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A New Mechanism of Action for the Anticancer Drug Mitomycin C: Mechanism-Based Inhibition of Thioredoxin Reductase

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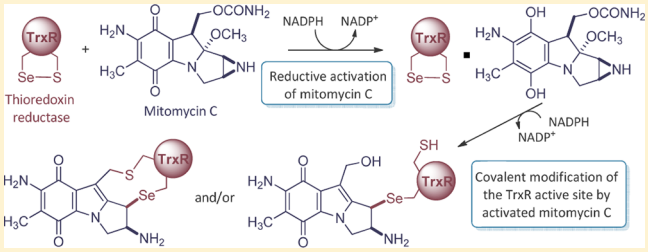
Supporting Information

ABSTRACT: Mitomycin C (MMC) is a chemotherapeutic drug that requires an enzymatic bioreduction to exert its biological effects. Upon reduction, MMC is converted into a highly reactive bis-electrophilic intermediate that alkylates cellular nucleophiles. Alkylation of DNA is the most favored mechanism of action for MMC, but other modes of action, such as redox cycling and inhibition of rRNA, may also contribute to the biological action of the drug. In this work, we show that thioredoxin reductase (TrxR) is also a cellular target for MMC. We show that MMC inhibits TrxR in vitro, using purified enzyme, and in vivo, using cancer cell cultures. The inactivation presents distinctive parameters of mechanism-based inhibitors: it is time- and concentration-dependent and irreversible. Additionally, spectroscopic experiments (UV, circular dichroism) show that the inactivated enzyme contains a mitosene chromophore. On the basis of kinetic and spectroscopic data, we propose a chemical mechanism for the inactivation of the enzyme that starts with a reduction of the quinone ring of MMC by the selenolthiol active site of TrxR and a subsequent alkylation of the active site by the activated drug. We also report that MMC inactivates TrxR in cancer cell cultures and that this inhibition correlates directly with the cytotoxicity of the drug, indicating that inhibition of TrxR may play a major role in the biological mode of action of the drug.

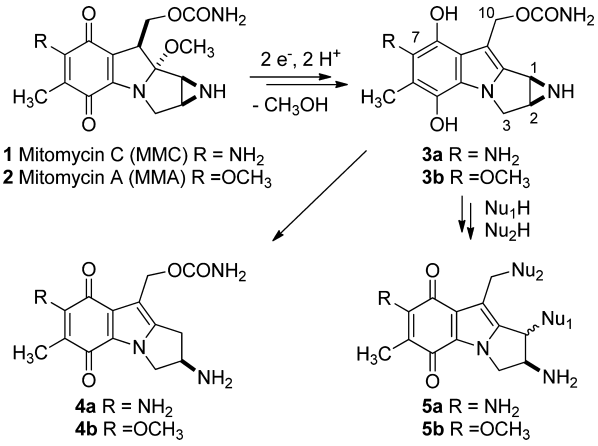


INTRODUCTION

Mitomycin C (MMC, 1) is an antitumor antibiotic that has been used in combination chemotherapy for a variety of tumors since 1974[1] and as an antifibrotic agent for several surgical procedures.[2−4] The vast majority of the research on its biological mode of action has been focused on its ability to bind covalently to DNA,[5] with a less-favored hypothesis proposing redox cycling to explain the cytotoxic effects of the drug.[6] MMC is considered the prototype bioreductive drug: it is inert toward nucleophiles in its original structure but is converted to an extremely reactive bis-electrophile after a cascade of reactions initiated by reduction of the quinone ring (Scheme 1).[7] The reductive activation of MMC generates the reactive intermediate **3a** that contains two electrophilic positions—at C1 and C10—and is able to alkylate biological nucleophiles, in particular DNA, to form four monoadducts and two cross-links.[8] The formation of an interstrand DNA−DNA cross-link is consistently considered the cause of its cytotoxic effects.[9] MMC has been shown to alkylate other cellular nucleophiles in addition to DNA, such as glutathione (GSH).[10,11] A recent novel hypothesis postulates rRNA as a target for alkylation by reductively activated MMC.[12]

The cellular activation of MMC has been attributed to several reductases, and recent reports indicated that GRP58, a protein containing a dithiol active site in its thioredoxin (Trx)-like domains, was implicated in the reductive activation of

Scheme 1. Mechanism for the Reductive Activation of Mitomycins[a]



[a]Nu_1H and Nu_2H represent two nucleophilic compounds.

MMC in several cancer cells.[13−15] Prompted by those findings, we recently studied the reactions of MMC with simple dithiols as models for proteins containing a dithiol active site. We

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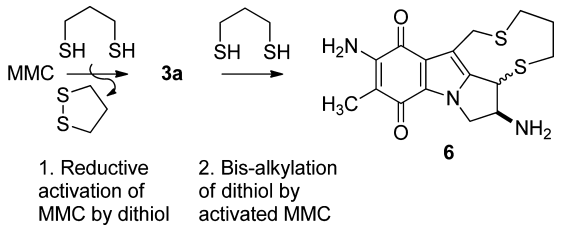
discovered that simple dithiols [1,3-propanedithiol, D,L-dithiothreitol (DTT), dihydrolipoic acid] could activate MMC by reduction[16] and that the metabolites formed by reductively activated-MMC reacted further with additional dithiol to form cross-links (e.g., **6**) by binding covalently the two sulfur atoms of the dithiol (Scheme 2).[17] As a conclusion

Scheme 2. Mechanism for the Reductive Activation of MMC by Dithiols and Subsequent Formation of a Dithiol Cross-Link



of our previous work, we speculated that MMC could be a mechanism-based inhibitor of enzymes containing a dithiol active site: the dithiol active site first would reduce MMC to form a bis-electrophilic mitosene that afterward would alkylate the newly reduced dithiol site, resulting in inactivation of the enzyme.

To test if the chemical reactivity observed with simple dithiols could be mimicked in biological proteins, we considered that thioredoxin reductase (TrxR) was an ideally suited enzyme. One reason to select TrxR was based on chemical reactivity: TrxR contains a redox active cysteine-selenocysteine (Sec) active site at a sequence located at the C-terminal sequence −Gly−Cys−Sec−Gly−OH.[18] This selenolthiol active site could, in theory, react with MMC by the same mechanisms that we had elucidated previously for simple dithiols: a reductive activation initiated by conjugate addition of selenoate to the quinone of MMC followed by alkylation of the selenolthiol site by the activated drug. A precedent for this type of mechanism can be found in the inactivation of parasitic TrxR by Mannich bases, which occurs by bis-alkylation of the C-terminal dithiol active site of the enzyme by two sequential Michael additions after activation of the prodrug.[19] Another motive to select TrxR to test our hypothesis was the well-known involvement of the Trx system in cancer development.[20] In fact, TrxR is a direct target for several anticancer drugs in clinical use.[21−23]

Here, we present our findings on the inhibition of TrxR by MMC, where we show that the enzyme is irreversibly inactivated by the drug. We characterize the inactivation as mechanism-based inhibition based on kinetic and spectroscopic data, and we show that MMC inhibits TrxR in cell cultures in a concentration-dependent manner that correlates the concentration-dependent cytotoxicity of the drug, thereby suggesting that TrxR inhibition plays a major role in the biological mode of action of the drug.

EXPERIMENTAL PROCEDURES

Materials and Methods. Recombinant rat TrxR1 was prepared as described previously.[24] The enzyme had a specific activity of 30−50% as determined by the 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) assay, and concentrations used are reported for active enzyme. NADPH was from Applichem; insulin, trypsin, and DTNB were from Sigma. MMC was dissolved in H_2O, and its concentration was determined spectrophotometrically using $\in_{367}$ (H_2O) 21840 cm^{-1} M^{-1}. The enzyme activity was measured using a VersaMax microplate reader (Molecular Devices) or an Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech). UV spectra were obtained with a Cary 300 or Shimadzu CPS-260 UV−visible spectrometers. Circular dichroism (CD) spectra were obtained with a Jasco 715 spectropolarimeter using a 2 mm path length rectangular cell in MeOH. LC/MS was performed with a HP Agilent Series 1100 diode array HPLC system connected to a HP Agilent Series 1100 MSD mass spectrometer. A Macherey Nagel (Nucleodur C18, 4.6 mm × 125 mm, 5 μm) column was used. The elution system was 5−30% v/v B in 20 min, 30−55% v/v B in 10 min (A = 10 mM ammonium acetate, pH 5.5; B = acetonitrile), with a flow rate of 1 mL/min. The determination of k_{obs} and nonlinear regression analysis was performed with the curve fitting program BioDataFit (version 1.02; Chang Bioscience Inc., Castro Valley, CA).

Enzyme Activity Assays. The activity of the enzyme was determined at room temperature in a buffer containing 50 mM Tris-Cl and 1 mM EDTA, pH 7.5 (TE buffer). TrxR was first reduced by incubation with excess NADPH at room temperature for 5 min. The preincubated mixture was divided in aliquots that were treated with increasing amounts of MMC. The final concentrations were 100 nM TrxR and 200 μM NADPH. The enzyme activities were then measured after incubation at room temperature for the appropriate time by using both the DTNB reduction assay and the insulin disulfide reduction assay following published protocols.[25]

Requirement of NADPH for Inhibition. TrxR (100 nM) was incubated with MMC (40 μM) with or without NADPH (200 μM). Aliquots were removed at time intervals, and the activity of TrxR was measured using the DTNB assay. A control sample containing TrxR and NADPH was included. The TrxR activity was measured against an incubation that included neither MMC nor NADPH.

Determination of Time- and Concentration-Dependent Inhibition of TrxR by MMC. The incubation mixtures were prepared similarly to those described above for the end point assay but using higher enzyme concentrations (in the range 200−400 nM). Aliquots (4 μL) were removed at time intervals and assayed for TrxR activity using the DTNB reduction assay, using a final volume of 200 μL. The activity was measured against control samples that contained all components excepting MMC. The decay of activity for each concentration of MMC was fitted to the equation % activity = $a \times e^{-k_{obs}[MMC]} + b$. The values of K_I and k_{inact} were calculated using the Kitz-Wilson plot or nonlinear regression analysis. For the Kitz-Wilson model, a linear plot of $1/k_{obs}$ versus 1/[MMC]) was constructed, and the y-intercept value afforded $1/k_{inact}$, while the x-intercept value gave $1/K_I$.[26] For nonlinear regression, the data were adjusted to the equation $k_{obs} = (k_{inact} \times [MMC])/(K_I + [MMC])$ using BioDataFit software.

Irreversibility of the Inactivation of TrxR by MMC. TrxR (200 nM) was incubated with MMC (40 μM) and NADPH (250 μM) for 6 h. After this period, only 10−15% activity remained. The enzyme was purified using either gel filtration or dialysis. Control samples containing TrxR and NADPH, but no MMC, were processed in parallel. The TrxR activity was determined using the DTNB and insulin reduction assays.

MMC as a Substrate for TrxR. The activity of TrxR using MMC as substrate was determined at 25 °C in a buffer containing 50 mM Tris-Cl and 1 mM EDTA, pH 7.5 (TE buffer). The final concentrations were 50 nM TrxR, 200 mM NADPH, and increasing concentrations of MMC. The reaction was monitored by measuring either A_{340} (for consumption of NADPH) or A_{550} (for appearance of the 7-aminomitosene chromophore resulting from reductive activation of MMC). Control incubations omitting one of the three components were included for reference. Mitomycin A (MMA) served as a positive control [MMA is a substrate for TrxR (Paz, M. M., Zhang, X., Lu, J., and Holmgren, A. Unpublished data).].

Time- and Concentration-Dependent Inhibition of TrxR by MMC in the Presence of Ethyl Xanthate or GSH. The assays were performed essentially as described above for time and concentration dependence experiments, except that ethyl xanthate or GSH were included in the incubations. The activity of TrxR was determined at

time intervals using the insulin disulfide reduction assay, as both GSH and ethyl xanthate interfered in the DTNB assay. Appropriate controls without MMC were included for every concentration of GSH or ethyl xanthate used in the experiment. The concentration of MMC was 60 μM.

pH Dependence of the Inactivation of TrxR by MMC. TrxR (100 nM) was incubated with MMC (60 μM) and NADPH (200 μM) in 100 mM phosphate buffer at six different pH values: 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. Aliquots were removed at time intervals, and the activity of TrxR was measured using the DTNB assay. The activity for each sample was measured against a control sample that contained TrxR and NADPH in the same buffer.

LC/MS Analysis of Reactions of MMC with TrxR and NADPH. Reaction mixtures prepared similarly to those described for end point assays were analyzed directly by LC/MS. Authentic samples of mitosenes derived from MMC were prepared as reported.[27]

Preparation of Samples of MMC-Modified TrxR for UV and CD Analysis. A solution containing 150 nM TrxR, 200 μM NADPH, and 60 μM MMC (5 mL total volume) in TE (pH 7.4) was incubated for 16 h. The activity of the inhibited enzyme was 83−90% as determined by the DTNB reduction assay. The modified enzyme was purified by gel filtration chromatography using prepacked Sephadex G-25 columns (PD-10, Amersham Biosciences, Uppsala, Sweden), using 10 mM ammonium acetate buffer as eluent. Fractions containing the protein were identified using a Bradford assay and concentrated by centrifugal membrane filtration (Microcon-10, Millipore, Bedford, MA) to a final volume of 300 μL, and the resulting solution was used directly to measure the UV and CD spectra.

Cell Culture and Cell Viability Assay. Du145 cells, A549 cells, and Hela cells were cultured with 1 g/L glucose Dulbecco's modified Eagle's medium (Gibco). MCF7 cells were grown in RPMI 1640 medium. Du145 cells were plated at a density of 1×10^6 in 100 mm Petri dishes and treated with various concentrations of MMC. After the treatment, cell lysates were prepared for Western blotting and TrxR activity assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the cell viability in 96-well plates.[28]

Measurement of TrxR Activity in Du145 Cells. The TrxR activity was determined in 96-well plates using an end point insulin assay.[25] The cell lysate (25 μg) was mixed thoroughly with 55 mM Hepes (pH 7.6), 0.2 mM insulin, 0.4 mM NADPH, 2 mM EDTA, and 2 μM human Trx1 in a final volume of 50 μL. Incubations without human Trx1 were used as the control. After the reactions proceeded at 37 °C for 30 min, 200 μL of 1 mM DTNB in 6 M guanidine hydrochloride solution was added. The concentration of free thiols from reduced insulin was determined by measuring the 2-nitro-5-thiobenzoate absorbance at 412 nm.

RESULTS

MMC Inhibits TrxR. After the NADPH-reduced rat TrxR was incubated with increasing concentrations of MMC, the TrxR activity was measured using two methods: The DTNB reducing assay, in which the formation of 5-thio-2-nitrobenzoic acid by reduction of the disulfide bond of DTNB catalyzed by TrxR/NADPH was measured, or the insulin assay, in which the NADPH consumption in a mixture containing NADPH and Trx after addition of TrxR was recorded. Both assays showed that MMC inhibited TrxR in a concentration-dependent manner (Figure 1). The IC_{50} values inferred from these experiments were 3 μM in the DTNB assay (performed after 1 h of incubation) and 1 μM in the insulin assay (performed after 3 h of incubation). The disparity in the inhibition observed in these two experiments indicated that MMC was a slow-acting inhibitor of TrxR, an aspect that was further investigated in experiments described below. The partition ratio for the inhibition of TrxR by MMC determined from the data obtained

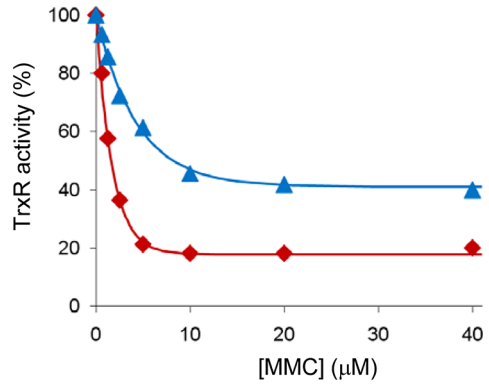


Figure 1. End point assays for the inhibition of TrxR by MMC. TrxR (100 nM) and NADPH (200 μM) were incubated with increasing concentrations of MMC. The enzyme activities were measured after 1 h by the DTNB assay (blue triangle) and after 3 h by using the insulin disulfide reduction assay (red diamond). The activity was measured against an incubation lacking MMC.

in this experiment[29] gave values ranging between 15 and 25 (see the Supporting Information for details).

NADPH Is Required for Inhibition of TrxR by MMC. The disulfide reducing activity of TrxR was virtually unaffected when TrxR was incubated with MMC in the absence of NADPH (Figure 2), indicating that the inhibition requires a selenolthiol active site in the reduced state.

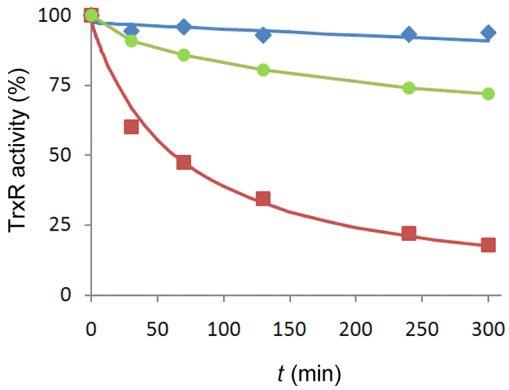


Figure 2. Requirement NADPH for the inactivation of TrxR by MMC. TrxR (200 nM) was incubated with 60 μM MMC (blue diamond), 200 μM NADPH (green dot), or 60 μM MMC, 200 μM NADPH (red square). Aliquots were removed at time intervals, and the activity of TrxR was measured using the DTNB assay. The activity was measured against a control incubation containing only TrxR.

Inhibition of TrxR by MMC Is Time- and Concentration-Dependent. Incubation-dilution assays are one of the defining experiments to identify mechanism-based inhibitors.[30] In these experiments, the enzyme is incubated with increasing concentrations of inhibitor, and the enzymatic activity is determined at time intervals for each of the incubations. The kinetic parameters k_{inact} and K_I can be derived from the rate of enzymatic activity decay observed at each concentration of inhibitor by using Kitz-Wilson plots[26] and nonlinear regression analysis. MMC showed a time- and concentration-dependent inhibition of TrxR (Figure 3a), with a k_{inact} of 0.017 (±0.005) min^{-1} and a K_I of 35 (±10) μM (Figure 3b).

Addition of Ethyl Xanthate as External Nucleophile Does Not Affect Inhibition of TrxR by MMC. Another

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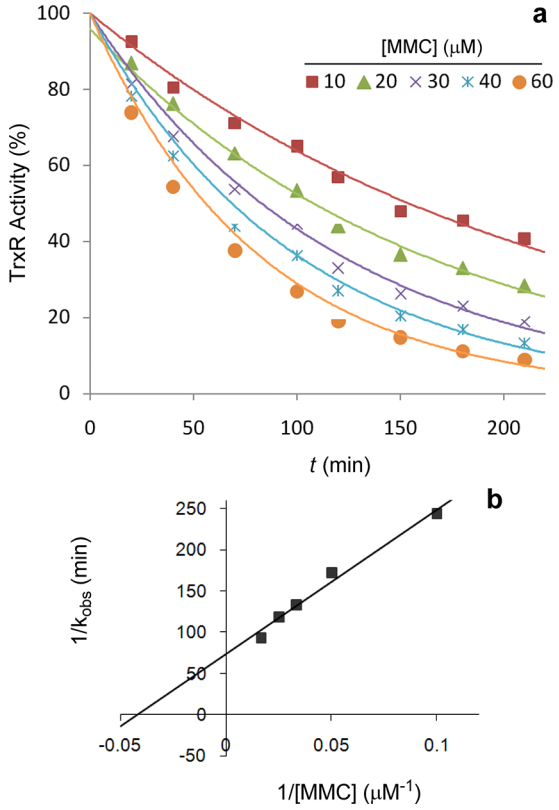

Figure 3. Time- and concentration-dependent inhibition of TrxR by MMC. (a) Incubations containing 200 nM TrxR and 200 μM NADPH were treated with MMC at concentrations as shown in the inset. Aliquots were removed at time intervals, and the activity of TrxR was measured using the DTNB assay. The activity was determined against an incubation without MMC. (b) Kitz-Wilson plot for the calculation of k_{inact} and K_I.

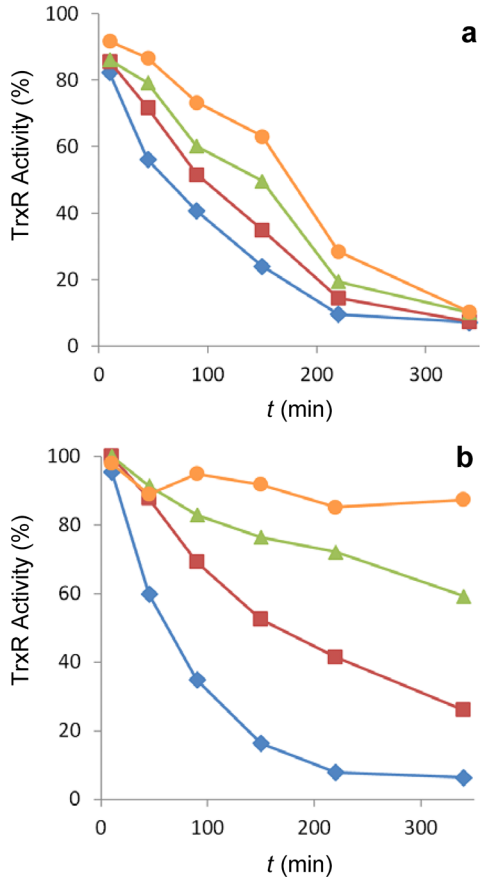

Figure 4. Time- and concentration-dependent inhibition of TrxR by MMC in the presence of ethyl xanthate (a) or GSH (b). Incubations containing 200 nM TrxR, 200 μM NADPH, 60 μM MMC and 0 (blue diamond), 100 (red square), 300 (green triangle), or 1000 μM nucleophile (yellow dot). Aliquots were removed at time intervals, and the activity of TrxR was measured using the insulin disulfide reduction assay. The activity was measured against a series of incubations that contained all components except MMC.

diagnostic experiment to identify mechanism-based inhibitors is the addition of an external nucleophile in the enzyme−inhibitor incubations. True mechanism-based inhibitors must inactivate the enzyme without leaving the active site, and the addition of a nucleophile must not influence the inhibitory effect. In our case, these assays were performed by adding ethyl xanthate or GSH as nucleophiles. The addition of ethyl xanthate resulted in a mild concentration-dependent recovery of TrxR activity at short reaction times, but after longer incubation periods, it had no effect (Figure 4a). However, when increased concentrations of GSH were added to incubation mixtures containing TrxR, MMC, and NADPH, a recovery of enzyme activity proportional to the concentration of added GSH was observed (Figure 4b).

Inhibition of TrxR by MMC Is Irreversible. To investigate the reversibility of the inhibition, MMC-inactivated TrxR was purified either by gel filtration or dialysis, and the activity of the purified samples was measured against samples of unmodified TrxR that were processed in parallel. No significant recovery of activity was observed, indicating that the inhibition is irreversible (data not shown).

Inhibition of TrxR by MMC Is Not pH-Dependent. The effect of pH in the inactivation of TrxR by MMC was studied at six different pH values covering a pH range from 6 to 8.5. It was found that TrxR was equally inhibited at all of the pH values studied (Figure S1 in the Supporting Information).

MMC Is Not a Substrate for TrxR. Two experiments indicated that MMC is not a substrate for TrxR: NADPH was not consumed in incubations with TrxR, MMC (Figure S2 in the Supporting Information), and no metabolites resulting from reduction of MMC were formed in these incubations as determined by monitoring the increase of A550 absorbance characteristic of the mitosenes derived from MMC (Figure S3 in the Supporting Information). Also, LC/MS analysis of reaction mixtures containing TrxR, MMC, and NADPH confirmed the lack of formation of mitosene metabolites (**4a** and **5a**) known to be irreversibly formed after reduction of MMC quinone[7] (Figure S4 in the Supporting Information). Parallel experiments showed that MMA (**2**), a member of the mitomycin family with a higher redox potential than that of MMC, showed concentration-dependent consumption of NADPH as determined by the decrease of A_{340} in incubation mixtures containing MMA, TrxR, and NADPH. Also, the formation of mitosene metabolites derived from MMA was observed when the increase of A_{550} caused by the formation of the mitosene chromophore was measured. Additionally, LC/MS analysis of reactions containing MMA, TrxR, and NADPH showed the formation of the **4b** as a major mitosene metabolite, indicating that MMA undergoes a bifunctional activation after reduction by TrxR.[31]

Inactivated Enzyme Incorporates a Mitosene Chromophore Derived from Reductively Activated MMC. The

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difference UV spectrum of MMC-inactivated TrxR was obtained by subtracting the UV spectrum of unmodified enzyme (normalized at 460 nm), and it showed the characteristic features of a 7-aminomitosene chromophore: a strong band at 306 nm (Figure 5b) with a shoulder at 355 nm

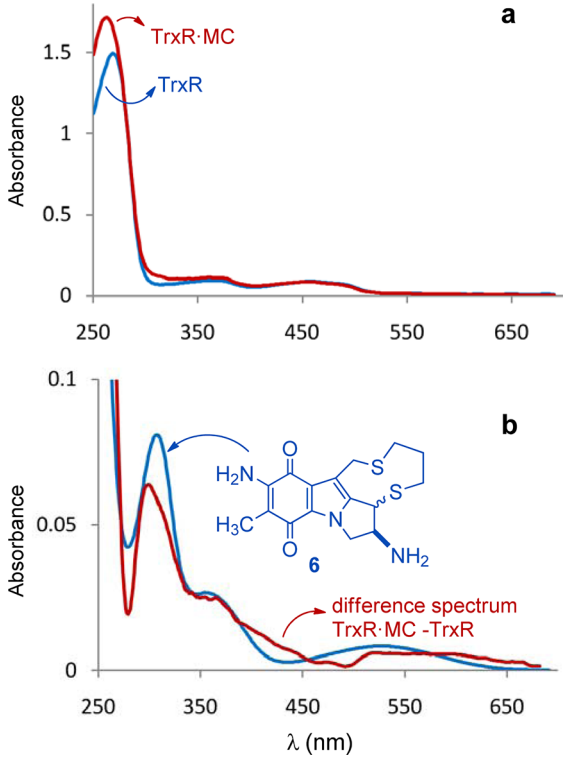

Figure 5. Characterization of the TrxR-MMC complex by UV spectroscopy. (a) UV spectrum of unmodified TrxR (blue) and MMC-inactivated TrxR (red). (b) Difference spectrum of MMC-modified TrxR minus TrxR (red); the spectrum of the mitosene **6** (blue) is shown for comparison.

and a weaker band centered at about 500 nm. This experiment also permitted the calculation of the stoichiometry mitosene/TrxR in the inactivated enzyme, by comparing the absorbance of the flavin (460 nm, ε 11300 M^{-1} cm^{-1}) and mitosene (305 nm, ε 11500 M^{-1} cm^{-1}) chromophores.[27] The observed ratio mitosene/flavin was 0.6:1 (original samples of TrxR had 50% specific activity), indicating that each molecule of inactivated enzyme contains a single mitosene molecule. CD is a technique that has been frequently used to ascertain the stereochemistry at C1 mitosenes substituted at that carbon, by determining the sign of the Cotton effect (CE) at a diagnostic band centered at around 550 nm.[32] Unmodified TrxR was not active in the diagnostic region; therefore, any CE observed for the modified enzyme in that region must be attributed to a mitosene-bound molecule. The CD spectrum of MMC-inactivated TrxR showed a positive band at about 600 nm, thus indicating a 1,2-*cis* configuration for the bound mitosene (Figure 6).

TrxR Is Inhibited in Cancer Cell Cultures Treated with MMC. We studied the toxicity of MMC on four cancer cell lines, Du145, Hela, A549, and MCF7, using the MTT assay to measure cell viability. Human prostate cancer cell Du145 cells were the most sensitive to MMC with an IC_{50} of 6 μM, and they were selected for the studies of cellular TrxR inhibition. The relative activity of TrxR in Du145 cells decreased with an increase in concentrations of MMC (Figure 7b) in a tendency

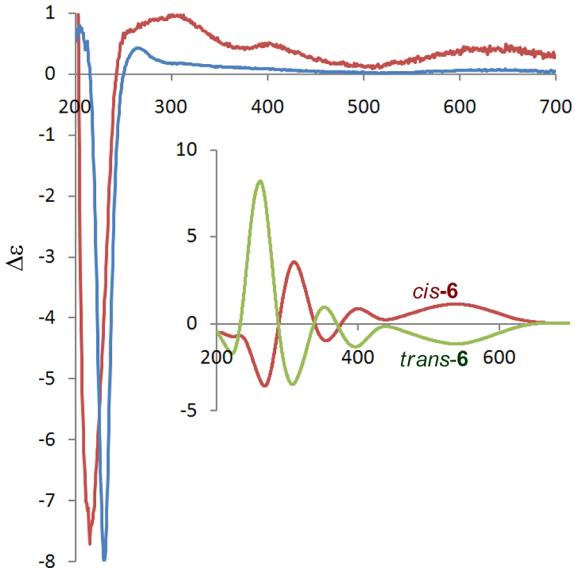

Figure 6. CD spectra of unmodified TrxR1 (blue) and MMC-inhibited TrxR1 (red). The CD spectra of *cis*-**6** (red) and *trans*-**6** (green) are shown in the inset for comparison.

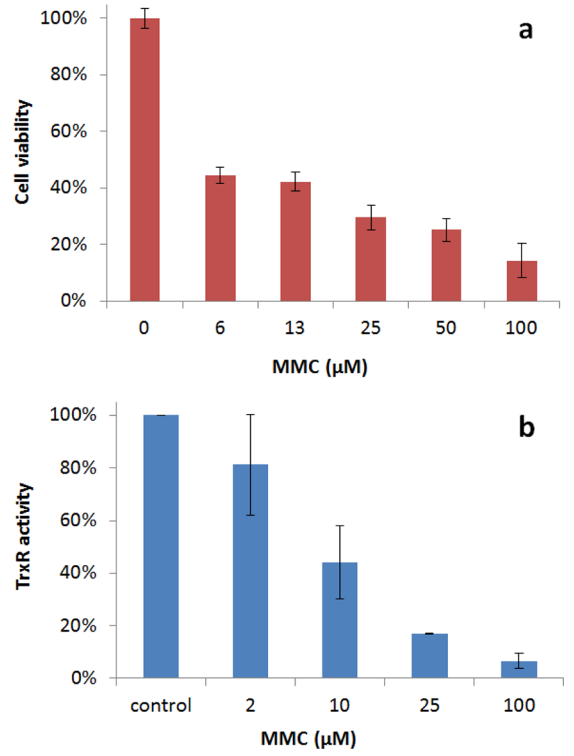

Figure 7. (a) Cell viability of cancer cells against MMC treatment. A549, Du145, Hela, and MCF7 cells were grown in a 96-well plate for 24 h and then treated with various concentrations of MMC. After another 24 h of treatment, the MTT assay was used to measure the cell viability. (b) Effect of MMC on TrxR activity in the cells. Du145 cells were treated with various concentrations of MMC for 24 h. The TrxR activity assay was performed in 96-well plates by an end point insulin assay with 25 μg of cell lysates.

that correlated the observed concentration-dependent cytotoxicity of the drug (Figure 7a). The protein level on MMC-treated cells was also analyzed, and only a slight decrease of TrxR1 was observed (Figure S7 in the Supporting Informa-

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tion), indicating that the observed low TrxR activity was not caused by a reduced expression of the enzyme and must therefore be attributed to TrxR inhibition by MMC

DISCUSSION

On the Kinetics of the Inhibition of TrxR by MMC. Our initial experiments on the inactivation of TrxR by MMC showed that the activity of TrxR was inhibited by increased concentrations of MMC, as determined by two experiments measuring the TrxR-catalyzed reduction of disulfide bonds using DTNB or insulin as substrates (Figure 1). This result contrasts markedly with a previous study that found that 10 mM MMC did not inhibit TrxR after incubation for 0 and 60 min.[33]

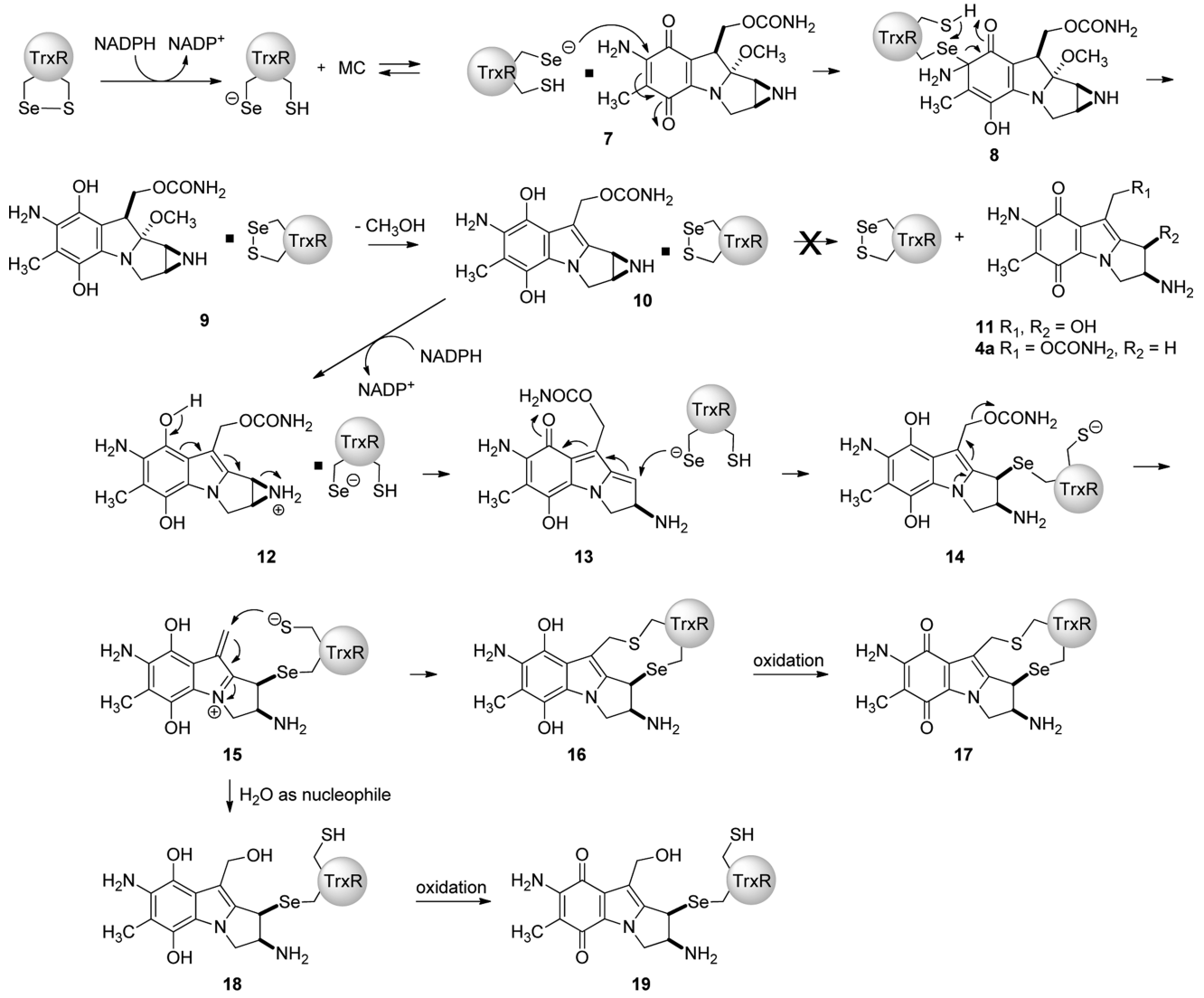
The working hypothesis that prompted this research was that TrxR could be inhibited by MMC by a mechanism analogous to the one that we proposed for the reductive activation of MMC by dithiols and the subsequent alkylation of dithiols by the activated drug (Scheme 2). If this hypothesis was correct, then the inactivation TrxR by MMC would be classified as mechanism-based inhibition, also known as suicide inhibition. Mechanism-based inhibition is characterized by being time- and concentration-dependent, irreversible, active-site directed, and requiring the conversion of enzyme-bound inhibitor to an active form. The inhibition of TrxR by MMC showed all of these characteristics. The inhibition is time- and concentration-dependent (Figure 3a), and it allowed the calculation of the two principal kinetic constants used to describe mechanism-based inhibitors: k_{inact} (rate constant for inactivation) and K_I (inhibitor concentration that produces half-maximal rate of inactivation). Data from several experiments gave an average k_{inact} of 0.017 min^{-1} and a K_I of 35 μM (Figure 3b). The inactivation of TrxR by MMC requires a selenolthiol active site in the reduction form, as evidenced by the full disulfide reducing activity presented by TrxR treated with MMC in the absence of NADPH (Figure 2).

Another diagnostic experiment to identify mechanism-based inhibitors is the addition of an external nucleophile in the inhibition reactions to compete with the enzyme for reaction with the inhibitor.[34] For mechanism-based inhibitors, the presence of this external nucleophile must have no effect in enzyme inhibition.[29] In our case, we used ethyl xanthate and GSH, two nucleophiles that are known to react very efficiently with reductively activated MMC.[10,11,35,36] The addition of ethyl xanthate produced a slight decrease in the rate on inhibition, but after longer incubation periods, the inactivation was equally efficient. Unexpectedly, the addition of GSH had a significant impact on the inhibition of TrxR by MMC, as the inactivating effect of MMC was almost completely abolished with the addition of 1 mM GSH (Figure 4b). This anomalous result can be explained considering that the C-terminal region of TrxR containing the selenolthiol active site is readily accessible, rather than buried, as it is the case for most enzyme active sites.[37] This ease of access may facilitate the addition of GSH to the enzyme-bound activated MMC, thus preventing inactivation. This result cannot be attributed to the reaction of GSH with the reactive species accumulating in solution after leaving the active site. If this was the case, then one should observe the formation of mitosene compounds (e.g., **4a** and **5a**), and this was not the case, as shown in experiments discussed below.

A relevant parameter in mechanism-based inhibition is the partition ratio, a value that represents the number of inactivator molecules metabolized per each molecule of enzyme inactivated. Its value can vary from zero, when the rate of metabolite formation is negligible, to infinity, when the rate of enzyme inactivation is negligible. One of the methods used for the determination of this parameter is the ratio k_{cat}/k_{inact}.[34] In our case, the value of k_{cat} is estimated to be close to zero, as all the experiments performed to learn if MMC was a substrate for TrxR were negative: If MMC was a substrate, we would expect to see TrxR-catalyzed oxidation of NADPH in the presence of MMC, but this was not the case. Additionally, if activated MMC was released from the active site, one should see a certain degree of formation of the known MMC-derived metabolites (1-hydroxymitosenes **11** and 2,7-diaminomitosene **4a**). The formation of **11** and **4a** after incubation of MMC with TrxR and NADPH was analyzed using two different methods: by monitoring the increase of absorbance at 550 nm characteristic of the 7-aminomitosene chromophore and by analyzing the formation of **11** and **4a** using HPLC. Both experiments indicated that mitosenes were not formed even after long reaction times and high concentrations of TrxR. Similar experiments were performed in parallel using MMA as substrate, and they did show that mitosene derivatives were formed. That the metabolites **11** or **4a** are not formed is evidence that activated MMC does not escape the active site prior to inactivation. Therefore, we must conclude that the value for the partition ratio for MMC must be close to zero, indicating that virtually every single molecule of MMC that is activated by the enzyme ends up covalently bound to the active site. [The value for the partition ratio calculated from end point assays ranged from 15 to 20 (Figures S6A,B in the Supporting Information), but we consider that partition ratios calculated using this method are overestimated: If these values were correct, then the experiments performed to determine if MMC is a substrate for TrxR should give a positive result, but this was not the case. Furthermore, the data obtained during the investigation of the parent drug MMA casted some doubts about the veracity of the partition ratio for MMC derived from end point assays. MMA was also an inhibitor for TrxR, and it gave a partition around 10 using the same methodology (Figures S6C,D in the Supporting Information). However, as opposed to MMC, MMA was a substrate for TrxR, as demonstrated by two experiments: Oxidation of NADPH was observed when MMA was treated with TrxR and NADPH (Figure S2 in the Supporting Information), and LC/MS analysis indicated full conversion of MMA to metabolites resulting from reductive activation of the drug (Figure S5 in the Supporting Information). This proves that MMA, after its reduction by TrxR, is released from the active site. It is obvious that the real value for the partition ratio for MMC *must* be lower than that of MMA, and we propose that the zero value inferred from k_{cat}/k_{inact} gives a more accurate estimation than the values derived from end point assays.]

The effect of pH on the inhibition of TrxR by MMC was also studied, and we found that the inactivation was pH-independent in the pH range from 6 to 8.5. This result contrasts markedly with the observed pH dependence of the inactivation of DT-diaphorase, an enzyme for which MMC is both a substrate and an inactivator.[38] We attribute this pH independence to the fact that the key step for both the reductive activation of MMC by TrxR and the inactivation involve a nucleophilic addition of selenoate. The pK_a of the selenol group is about 5; therefore, it will be fully deprotonated at all of the pH values studied,[39] reacting at very similar rates as

Scheme 3. Proposed Mechanism for the Mechanism-Based Inhibition of TrxR by MMC



a nucleophile independently of the pH in the two mechanistic steps of the inactivation where selenoate is involved.

Structural Characterization of the Modified Enzyme. Differential UV spectroscopy and CD spectroscopy are two ideally suited tools for the structural characterization of biological adducts formed by MMC, as both the MMC and the mitosenes resulting from activation of the drug show distinct bands in spectral regions where biological molecules do not interfere significantly.[40−43] The UV spectrum of MMC shows a characteristic absorption band at 364 nm that disappears when MMC is converted to mitosene derivatives, while the 7-aminomitosene chromophore presents a distinctive band at 300−320 nm. Subtracting the spectrum of unmodified TrxR from the UV spectrum of MMC-inactivated, TrxR should therefore reveal if the inactivated enzyme contains MMC or mitosene chromophores in its structure. The difference UV spectrum (Figure 5b) showed the characteristic spectrum of 7-aminomitosenes, with a main band at 306 nm, thus demonstrating that the inhibition had occurred by the formation of a covalent complex and that MMC had undergone a reductive activation process. Furthermore, this experiment allowed the calculation of the number of molecules of mitosene bound per molecule of enzyme in the inactivated enzyme. The calculated stoichiometry mitosene:TrxR was roughly 1:1 (based on active TrxR), thus fulfilling one additional attribute of mechanism-based inhibitors.

The addition of nucleophiles at C1 of activated MMC occurs by an S_N1 mechanism, resulting in the potential formation of two isomers at that carbon. CD spectroscopy allows us to distinguish the stereochemistry of mitosenes substituted at C1 by using the sign of the CE of the band at 500−600 nm: a positive CE is associated with a relative 1,2-*cis* configuration, while a negative CE is associated with a 1,2-*trans* configuration.[32] The CD spectrum of TrxR modified by MMC showed a positive CE in the diagnostic region (Figure 6), thus indicating a relative *cis*- configuration. As the aziridine ring-opening reactions of MMC occur with retention of configuration at C2, the absolute configuration at C1 in MMC-TrxR is established as *S*. It must be remarked that this stereochemistry is the opposite of the one observed in the alkylation reactions of DNA with MMC, where C1 adducts of dG are formed with *trans* configuration.[44]

On the Mechanism of Inhibition of TrxR by MMC. In accordance with the results discussed above, we propose the mechanism for the inactivation of TrxR by MMC shown in Scheme 3, which is analogous to the mechanism that we

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proposed previously for the reductive activation of MMC by dithiols[16] and the subsequent formation of dithiol cross-links by activated MMC[17] (Scheme 2).

The mechanism that we propose for the inhibition of MMC by TrxR starts by a conjugate addition of selenoate from the Sec-498 residue to the quinone ring of MMC to form the covalent complex **8**. The intermediate **8** then undergoes an internal redox reaction involving hydrogen transfer from Cys-497 to the bound drug to form the noncovalent complex **9**, containing the hydroquinone of MMC and an oxidized selenolthiol active site. The hydroquinone of MMC then rapidly experiences the known cascade of reactions that generate the active intermediate (**9** → **10** → **12** → **13**),[45] while NADPH reduces the active site of TrxR back to the selenolthiol form. Michael addition at C1 of selenoate from Sec-498 forms the monoadduct **14**. Elimination of carbamate in **14** gives **15**, which undergoes a second-arm addition of thiolate from Cys-497 forming the cross-link at the active site **16**. Lastly, oxidation of the hydroquinone ring generates the final adduct **17**. Alternatively, the intermediate **15** could add H_2O at the second electrophilic position, and in that case, the enzyme would be inactivated by the formation of **18**, which then oxidizes to form the monoadduct **19**. Further work will be necessary to ascertain the precise structure of the modified enzyme. A mechanism directly related to the one presented above for MMC may be responsible for the inactivation of TrxR by indoloquinones structurally related to 2,7-diaminomitosene **4a**.[46] TrxR has been proposed as the likely cellular target for these compounds, and in similitude to the mechanism that we propose here for MMC, the inactivation of TrxR by these compounds also involves a reductive activation that generates a reactive electrophile that inhibits the enzyme by alkylation of the Sec residue in the active site.

On the Biological Implications of the Inhibition of TrxR by MMC. The biological mode of action of MMC has been the subject of a vast amount of research in the last 5 decades, starting with the pioneering work of Iyer and Szybalski, who first proposed that MMC was able to cross-link complementary strands of DNA[47] and a chemical mechanism to explain the conversion of an initially inert drug to a highly reactive bis-electrophile by a cascade of reactions initiated by reduction of the quinone ring.[48] The cytotoxic effects of MMC are regularly attributed to the formation of a DNA−DNA interstrand cross-link, a lesion that is formed after two sequential alkylations of DNA by leucoaziridinomitosene **3a** at positions 1 and 10 (Scheme 1).[5] This lesion is formed exclusively at CpG sites of double strand DNA by binding the exocyclic N-2 amino group of deoxyguanosine residues in opposite strands.[8] The cytotoxicity of the interstrand cross-link lesion is manifested by the observation that a single cross-link per genome suffices to kill a bacterial cell.[47] However, considering the high reactivity of reductively activated MMC, it is most likely that there are other cellular targets for alkylation that contribute to the biological effects of the drug. In this context, it was recently found that rRNA is a target for MMC.[12]

The finding that TrxR is inhibited by MMC in vitro and in cell cultures leads us to postulate that TrxR is a major cellular target for MMC in cancer cells. TrxR is known to be involved in cancer development and progression.[20−23] Recent research has evidenced that TrxR is critical for the self-sufficient growth of malignant cells, indicating that it acts as a pro-cancer protein.[49] It has been hypothesized that TrxR plays a role in drug resistance by eliciting pro-survival signals that help cancer cells evade the effects of antitumor drugs and that TrxR inhibition may reverse this effect.[50,51] It was also found recently that TrxR blocks the generation of reactive oxygen species (ROS) in cells grown under hypoxic conditions.[52] The inhibition of TrxR by MMC may promote the cytotoxic effects of the drug by circumventing the resistance pathways induced by high TrxR activity. TrxR inhibition might prevent the antioxidant defense that this enzyme provides, and it could lead to an increase in the damage inflicted by ROS, known to be generated by redox cycling of the MMC quinone after its reduction by 1-electron reductases.[53,54] It has also been shown that low cellular levels of Trx increase the sensitivity of tumor cells to MMC.[55] The inhibition of TrxR will result in low levels of Trx activity and, therefore, render the cell more susceptible to the cytotoxic effects of the drug.

The inhibition of TrxR by DNA alkylating agents is well precedented.[56] Other antitumor drugs in clinical use that target both DNA and TrxR include cisplatin,[57] carmustine,[58] ifosfamide,[59] and doxorubicin.[60] The Trx system is emerging as a novel target in cancer therapy, and several drugs that target Trx or TrxR are currently under development.[61−64] The finding that MMC inhibits TrxR lends further strength to the validation of TrxR as a target for anticancer drugs.

Our results indicate that MMC might function as a multitarget drug, exerting its anticancer activity by binding several cellular targets, including DNA, RNA, and TrxR. The notion that "dirty" or "promiscuous" drugs are more efficient as antitumor agents than single-target drugs is recently gaining strong support among the scientific community.[65−68] The proven therapeutic effectiveness of MMC in the treatment of cancers is likely a consequence of a dirty mode of action, impacting simultaneously several cellular events, including the inhibition of TrxR described here.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CD, circular dichroism; CE, cotton effect; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); DTT, D,L-dithiothreitol; GSH, glutathione; MMA, mitomycin A; MMC, mitomycin C; mitosene, structure 4 without substituents in the 1-, 2- and 7-positions; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Trx, thioredoxin; TrxR, thioredoxin reductase; Sec, selenocysteine

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