained for MGd (Fig. 7). Thus, MGd is a relatively strong direct inhibitor of TrxR activity.

**MGd Inhibits the Activity of Ribonucleotide Reductase** — RNR is composed of two non-identical subunits, which, when combined, demonstrate activity (12, 27). Initial experiments using E. coli RNR (32) showed that MGd was a strong inhibitor (data not shown). We then produced mouse RNR by separately expressing the two subunits (33, 34) in E. coli and reconstituting the tyrosol-free radical of the R2 enzyme subunit. RNR activity was measured by using conversion of $[3H]$CDP into $[3H]$dCDP. Using the Trx system as an electron donor, MGd was a strong inhibitor of mouse RNR activity, with an IC$_{50}$ of 2 $\mu M$ (Fig. 8). Alternatively, 4 mM DTT was used as reducing substrate. Again, RNR activity was inhibited with an IC$_{50}$ of 6 $\mu M$ (Fig. 8). These results indicate a strong direct inhibitory effect of MGd on RNR.

**DISCUSSION**

MGd is a novel and promising chemotherapeutic drug acting by a redox cycling mechanism. The present results show that MGd acts as a substrate of the cytosolic selenocysteine-dependent mammalian TrxR (19, 20, 38–40), generating ROS from NADPH in the presence of oxygen. We demonstrate that the reaction end product was hydrogen peroxide. As with other flavoproteins, TrxR has a low inherent NADPH oxidase activity ($k_{cat}$ 0.14 $s^{-1}$), which is stimulated up to 34-fold ($k_{cat}$ 4.7 $s^{-1}$) following alkylation of the selenol thiol -Cys-Sec- in the active site by DNBC (36, 37). In the presence of 8 $\mu M$ MGd, there was a similar turnover rate of mammalian TrxR. Obviously, this is responsible for generating oxidative stress in cells as observed (10, 11). The induced activity is of the same magnitude as that of naturally occurring NADPH oxidases (Nox enzymes) involved in redox signaling or transformation of cells (41). Tumor cells often have up to 10-fold higher expression of TrxR compared with normal cells (38, 39); it can be
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FIGURE 7. The inhibition of TrxR by MGd is independent of Trx. Using DTNB as a direct substrate for the mammalian TrxR, this assay was done with 5 mM DTNB, 200 μM NADPH, 15 mM rat-TrxR, and 0–16 μM MGd to study the effect of MGd on the catalytic activity of rat TrxR. Results show that MGd is an efficient inhibitor of mammalian TrxR with an IC50 of about 6 μM.

FIGURE 8. MGd inhibits the activity of ribonucleotide reductase. The enzymatic activity of 0.5 μM mouse RNR was measured in the presence of either 3 μM Trx, 0.1 μM TrxR, and 1 mM NADPH (A) or 4 mM DTT (B) as described for the RNR assay. Incubation was performed with varying concentrations of MGd (0–10 μM).

hypothesized that transformed cells are more susceptible to the MGd-induced oxidative stress response, leading to cell death predominantly in tumor cell populations (42–44). Previous results had reported that MGd reacts with reducing metabolites by using oxygen electrode measurements (6). A comparison of the calculated reaction rates is shown in Table 1. Obviously, mammalian TrxR reactivity is orders of magnitude faster than that of NADPH, GSH, or ascorbate. The reactivity with NADPH was usually negligible in our experiments.

Surprisingly, MGd did not react with E. coli or human Trx under any assay conditions. The reactivity is so slow that it should not play any role physiologically. Another remarkable fact is that the E. coli TrxR, which displays a high degree of substrate specificity (36), showed a high reactivity with MGd. Furthermore, MGd was a powerful inhibitor of the E. coli Trx system. This effect was also detectable when assessing the mammalian Trx system; however, the inhibition was much more moderate than for the E. coli TrxR. The effect of MGd on rat TrxR1 was demonstrated using DTNB as a direct substrate. Results showed an IC50 value of 6 μM. TrxR is an enzyme directly reducing, e.g. dehydroascorbate, by NADPH and this reaction should be blocked. Ascorbate is known to potentiate the effect of MGd (6, 10, 11). In summary, the reactivity with Trx system will induce oxidative stress in cells through a major production of ROS. Inhibition of TrxR as a disulfide reductase involved in Trx-dependent reactions such as peroxiredoxin activity may also play a major role.

The other major effect of MGd discovered in our studies is a direct inhibition of the activity of RNR, an essential enzyme required for DNA synthesis and repair (12, 27). In the case of the mammalian RNR, inhibition was direct on the enzyme and required low amounts of MGd. It occurred both with Trx and TrxR as well as with the artificial electron donor DTT, as electron donors, clearly demonstrating a direct effect on RNR. Inhibition of RNR will stop DNA synthesis, DNA repair, and cell growth. This may be an important component of the anti-tumor activity of MGd. The low IC50 of 2 μM of MGd for RNR makes it a strong inhibitor of the enzyme (Fig. 8). Further studies are required to assess whether MGd binds to RNR and to analyze which of the two RNR subunits is targeted by MGd. RNR activity could also be indirectly affected as a downstream consequence of inhibition of the Trx system.

Two recent publications of interest for understanding MGd (10, 11) have appeared. One study shows that MGd disrupts zinc metabolism in human cancer cell lines and that metallothionein genes are among the most strongly induced, indicating that MGd can mobilize intracellular zinc. Disruption of zinc metabolism and increases of free zinc would also inhibit TrxR (11). Magda et al. (45) show an assay for TrxR using cellular lipoate reduction, which is inhibited in the presence of MGd, corroborating our results. Free zinc has obvious effects on enzymes with redox active SH groups like TrxR, Trx, or RNR. Interestingly, it was reported that the R2-subunit RNR was also induced in the presence of MGd (45).

In summary, we have described the effect of MGd on the thioredoxin system and RNR. We have demonstrated that MGd and NADPH react in the presence of TrxR, producing ROS, which will generate oxidative stress in cells, as has been demonstrated (46). This may lead to induction of apoptosis through oxidation of Trx, via ASK1 mediated cell death (47, 48). Additional redox-dependent pathways may be disrupted as a result of inhibition of the Trx system as a disulfide reductase. We also demonstrate a direct inhibitory effect of MGd on RNR. Our results suggest that MGd may act on tumor cells by disrupting the redox balance and inducing oxidative stress apart from inhibiting DNA synthesis due to lack of deoxyribonucleotides.

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TABLE 1
Oxygen dependent reaction rates of MGd with cellular reductants

| Substrate       | Rate  |
|-----------------|-------|
| NADPH<sup>a</sup> | 0.5   |
| Ascorbate<sup>a</sup> | 5     |
| GSH<sup>a</sup>   | 0.12  |
| TrxR E.coli<sup>a</sup> | 4.9 × 10<sup>4</sup> |
| TrxR human<sup>a</sup> | 2.2 × 10<sup>5</sup> |

<sup>a</sup> Data from Ref. 6.
<sup>a</sup> Calculated as k<sub>cat</sub>/K<sub>m</sub> (this study).
