Maleimide Is a Potent Inhibitor of Topoisomerase II in Vitro and in Vivo: A New Mode of Catalytic Inhibition

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ABSTRACT

Maleimide, N-ethyl-maleimide (NEM), and N-methyl-maleimide (NMM) were identified as potent catalytic inhibitors of purified human topoisomerase IIα, whereas the ring-saturated analog succinimide was completely inactive. Catalytic inhibition was not abrogated by topoisomerase II mutations that totally abolish the effect of bisdioxopiperazine compounds on catalytic inhibition, suggesting a different mode of action by these maleimides. Furthermore, in DNA cleavage assay maleimide and NEM could antagonize etoposide-induced DNA double-strand breaks. Consistently, maleimide could antagonize the effect of topoisomerase II poisons in three different in vivo assays: 1) In an alkaline elution assay maleimide protected against etoposide-induced DNA damage. 2) In a band depletion assay maleimide reduced etoposide-induced trapping of topoisomerase IIα and β on DNA. 3) In a clonogenic assay maleimide antagonized the cytotoxicity of etoposide and daunorubicin on four different cell lines of human and murine origin. at-MDR cell lines with reduced nuclear topoisomerase IIα content are fully sensitive to maleimide, indicating that it is not a topoisomerase II poison in vivo. Our finding that topoisomerase II is sensitive to maleimide, NMM, and NEM but insensitive to succinimide demonstrates a strict requirement for the unsaturated ring bond for activity. We suggest that the observed antagonism in vitro and in vivo is caused by covalent modification of topoisomerase II cysteine residues reducing the amount of catalytically active enzyme sensitive to the action of topoisomerase II poisons.

Type II topoisomerases are nuclear enzymes that regulate the topology of DNA in all living cells (Wang, 1996) by transferring one DNA double helix (the transfer-DNA segment) through a transient break in another DNA double helix (the gate-DNA segment) (Roca and Wang, 1992, 1994).

Cellular topoisomerase II continuously generates transient protein-linked double-strand breaks in DNA, and it is this conformation called the cleavable complex, that is the target of many clinically important anticancer drugs used today, such as the anthracyclines (daunorubicin and doxorubicin), the epipodophyllotoxins (etoposide and teniposide), and the aminoacridines (m-AMSA). Because these drugs stabilize the cleavable complex that leads to the accumulation of cytotoxic DNA breaks, they are referred to as DNA topoisomerase II poisons (Liu, 1989; Chen and Liu, 1994).

Another major class of topoisomerase II-directed drugs is the catalytic inhibitors. They inhibit the catalytic activity of topoisomerase II without generating DNA breaks, thus depriving cells of essential topoisomerase II catalytic activity also leading to cell death (Andoh and Ishida, 1998). Aclarubicin is known to intercalate into DNA, thereby preventing the binding of topoisomerase II to DNA (Jensen et al., 1990, 1991). Chloroquine, which also intercalates into DNA, displays a pH-dependent inhibition of cellular topoisomerase II activity as its diffusion across the cell membrane is facilitated at lowered pH (Langer et al., 1999). Finally, merbarone prevents cleavage of the gate-DNA strand acting at the same stage in the catalytic cycle as the topoisomerase II poisons (Fortune and Osheroff, 1998).

The bisdioxopiperazine compounds stabilize a transient configuration of topoisomerase II on DNA called the poststrands passage closed-clamp configuration, thereby preventing enzymatic turnover (Ishida et al., 1991; Tanabe et al., 1991; Roca et al., 1994; Morris et al., 2000). Due to the inhibition of enzymatic turnover, the bisdioxopiperazine compounds are classified as catalytic topoisomerase II inhibitors. However, recent data suggest that the closed-clamp configuration of topoisomerase II on DNA may act as a new kind of noncovalent poison interfering with metabolic DNA processes (van Hille and Hill, 1998; Wessel et al., 1999; Jensen et al., 2000a,b; Kobayashi et al., 2001). Despite this

ABBREVIATIONS: m-AMSA, amsacrine; NMM, N-methyl-maleimide; NEM, N-ethyl-maleimide; wt, wild-type; kDNA, kinetoplast DNA; BSA, bovine serum albumin; DTT, dithiothreitol; DSB, double-strand break; SSB, single-strand break.
newly recognized aspect of the bisdioxopiperazine compounds, ICRF-187 can antagonize the cytotoxicity of topoisomerase II poisons in cultured mammalian cells (Sehested et al., 1998; Wessel et al., 1999) as well as in mice. The latter has been used to target topoisomerase II poisons to the central nervous system (Holm et al., 1998). It was further recently demonstrated that systemic ICRF-187 administration protects against necrosis induced by subcutaneous doxorubicin and daunorubicin injection in mice (Langer et al., 2000).

Due to structural similarities between maleimide/succinimide (Fig. 1, A and B) and the 2,4-dioxopiperazine rings found in the bisdioxopiperazine compounds (Fig. 1, C and D), these drugs as well as the N-substituted methyl- and ethyl maleimide analogs NMM and NEM were assessed as inhibitors of topoisomerase II catalytic activity. In the present study we present evidence that maleimide, NMM, and NEM but not succinimide inhibit topoisomerase II catalytic activity in vitro and that maleimide but not succinimide is capable of antagonizing the effect of topoisomerase II poisons in vivo.

NMM and NEM have been extensively used for enzyme modification and labeling by reacting with the SH group of cysteine residues in a process known as Michael addition, thereby alkylating these. It has recently been shown that the in vitro cleavage activity of human topoisomerase II was stimulated by a variety of compounds capable of acting as Michael acceptors, including NEM (Wang et al., 2001), suggesting that topoisomerase II is sensitive to thiol alkylation. Our finding that maleimide, NMM, and NEM but not succinimide inhibit topoisomerase II catalytic activity in vitro and that maleimide but not succinimide is capable of antagonizing the effect of topoisomerase II poisons in vivo.

**Materials and Methods**

**Drugs.** Maleimide, NMM, and NEM (all from Sigma-Aldrich, St. Louis, MO), ICRF-187 (Cardioxane; Chiron Corp., Amsterdam, The Netherlands), doxorubicin (Pharmacia, Copenhagen, Denmark), idarubicin, (Pharmacia), and daunorubicin (Cerubin; Aventis, Birked, Denmark) were dissolved in sterile distilled water. Etoposide (Bristol-Myers Squibb Co., Lyngby, Denmark), teniposide (Bristol-Myers Squibb Co.), and mitoxantrone (Lederle, Glostrup, Denmark) were in solution for infusion. Maleimide, NMM, and NEM were dissolved in water just before use, due to rapid hydrolysis.

**Cells.** The classical human SCLC cell line NCI-H69 is described in (Carney et al., 1985). The two derivatives of this cell line, NCI-H69/DAU and NCI-H69/VP, have been developed in our laboratory by selection to gradually increasing concentrations of daunorubicin and etoposide, respectively, as described previously (Jensen et al., 1989, 1993b). The variant human SCLC cell line OC-NYH is described in de Leij et al. (1985). The murine cell lines EHR2 and L1210 have been described in Friche et al. (1991) and Jensen et al. (1993a, respectively.

**Preparation of ³H-Labeled Kinetoplast DNA (kDNA).** Tritium-labeled kDNA was isolated from Crithidia fasciculata as described in Sahai and Kaplan (1986).

**Purification of Human Topoisomerase IIα.** The purification of wt and mutant human topoisomerase IIα from overexpressing yeast cells was carried out as described in Wessel et al. (1999).

**Determination of Topoisomerase II Catalytic Activity.** Topoisomerase II catalytic activity was measured by kDNA decatenation assay. ³H-Labeled kDNA (200 ng) isolated from C. fasciculata was incubated with increasing concentrations of drug in 20 μl of reaction buffer containing 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 15 μg/ml BSA, and 1 mM Na₂ATP (all from Sigma-Aldrich) with 1 U of purified wt or mutant topoisomerase IIα for 20 min at 37°C. One unit of activity was defined as the amount of enzyme required for complete decatenation in the absence of drug. After addition of stop buffer containing 5% Sarkosyl, 0.0025% bromphenol blue, and 25% glycerol (all from Sigma-Aldrich), unprocessed kDNA network, and decatenated DNA circles were separated by filtering, and the amount of unprocessed kDNA in each reaction was determined by scintillation counting (Packard BioScience, Meriden, CT).

**Plasmid Cleavage Assay.** A modification of the protocol described in Burden et al. (2001) was used. Purified human topoisomerase IIα (350 ng), 400 ng of pUC18 DNA, and increasing concentrations of drugs were incubated for 6 min at 37°C in 20 μl of topoisomerase II cleavage buffer (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 15 μg/ml BSA, and 1 mM Na₂ATP; all from Sigma-Aldrich). Next, the cleavable complex was trapped by adding 2 μl of 10% SDS. After vigorous vortexing 1.5 μl of 0.25 M EDTA and 2 μl of proteinase K (0.8 μg/ml) in proteinase buffer (50 mM Tris-HCl pH 7.9 and 1 mM CaCl₂) was added and the samples vortexed. After a 30-min incubation at 45°C, 5 μl of loading buffer (5% Sarkosyl, 0.0025% bromphenol blue, and 25% glycerol) was added and the samples were exposed to 70°C for 5 min. Next, samples were run through a 0.8% agarose gel in 1× Tris/acetate/EDTA buffer for 3 h at 9 V/cm to separate different topological forms of plasmid DNA. Finally the gels were stained in distilled water containing 10 μg/ml ethidium bromide for 15 min followed by destaining in distilled water for 1 h before they were photographed in UV light.

**Clonogenic Assay.** A 3-week clonogenic assay using soft agar on a sheep red blood cell feeder layer was used as described in Jensen et al. (1993b). For the study of maleimide-mediated antagonism of topoisomerase II poison-induced cytotoxicity cells were incubated with the appropriate drugs for a fixed time, washed twice, and then plated in agar in the absence of drugs. For the study of cytotoxicity itself continuous drug exposure was used.

**Alkaline Elution.** For the determination of drug-induced DNA damage in vivo the alkaline elution assay (Kohn et al., 1976) was used with modifications as described in Sehested et al. (1998).

**Depletion of Cellular ATP.** Cellular ATP depletion was obtained by incubating cells with azide and 2-deoxyglucose (both from Sigma-Aldrich) as described in Sorensen et al. (1999).

**Band Depletion Assay.** Band depletion assay was performed as described in Sehested et al. (1998) by using the enhanced chemilu-
minescence detection method (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). In experiments assessing maleimide-mediated antagonism of etoposide-induced band depletion, maleimide was added 20 min before etoposide to exert its effect on cellular topoisomerase II before the addition of the poison. For detection of topoisomerase IIα and β two polyclonal primary antibodies (BioTrend, Cologne, Germany) were used. For the detection of these antibodies the same horseradish peroxidase linked anti-rabbit antibody (Amersham Biosciences UK, Ltd.) was used. For the detection of topoisomerase I a mouse monoclonal primary antibody kindly provided by Dr. Y. C. Chang (Yale University, New Haven, CT) was used. Herein, horseradish peroxidase-linked anti-mouse antibody (Amersham Biosciences UK, Ltd.) was used as secondary antibody.

Results

Maleimide NMM and NEM Are Potent Inhibitors of Human Topoisomerase IIα Catalytic Activity. Decatena-
tion of 3H-labeled C. fasciculata kDNA by human topoi-
somerase IIα wt protein as well as Y50F (Sehested et al., 1998), Y165S (Wessel et al., 2000), and L169F (Jensen et al., 2000b) mutant proteins in the presence of increasing concentrations of maleimide, NMM, and NEM shows that these maleimides are potent concentration-dependent inhibitors of human topoisomerase IIα catalytic activity. From Fig. 2, A to C, it can be seen that Y50F, Y165S, and L169F proteins, which are all completely insensitive toward catalytic inhibition by bisdioxopiperazine compounds (Fig. 2F), although retaining their sensitivity toward etoposide (Fig. 2E), all display maleimide, NMM, and NEM sensitivities comparable with wt protein. This suggests a mode of catalytic inhibition by these drugs different from that of the bisdioxopiperazines. When decatenation of wt and mutant human topoisomerase IIα was performed in the presence of succinimide, a ring-saturated analog of maleimide having no double bond and thus incapable of serving as a Michael acceptor, no inhibition of decatenation was observed (Fig. 2D), demonstrating a strong requirement for the unsaturated ring bond for catalytic topoisomerase II inhibition. DTT had to be omitted in these decatenation reactions because it reacts with the maleimide compounds, thereby reducing their concentration. Much higher and highly irreproducible IC_{50} values were obtained when DTT was included (data not shown). The BSA concentration used in the reactions is adapted from a commercially available topoisomerase II buffer (U.S. Biochemical Corp., Cleveland, OH). Apparently, the cysteines derived from BSA do not compete with those derived from topoisomerase II to an extent that precludes efficient catalytic inhibition of topoisomerase II.

Maleimide and NEM Antagonize Etoposide-Induced DNA Damage In Vitro. To evaluate whether maleimide and NEM promote topoisomerase II-induced DNA damage in vitro, we used a plasmid cleavage assay described in Burden et al. (2001) with minor modifications (see Materials and Methods). By using this assay etoposide clearly induced an inhibition of topoisomerase II catalytic activity by various drugs by using purified wt and mutant human topoisomerase IIα. Decatena-
tion of C. fasciculata kDNA was used to determine the inhibition of topoisomerase II catalytic activity by various drugs by using purified wt and mutant human topoisomerase IIα. One unit of activity (defined as the amount required to decatenate 200 ng of kDNA at 37°C in 20 min) was incubated with 200 ng of kDNA and increasing concentrations of maleimide in a 20-μl reaction volume at 37°C for 20 min as described under Materials and Methods. After separation from decat- enated mini circles, the amount of unprocessed kDNA expressed as percentage not decatenated, i.e., percentage not processed by the enzyme was plotted against the drug concentration to reveal dose-response curves, where 100% corresponds to the radioactivity present when no enzyme is applied. Effect of maleimide (A), effect of NMM (B), effect of NEM (C), effect of succinimide (D), effect of etoposide (E), and effect of ICRF-187 (F). In Fig. 3, A to D, error bars represent S.E.M. of five independent experiments, whereas error bars in Fig. 3, D to F, represent S.E.M. of two to three independent experiments.
Maleimide Protects against Etoposide-Induced DNA Damage in Vivo. To assess the effect of maleimide and NEM on DNA damage in living cells, we used an alkaline elution assay (Kohn et al., 1976) to test the ability of equimolar concentrations of NEM and maleimide to induce DNA damage (Fig. 4A). Because the assay is performed at alkaline pH, the DNA damage detected is both DNA DSBs and SSBs. Although NEM was found to cause a slight induction of DNA damage at all concentrations tested, the level of DNA damage is much lower than what is caused by 3 μM etoposide, a typical topoisomerase II poison. The amount of DNA damage induced by maleimide is even lower but can be detected, because the “no drug” graph is clearly the most flat. It is concluded that maleimide and NEM do not cause substantial DNA damage in vivo at concentrations up to 500 μM.

Maleimide Causes Extensive Band Depletion of Topoisomerase II at High Concentrations. The band depletion assay can be used to assess drug-mediated binding of proteins to DNA in vivo. In this assay protein is extracted from cells after preincubation with drug. If a drug causes an increase in DNA binding, less protein will appear in the cell extract due to fixation on DNA. Figure 6A, lanes 1 to 4, shows the extractable amount of topoisomerase IIα after incubation with increasing concentrations of maleimide. It is evident that maleimide causes a pronounced dose-dependent depletion of the α-isoform. Figure 6C, lanes 1 to 4, shows band depletion of topoisomerase IIβ. The β-isoform is also depleted by maleimide, but to a lesser extent (compare lanes 1–4 in

Maleimide and NEM Cause Low Levels of DNA Damage in Vivo. To assess the effect of maleimide and NEM on DNA damage in living cells, we used an alkaline elution assay (Kohn et al., 1976) to test the ability of equimolar concentrations of NEM and maleimide to induce DNA damage (Fig. 4A). Because the assay is performed at alkaline pH, the DNA damage detected is both DNA DSBs and SSBs. Although NEM was found to cause a slight induction of DNA damage at all concentrations tested, the level of DNA damage is much lower than what is caused by 3 μM etoposide, a typical topoisomerase II poison. The amount of DNA damage induced by maleimide is even lower but can be detected, because the “no drug” graph is clearly the most flat. It is concluded that maleimide and NEM do not cause substantial DNA damage in vivo at concentrations up to 500 μM.
Fig. 6, A and C). Coincubation with azide and 2-deoxyglucose, which together reduce the level of ATP in OC-NYH cells to less than 5% of the normal value (Sorensen et al., 1999), exerts only minor effects on the extent of band depletion (compare lanes 1–4 with lanes 5–8 in Fig. 6, A and C). In contrast, band depletion of topoisomerase IIα as well as topoisomerase IIβ caused by 50 μM ICRF-187 was clearly reduced at low ATP levels (compare lanes 2 and 4 in Fig. 6, B and D). We are thus able to extend the lack of ATP dependence seen with thiol-reactive compounds in Wang et al. (2001) to an in vivo setting. Figure 6E, lanes 1 to 4, show the amount of extractable topoisomerase I after preincubation with increasing concentrations of maleimide. No band depletion is evident.

Maleimide Antagonizes Etoposide-Mediated Band Depletion of Topoisomerase II at Low Concentrations. Etoposide is capable of band depleting topoisomerase II by trapping the enzyme on DNA in the form of covalent cleavable complexes. We therefore assessed the effect of increasing concentrations of maleimide on etoposide-induced band depletion of topoisomerase IIα and β. Antagonism of etoposide-induced depletion of topoisomerase IIα and β (by 100 μM etoposide) could be observed between 4 and 50 μM maleimide. At 100 and 200 μM (lanes 11–14, Fig. 7), maleimide causes band depletion per se in accordance with the data presented in the previous section.

At-MDR Cell Lines Display wt Sensitivity to Maleimide. The results outlined in the previous sections clearly demonstrate that maleimide inhibits the catalytic activity of topoisomerase II both in vitro and in vivo. We therefore tested the sensitivity of two at-MDR cell lines both displaying cross-resistance to typical topoisomerase II poisons against maleimide. Figure 8 shows the sensitivity toward maleimide of the SCLC cell line NCI-H69 and its two derivatives, NCI-H69/DAU (Jensen et al., 1997) and NCI-H69/VP (Wessel et al., 1997). Both sublines display reduced nuclear topoisomerase II levels due to down-regulation (NCI-H69/DAU) or due to extranuclear localization (NCI-H69/VP). In addition to the reduced topoisomerase II levels NCI-69H/DAU overexpresses P-glycoprotein in the cell membrane, whereas NCI-
69H/VP overexpresses both P-glycoprotein and multidrug resistance protein. It is seen that these three cell lines display equal sensitivity toward maleimide, thus demonstrating that maleimide is not a substrate for the P-glycoprotein and multidrug resistance protein drug efflux pumps, and that maleimide does not act as a topoisomerase II poison. It is also noted that NCI-H69 and its derivatives are slightly less sensitive to maleimide than OC-NYH cells when continuous drug exposure is used (compare Figs. 5E and 8).

Discussion

It has been shown recently (Wang et al., 2001) that diverse classes of thiol-reactive compounds, including NEM, stimulate the DNA cleavage activity of purified topoisomerase II. It was also shown that a “cysteineless” yeast topoisomerase II obtained by site-directed mutagenesis was insensitive to the thiol-reactive compound jugalbone. Finally, in the same study the potency of DNA cleavage stimulation among a panel of quinone congeners correlated well with their electrophilic addition potential. Together, these findings lead the authors to suggest that alkylation of human topoisomerase II via Michael addition to cysteine residues was the common mechanism of action leading to stimulation of DNA cleavage when these drugs were coincubated with topoisomerase II and DNA, as well as inhibition of catalytic activity in the case of preincubation of topoisomerase II and drugs before DNA addition.

We found that decatenation of C. fasciculata kDNA by human topoisomerase IIα was strongly inhibited by maleimide, NMM, and NEM with IC₅₀ values in the range of 5 to 10 μM (Fig. 2, A–C). Furthermore, the presence of amino acid substitutions in human topoisomerase IIα, which completely abrogate the inhibitory effect of bisdioxopiperazine compounds on decatenation Y50F, Y165S, and L169F (Fig. 2F) had little effect on maleimide, NMM, and NEM sensitivity (Fig. 2, A–C), suggesting that these maleimides inhibit topoisomerase II catalytic activity by a different mechanism than...
the bisdioxopiperazines, although structural similarities exist.

Succinimide, which is the ring-saturated analog of maleimide, did not inhibit the catalytic activity of wt and mutant topoisomerase IIα protein even at micromolar concentrations (Fig. 2D), suggesting that maleimide as well as NMM and NEM interact with topoisomerase II via their unsaturated ring bond due to Michael addition. The fact that DTT has to be omitted in the reactions to achieve efficient catalytic inhibition also supports this mechanism of action. Therefore, our data likewise suggest that protein alkylation plays an important role in the effect of thiol-reactive compounds on topoisomerase II.

Antagonism of Topo II Poisons by Thiol-Reactive Compounds

Fig. 6. Maleimide is able to band deplete topoisomerase IIα and β but not topoisomerase I. The formation of topoisomerase IIα, topoisomerase IIβ, and topoisomerase I complexes on DNA was measured using band depletion assay as described under Results and Materials and Methods, and references therein. A, detection of topoisomerase IIα in whole cell extracts after preincubation with maleimide. Lane 1, no drug; lane 2, 50 μM maleimide; lane 3, 100 μM maleimide; lane 4, 250 μM maleimide; lane 5, azide (AZ) + 2-deoxyglycosyl (2-DOG); lane 6, AZ + 2-DOG + 50 μM maleimide; lane 7, AZ + 2-DOG + 100 μM maleimide; and lane 8, AZ + 2-DOG + maleimide 250 μM. B, detection of topoisomerase IIα in whole cell extracts after preincubation with ICRF-187. Lane 1, no drug; lane 2, 50 μM ICRF-187; lane 3, AZ + 2-DOG; and lane 4, AZ + 2-DOG + 50 μM ICRF-187. C, detection of topoisomerase IIβ in whole cell extracts after preincubation with maleimide. Lane 1, no drug; lane 2, 50 μM maleimide; lane 3, 100 μM maleimide; lane 4, 250 μM maleimide; lane 5, AZ + 2-DOG; lane 6, AZ + 2-DOG + 50 μM maleimide; lane 7, AZ + 2-DOG + 100 μM maleimide; and lane 8, AZ + 2-DOG + maleimide 250 μM. D, detection of topoisomerase IIβ in whole cell extracts after incubation with ICRF-187. Lane 1, no drug; lane 2, 50 μM ICRF-187; lane 3, AZ + 2-DOG; and lane 4, AZ + 2-DOG + 50 μM ICRF-187. E, detection of topoisomerase I in whole cell extracts after incubation with maleimide. Lane 1, no drug; lane 2, 50 μM maleimide; lane 3, 100 μM maleimide; and lane 4, 250 μM maleimide. A, representative data from four independent experiments performed with individually prepared extracts. B to D, representative data from two independent experiments also performed with individually prepared extracts.

Fig. 7. Maleimide causes concentration-dependent antagonism of etoposide-induced band depletion of topoisomerase IIα and β. OC-NYH cells were incubated with increasing concentrations of maleimide for 10 min before the addition of 100 μM etoposide followed by coincubation during 30 min before the cells were lysed. Western blot was performed as described under Materials and Methods. A, effect of maleimide on etoposide-induced depletion of topoisomerase IIα. Lane 1, no drug; lane 2, 100 μM etoposide; lane 3, 4 μM maleimide; lane 4, 4 μM maleimide + 100 μM etoposide; lane 5, 10 μM maleimide; lane 6, 10 μM maleimide + 100 μM etoposide; lane 7, 20 μM maleimide; lane 8, 20 μM maleimide + 100 μM etoposide; lane 9, 50 μM maleimide; lane 10, 50 μM maleimide + 100 μM etoposide; lane 11, 100 μM maleimide; lane 12, 100 μM maleimide + 100 μM etoposide; lane 13, 200 μM maleimide; and lane 14, 200 μM maleimide + 100 μM etoposide. B, effect of maleimide on etoposide-induced depletion of topoisomerase IIβ. Drug treatments are similar to that in A. This figure shows representative data of two independent experiments.

Fig. 8. Human SCLC cells with reduced topoisomerase II show wt sensitivity to maleimide. The SCLC cell line NCI-H69 and its two derivatives NCI-H69/DAU and NCI-H69/VP, which both contain reduced topoisomerase II amounts in the nucleus, were exposed to increasing concentrations of maleimide in clonogenic assay as described under Materials and Methods by using continuous drug exposure. It is evident these two at-MDR cell lines are fully sensitive to maleimide. This figure shows representative data of one of two experiments with similar results.
Although it was previously shown that preincubation of topoisomerase II and the thiol-reactive compounds menadione and plumbagin before the addition of DNA reduces the catalytic activity of topoisomerase II as well as its ability to cleave DNA (Wang et al., 2001), the present decatenation experiments demonstrate that addition of topoisomerase II to a mixture of DNA and a thiol-reactive compound can also result in inhibition of catalytic activity. It appears that the covalent modification of topoisomerase II leading to enzymatic inactivation happens very fast.

Our results concerning stimulation of DNA cleavage by maleimide and NEM indicate that these drugs do not stimulate DNA damage at concentrations up to 10 mM (Fig. 3, B and C), whereas etoposide and m-AMSA was found to stimulate DNA damage in accordance with their known function (Fig. 3A; data not shown). Thus, our cleavage data concerning NEM is in contrast to the cleavage data presented in Wang et al. (2001) where DNA DSB cleavage was clearly stimulated by 800 μM NEM. The reason for this discrepancy remains unclear. However, in Wang et al. (2001) data were obtained using another cleavage assay detecting double-strand cleavage of a linear end-labeled DNA fragment by autoradiography. It may be that our assay is simply not sensitive enough to detect the level of DNA DBSs induced by NEM (and maleimide). The fact that low levels of DNA damage are seen with both NEM and maleimide in alkaline elution assay supports this notion.

Addition of topoisomerase II to a mixture of DNA, maleimide, and etoposide or to a mixture of DNA, NEM, and etoposide could antagonize the stimulation of DNA DSBs by etoposide (Fig. 3, D and E), which is in good accordance with the decatenation data. It seems that maleimide and NEM are able to modify topoisomerase II, thereby inhibiting its catalytic activity very efficiently so the protein is no longer capable of etoposide-induced DNA DSB stimulation.

We were able to extend the observed maleimide-mediated antagonism of etoposide-induced DNA damage to an in vivo setting. Using the alkaline elution assay that detects both DNA SSBs and DSBs, we demonstrated that the level of DNA damage induced by 3 μM etoposide could be antagonized by maleimide in a dose-dependent manner (Fig. 4B). The simplest explanation for this observation is that maleimide reduces the level of catalytically active cellular topoisomerase II available for the action of etoposide. When applied alone maleimide as well as NEM produced very limited DNA damage, supporting the in vitro DNA cleavage data (Fig. 4A). This in vivo antagonism was further confirmed using the band depletion assay. Herein, low concentrations of maleimide incapable of band depleting topoisomerase II by itself could antagonize etoposide-induced band depletion of both topoisomerase IIα (Fig. 7A) and β (Fig. 7B). Thus, a clear correlation between antagonism of etoposide-mediated DNA damage as assessed in alkaline elution assay, and inhibition of topoisomerase II catalytic activity as assessed in band depletion assay (antagonism of etoposide-mediated trapping) is established, because these effects are seen at similar maleimide concentrations.

Our finding that high concentrations of maleimide cause extensive band depletion of cellular topoisomerase II at concentrations where DNA damage is only limited in alkaline elution assay is interesting (Figs. 4A and 6, A and C). This suggests that maleimide/topoisomerase II complexes trapped on DNA do not represent covalent cleavable complexes, again supporting the notion that maleimide functions primarily as a catalytic topoisomerase II inhibitor and not as a topoisomerase II poison in living cells. The nature of the topoisomerase II/DNA interaction induced by maleimide in vivo remains unclear and it should be interesting to study these interactions further with in vitro settings.

We found that maleimide was more efficient in depleting topoisomerase IIα (Fig. 6A) than topoisomerase IIβ (Fig. 6B). Assuming that maleimide exerts its effect through cysteine alkylation, comparing the number and location of cysteine residues in the α- and β-isofoms seems relevant. Topoisomerase IIα contains 13 cysteine residues, whereas the number is 17 in topoisomerase IIβ. Twelve cysteines are conserved between the α- and β-isofom, and most are located within highly conserved functional protein domains. Although this explains why cysteine modification has such dramatic consequences for protein function, it fails to explain the observed isoform selectivity. We conclude that the observed difference in sensitivity is most likely not related to differences in the location of cysteine residues. It is possible that it is the consequences of cysteine modification that differs between the α- and the β-isofom, i.e., modification a homologous cysteine residue may have different effect on enzyme function in the two isoforms. This hypothesis is supported by the fact that topoisomerase I is not depleted at all, although it has eight cysteine residues.

Maleimide was able to antagonize the cytotoxicity of the two well characterized topoisomerase II poisons etoposide and daunorubicin by using four different cell lines of both murine and human origin, demonstrating that topoisomerase II is also a functional biological in vivo target for maleimide. This opens up the possibility for pharmacological regulation of topoisomerase II poisons by thiol-reactive compounds.

The apparent lack of correlation between nuclear topoisomerase II content and maleimide cytotoxicity seen with the two at-MDR cell lines is puzzling. This could indicate that topoisomerase II is not the primary cellular mediator of maleimide-induced cytotoxicity. It can therefore not be excluded that cytotoxicity, at least in part, is caused by the modification of multiple cysteine-containing proteins involved in basic metabolic cellular processes.

In summary, the present report describes for the first time protection against topoisomerase II poison-mediated cytotoxicity, etoposide-mediated DNA damage, and etoposide-induced band depletion by maleimide, a topoisomerase II-thiolating agent. This modality groups maleimide with such diverse drugs as aclacinomycine, chloroquine, and the bisdioxopiperazines, working at different steps in the topoisomerase II catalytic cycle. The main limitation of maleimide as protector is its high level of cytotoxicity that limits the window of protection. However, maleimide may serve as a lead in the search for other less toxic topoisomerase II-thiolating agents with a broader therapeutic window.

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References

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