Published in final edited form as:

Biochem Pharmacol. 2012 July 1; 84(1): 52–58. doi:10.1016/j.bcp.2012.03.021.

The anticancer thiosemicarbazones Dp44mT and triapine lack inhibitory effects as catalytic inhibitors or poisons of DNA topoisomerase II α

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Abstract

The thiosemicarbazones Dp44mT (di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone) and triapine have potent antiproliferative activity and have been evaluated as anticancer agents. While these compounds strongly bind iron and copper, their mechanism(s) of action are incompletely understood. A recent report (Rao et al., *Cancer Research* 69:948-957, 2009) suggested that Dp44mT may, in part, exert its cytotoxicity through poisoning of DNA topoisomerase II α . In the present report, a variety of assays were used to determine whether Dp44mT and triapine target topoisomerase II α . Neither compound inhibited topoisomerase II α decatenation or induced cleavage of pBR322 DNA in the presence of enzyme. In cells, Dp44mT did not stabilize topoisomerase II α covalent binding to DNA using an immunoblot band depletion assay, an ICE (immunodetection of complexes of enzyme-to-DNA) assay, and a protein-DNA covalent complex forming assay. Dp44mT did not display cross resistance to etoposide resistant K562 cells containing reduced topoisomerase II α levels. Synchronized Dp44mT-treated CHO cells did not display a G₂/M cell cycle block expected of a topoisomerase II inhibitor. A COMPARE analysis of Dp44mT using the NCI 60-cell line data indicated that inhibition of cell growth was poorly correlated with DNA topoisomerase II α mRNA levels. In summary, we found no support for the conclusion that Dp44mT inhibits cell growth through the targeting of topoisomerase II α . Since clinical trials of triapine are underway, it will be important to better understand the intracellular targeting and mechanisms of action of the thiosemicarbazones to support forward development of these agents and newer analogs.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Keywords

Dp44mT; triapine; topoisomerase II α ; cell cycle analysis; iron; thiosemicarbazone

1. Introduction

The thiosemicarbazones Dp44mT (di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone) and the structurally related triapine (Fig. 1) and their analogs have been evaluated as anticancer agents [1–4]. Triapine is undergoing and has undergone Phase 1 and Phase 2 clinical trials [5]. These semithiocarbazones typically inhibit the growth of cancer cells at nanomolar concentrations [4,6,7]. The thiosemicarbazones are strong iron [1,4] and copper [8] chelators. We previously showed that Dp44mT is able to chelate free intracellular iron in myocytes [9]. Unlike the iron chelator prodrug dexrazoxane, Dp44mT was unable to protect cardiac myocytes from doxorubicin-induced damage. On the contrary Dp44mT synergistically increased doxorubicin-induced myocyte damage [9]. Others showed that, in vivo, Dp44mT was unable to protect rats from doxorubicin-induced cardiotoxicity [10].

While it is well established that triapine inhibits ribonucleotide reductase [1,2], it has only recently been reported that Dp44mT also inhibits this enzyme [11]. Triapine is thought to inhibit ribonucleotide reductase through its preformed iron chelate, rather than directly by removing iron from the active site [1,2]. Dp44mT has been suggested to inhibit ribonucleotide reductase through its ability to affect crucial thiol systems required for enzyme activity mediated through redox active iron complexes [11]. Dp44mT has also been shown to affect multiple molecules in the mitogen-activated protein kinase (MAPK) pathway including several phosphatases [12].

Recent evidence has been presented that Dp44mT may, in part, be cytotoxic in breast cancer cells through its ability to poison topoisomerase II α [13]. The most compelling evidence in support of this conclusion involved using leukemia cell lines to demonstrate Dp44mT-induced topoisomerase II α -DNA complexes [13]. In addition, these authors demonstrated that siRNA-mediated knockdown of topoisomerase II α resulted in partial resistance to Dp44mT. Though these results are consistent with activity at the level of topoisomerase II, Dp44mT induced a G₁ cell cycle arrest in Nalm-6 leukemia cells, clearly distinct from the G₂/M phase block observed for the topoisomerase II poison etoposide in these cells [13] and as would be expected for a topoisomerase II poison [14–16].

We decided to further characterize Dp44mT and triapine as putative DNA topoisomerase II catalytic inhibitors and/or poisons and to determine the role of this target in the cytotoxicity observed in the presence of these iron chelators. Overall, our results did not provide support for DNA topoisomerase II α as a major direct target for Dp44mT and triapine. These results are discussed in light of other mechanisms demonstrated for the thiosemicarbazones and are put into perspective regarding previous findings of DNA topoisomerase II α poisoning induced by Dp44mT.

2. Materials and methods

2.1 Materials, cell culture and growth inhibition assays

Dp44mT was from EMD Biosciences (San Diego, CA) or was synthesized as described [3]. Triapine was synthesized as described [17] in one step from the reaction of thiosemicarbazide with (2-formyl-pyridin-3-yl)-carbamic acid tert-butyl ester obtained from Adesis (New Castle, DE). Both Dp44mT and triapine were characterized for identity and purity by ¹H-NMR and electrospray ionization mass spectrometry. DNazol was from

Invitrogen (Burlington, ON, Canada). Unless specified, all other reagents were obtained from Sigma-Aldrich (Oakville, Canada). The errors shown are SEMs. Where significance is indicated ($p < 0.05$), a t -test was used (SigmaPlot, San Rafael, CA).

An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, as previously described, was used to determine cell growth inhibition of CHO cells [18]. Human leukemia K562 cells, obtained from the American Type Culture Collection, and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived sub-line with decreased levels of topoisomerase II α mRNA and protein) [19,20] were maintained as suspension cultures in "MEM (Minimal Essential Medium Alpha, Invitrogen) containing 10% fetal calf serum (FCS). For growth inhibition assays, K562 and K/VP.5 cells were plated at a concentration of 1.5×10^5 cell/ml, and incubated 5 d with various concentrations of Dp44mT, triapine or vehicle (DMSO) for 48 h, after which cells were counted on a model ZBF Coulter counter (Beckman Coulter, Brea CA). The IC_{50} growth inhibitory concentration for each cell line was calculated from a non-linear least-squares fit to a 2-parameter logistic equation (SigmaPlot).

2.2 Topoisomerase II α kDNA decatenation, pBR322 DNA cleavage assays

An agarose gel DNA decatenation assay was used to determine if Dp44mT and triapine inhibited the catalytic decatenation activity of topoisomerase II α . In this assay, kinetoplast DNA (kDNA), which consists of highly catenated networks of circular DNA, is decatenated by topoisomerase II α in an ATP-dependent reaction to yield individual minicircles of DNA. The assay conditions and the expression, extraction and purification of recombinant full-length human topoisomerase II α were as previously described [21,22].

Topoisomerase II-cleaved DNA complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with sodium dodecyl sulfate (SDS) [23,24]. The drug-induced cleavage of double-stranded closed circular plasmid pBR322 DNA to form linear DNA at 37°C was followed by separating the SDS and proteinase K-treated reaction products by agarose gel electrophoresis in ethidium bromide (0.7 μ g/ml), essentially as described [21,24].

2.3 Cellular assays for detection of covalent DNA-topoisomerase II α and topoisomerase II β or DNA-protein complexes

The cellular ICE (immunodetection of complexes of enzyme-to-DNA) assay used for the detection of covalent complexes of topoisomerase II β and topoisomerase II β bound to DNA was based on the original cesium chloride ultracentrifugation gradient assay to isolate DNA with bound protein [25]. A modification, published in abstract form [26], used DNAzol (Invitrogen Life Technologies) for selective precipitation of genomic DNA to isolate topoisomerase I-DNA covalent complexes. Here, the DNAzol adaptation was further modified for determining topoisomerase II α - and topoisomerase II β -DNA covalent complexes. Mid-log phase K562 cells were pelleted, and subsequently washed, resuspended, and incubated at 37°C in buffer (pH 7.4) containing 25 mM HEPES, 115 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM NaH₂PO₄ and 10 mM glucose [Buffer A] at a final concentration of 1×10^6 cell/ml (5 million cells total). Cells were then incubated with DMSO (vehicle, 0.1% v/v), etoposide (20 and 50 μ M), or Dp44mT (20 and 50 μ M) for 1 h. The cells were washed once in cold Buffer A, pelleted and subsequently resuspended in cold PBS containing a cocktail of protease inhibitors. Cells were pelleted again and lysed with 1 ml of DNAzol by trituration ten times using a 1 ml pipet tip. Ethanol (0.5 ml, 100%) was then added and mixed with the lysate and the solution was incubated overnight at -20°C. The precipitated DNA was collected by centrifugation (13,000 g) at 25°C for 10 min followed by washing twice with 75% ethanol and air drying for 30 s. The DNA pellet was dissolved in

0.2 ml NaOH (8 mM) and then sonicated twice on ice for 5 s. HEPES (1 M, 27 μ l) was added to adjust the pH to 7.2. After centrifugation at 30,000 g for 10 min, the supernatant was used to quantify the DNA concentration. DNA (2 – 3 μ g) dissolved to a final volume of 100 μ l in NaPO₄ buffer (25 mM, pH 6.5) was then loaded onto nitrocellulose membranes using a slot blot apparatus under vacuum. Membranes were incubated overnight at 4°C with rabbit polyclonal antisera to human topoisomerase II α (1:5000) prepared as described previously [27] or with an anti-mouse topoisomerase II β antibody (1:200) (Santa Cruz Biotechnology, CA); then incubated for 1 h with donkey anti-rabbit or donkey anti-mouse secondary horseradish peroxidase-conjugated antibody (1:10000 dilution; Jackson Immunoresearch, West Grove PA), respectively. Reactive bands were detected using Immunostar chemiluminescence Western C kit reagents (Bio-Rad, Hercules, CA) on a Chemi-Doc XRS+ imager (Bio-Rad).

The cellular topoisomerase II α band depletion assay was modified from a previously described protocol [28]. K562 cells (5×10^5 /ml) were incubated with vehicle control (DMSO), etoposide (50 μ M), triapine (100 μ M), or Dp44mT (100 μ M) for 3 h at 37°. Isolated nuclear protein (20 μ g/well) was loaded onto 7% (v/v) SDS-PAGE gels. Resolved proteins were transferred electrophoretically to nitrocellulose and blocked overnight with 3% nonfat milk in PBS buffer with 0.05% Tween 30. Membranes were probed sequentially with rabbit polyclonal antisera to human topoisomerase II α (1:5000) prepared as described previously [27] and peroxidase-conjugated donkey anti-rabbit antisera (1:2000; Jackson Immunoresearch, West Grove PA). Bound antibody was detected using enhanced chemiluminescence (Perkin Elmer, Boston MA). Quantitation of autoradiographic signals was performed using a Molecular Dynamics Personal Densitometer SI (Amersham Biosciences, Piscataway NJ).

Protein-DNA covalent complex formation in intact K562 or K/VP.5 cells was measured as previously described [29]. Mid-log phase cells were labeled for 24 h with [*methyl*-³H]thymidine and [¹⁴C]leucine followed by a brief incubation in fresh media as described [29]. The cells were pelleted and incubated at 37°C in Buffer A at a final concentration of 1.5×10^6 cell/ml. Cells were then incubated with DMSO (vehicle, 0.4% v/v), etoposide (50 μ M), or Dp44mT (20 or 50 μ M) for 1 h. Cellular processes were quenched by adding aliquots of cell suspension to ice-cold PBS at 10 $\times$ volume. Cells were then pelleted, lysed, DNA was sheared, and protein-DNA complexes were precipitated with SDS and KCl as described [30]. Protein-DNA complexes were quantified by scintillation counting and [³H]DNA was normalized to cell number using the co-precipitated ¹⁴C-labeled protein as an internal control. Results are presented as protein-DNA complexes in the presence of drugs minus those found in DMSO controls.

2.4 Cell cycle synchronization and flow cytometry

The cell cycle synchronization experiments were carried out as previously described [18]. For the synchronization experiments CHO cells were grown to confluence in α -MEM supplemented with 10% FCS. Following serum starvation with α -MEM-0.5% FCS for 48 h, the cells were seeded at 3×10^5 cells/ml, repleted with α -MEM-10% FCS. Directly after repletion they were continuously treated with 100 nM Dp44mT in 35-mm diameter dishes for different periods of time. Cells were fixed in 70% ethanol and stained with a solution containing 20 μ g/ml propidium iodide, 100 μ g/ml RNase A in 0.1% (v/v) Triton X-100 at room temperature for 30 min. Flow cytometry was carried out on a BD FACSCanto II flow cytometry system (BD Biosciences, Mississauga ON, Canada) and analyzed with FlowJo software (Tree Star, Ashland OR) for the proportion of cells in sub-G₁, G₀/G₁, S, and G₂/M phases of the cell cycle.

3. Results

3.1 Comparison of the effect of Dp44mT on the growth of a K562 cell line with a K/VP.5 cell line with a reduced level of topoisomerase II α

The etoposide resistant K/VP.5 cell line contains one-fifth the amount of topoisomerase II α compared to the parent K562 cell line [29], and can be used to test whether a compound is a topoisomerase II poison [22]. We have previously shown that the topoisomerase II poison etoposide has an IC_{50} value for growth inhibition of 0.07 μ M and 1.8 μ M for K562 and K/VP.5 cells, respectively [19]. In cells containing less topoisomerase II α fewer DNA strand breaks will be produced, and thus topoisomerase II poisons will be less inhibitory in the K/VP.5 cell line. As shown in Fig. 2, the IC_{50} values for Dp44mT growth inhibition were 48 ± 9 nM and 60 ± 12 nM, for K562 and K/VP.5 cells, respectively. The IC_{50} values for triapine growth inhibition were 476 ± 39 nM and 661 ± 69 nM for K562 and K/VP.5 cells, respectively. The lack of cross resistance for both Dp44mT and triapine to the K/VP.5 cell line suggests that these thiosemicarbazones did not act as topoisomerase II poisons in a cellular context.

3.2 Effect of the Dp44mT and triapine on the decatenation activity of topoisomerase II α and on the stabilization of the covalent topoisomerase II α -DNA cleavable complex

Topoisomerase II α is able to decatenate the highly knotted circular catenated kDNA into open circular kDNA in an ATP-dependent reaction [15,31]. As shown in lane 8 (Fig. 3A), in the absence of topoisomerase II α the extremely high molecular weight catenated kDNA did not move from the gel origin. When decatenation was partially inhibited by the topoisomerase II catalytic inhibitor positive control dexrazoxane (lanes 17 and 18) [32], the amount of open circular kDNA was reduced and some kDNA remained at the gel origin. Also shown in Fig. 3A, neither Dp44mT nor triapine had an appreciable effect on the decatenation activity of topoisomerase II α at concentrations that were much higher than their IC_{50} values for growth inhibition [13]. In addition, at concentrations up to 200 μ M, Dp44mT did not inhibit the decatenation activity of topoisomerase II α using a spectrofluorometric-based decatenation assay [18] (Supplemental Fig. 2).

Covalent-topoisomerase II-cleaved DNA complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with SDS [24,33]. In order to trap and detect the normally transient covalent enzyme intermediate [14,15], high drug concentrations as well as a proteinase to cleave the enzyme-DNA complex to reveal DNA double strand breaks are typically used in this assay. Thus, in order to see whether Dp44mT or triapine stabilized the topoisomerase II α -DNA cleavable complex, *in vitro* DNA cleavage assay experiments were carried out using etoposide as a positive control. As shown in Fig. 3B, the addition of the positive control etoposide (100 μ M) in the reaction mixture induced formation of linear pBR322 DNA (lane 3), an indication of DNA double strand breaks. Linear DNA was identified by comparison with linear pBR322 DNA produced by action of the restriction enzyme *HindIII* acting on a single site on pBR322 DNA. Quantitation of the linear DNA bands indicated that etoposide enhanced DNA double strand breaks 12-fold compared to DMSO control. However, 50 – 200 μ M Dp44mT and triapine were essentially without effect (Fig. 3B). The relative lack of linear DNA produced in the presence of Dp44mT or triapine is also consistent with the lack of cross-resistance of Dp44mT and triapine to the K/VP.5 cell line containing a reduced level of topoisomerase II α (Fig. 2).

3.3 Effect of the Dp44mT on cellular topoisomerase II covalent complexes and topoisomerase II α band depletion in cells

Three different cellular assays were used to determine if Dp44mT could produce topoisomerase II α -covalent complexes (Fig. 4). In K562 cells, using the ICE assay, there

was a concentration dependent increase in the amount of topoisomerase II α and topoisomerase II β covalently bound to DNA after treatment with etoposide (20 and 50 μ M) (Fig. 4A). In contrast, Dp44mT (20 and 50 μ M) had no effect on the amount of complex formed (Fig. 4A). Similarly, in K562 and K/VP.5 cells containing radiolabeled DNA ([*methyl*- 3 H]thymidine) and protein ([U- 14 C]leucine), Dp44mT failed to induce protein-DNA covalent complexes (Fig. 4B). Under these experimental conditions, etoposide treatment induced robust formation of protein-DNA covalent complexes which were attenuated in the topoisomerase II α -deficient K/VP.5 cells (Fig. 4B), as we have previously demonstrated [19,29]. Topoisomerase II α immunoblot band depletion assays were used next as an additional cellular assay to indirectly assess the ability of Dp44mT and triapine to induce topoisomerase II-DNA covalent complexes. DNA covalently bound to topoisomerase II α prevents the enzyme from entering an SDS PAGE gel due to the increased size of the complex [28]. As shown in Fig. 4C, K562 cells treated with etoposide displayed a statistically significant decrease in the topoisomerase II α band compared to DMSO control. Neither Dp44mT nor triapine treated cells displayed any significant band depletion (Fig. 4C). These results are consistent with the lack of covalent complex formation in the cellular assays described above (shown in Figs. 4A and 4B) and the DNA cleavage assay employing purified topoisomerase II α (Fig. 3B).

3.4 Cell cycle analysis of Dp44mT treated synchronized CHO cells

CHO cells (normal doubling time of 12 h) that were synchronized to G₀/G₁ through serum starvation were treated with 100 nM Dp44mT to determine whether this agent induced a G₂/M cell cycle block that would be indicative of a topoisomerase II poison. Using an MTT growth assay, Dp44mT inhibited CHO cells with an *IC*₅₀ value of 30 $\pm$ 3 nM (Supplemental Fig. 1). CHO cells were chosen for these experiments as they are easily and effectively synchronized by serum starvation. Subsequent to this starvation, serum replent resulted in the control (DMSO vehicle) cells advancing to the S phase by 13 h; by 16 h cells were in the G₂/M phase (Figs. 5A and 5B). The control cells then went through several complete cell cycles as evidenced by the 12 h periodicity for peaks in the various cell cycle stages. The percentage of sub-G₀/G₁ cells that was characteristic of induction of apoptosis was small and not significantly different in both control- and Dp44mT-treated cells (2 – 3 %) and did not significantly increase with time (Fig. 5A). Dp44mT treatment of synchronized cells slowed progression out of G₀/G₁, compared to control cells. Dp44mT-treated cells showed both an increased block in S phase and a delay in progressing out of S phase. Only by 25 h did the Dp44mT treated cells achieve a normal (compared to control) percentage of cells in the G₂/M phase, some 10 h behind untreated cells. Thus, the lack of a G₂/M block after Dp44mT treatment is not consistent with Dp44mT functioning as a topoisomerase II inhibitor. However, these results are in accord with a previous cell cycle analysis of Dp44mT-treated Nalm-6 leukemia cells carried out at single time point which also showed a lack of accumulation of cells in the G₂/M phase [13]. However, it was reported that Dp44mT induced a sub-G₁ population at a single time point of 48 h in Nalm-6 leukemia cells. In contrast CHO cells did not display a sub-G₁ population that was statistically any different than the DMSO-treated controls. We have no ready explanation why Dp44mT-treated Nalm-6 cells displayed a sub-G₁ population, but CHO cells did not, other than that this might be due to basic differences between these two cell types.

3.5 Dp44mT NCI molecular targets COMPARE analysis

An NCI COMPARE analysis (<http://dtp.nci.nih.gov/compare>) of the NCI Dp44mT GI₅₀ endpoint 60-cell line data was correlated with the log of the relative topoisomerase II α mRNA cellular levels (TOP2A MoltId:GC13834) [34]. Cells that have more topoisomerase II α would be expected to be more sensitive to topoisomerase II α poisons due to a topoisomerase II α -mediated increase in cytotoxic DNA strand breaks. The log *GI*₅₀ values

for both Dp44mT and triapine growth inhibition were poorly and negatively correlated (r values of -0.046 and -0.043 , respectively) with the log of the mRNA levels. For comparison, growth inhibition and log mRNA levels for the topoisomerase II poisons amsacrine and etoposide were well and positively correlated (r values of 0.467 and 0.221 , respectively). Assuming that topoisomerase II α mRNA levels are correlated with cellular topoisomerase II α protein levels, the COMPARE results suggest that neither Dp44mT nor triapine inhibited cell growth through targeting of topoisomerase II α .

4. Discussion

Dp44mT is able to mobilize intracellular iron, form copper complexes [8] and exert a variety of effects on cells [4]. These include, among others, effects on signaling pathways [12] and inhibition of ribonucleotide reductase that may be mediated through redox active iron [11]. Dp44mT has also been suggested to act as a topoisomerase II α poison [13]. We have evaluated the latter claim with a variety of assays and tests and have found little evidence that Dp44mT, or its analog triapine, inhibited cell growth through targeting topoisomerase II α . Additionally, we showed that Dp44mT did not produce cellular topoisomerase II β -DNA covalent complexes. In this latter respect our results are in accord with those of Rao *et al.* [13]. The lack of Dp44mT and triapine cross resistance in etoposide-selected K/VP.5 cells that contain reduced levels of topoisomerase II α (Fig. 2) and the lack of correlation of growth inhibitory effects with mRNA levels in the NCI 60-cell line COMPARE analysis both suggested that Dp44mT antiproliferative activity was not due to topoisomerase II α targeting. Neither Dp44mT nor triapine were able to inhibit the decatenation activity of topoisomerase II α (Fig. 3A), a result that also suggests that these compounds failed to target topoisomerase II α . Dp44mT and triapine produced very little or no measurable linear DNA in the topoisomerase II α cleavage assay (Fig. 3B). What little linear DNA they did produce may have been due to the redox activity of the iron-drug complexes [3]. In two different covalent complex forming assays in K562 cells (Fig. 4A and 4B), Dp44mT failed to produce detectable topoisomerase II α /protein-DNA complexes. Likewise, in K562 cells, neither Dp44mT ($100\text{ }\mu\text{M}$) nor triapine ($100\text{ }\mu\text{M}$) were able to form topoisomerase II α -DNA complexes as assessed in a topoisomerase II α immunoblot band depletion assay (Fig. 4C).

Our ICE assay results demonstrating that Dp44mT does not induce topoisomerase II α covalent complexes with DNA are not in accord with those reported previously [13] in which a different cell line (Nalm-6 leukemia) and much longer Dp44mT treatment times were used (6 – 24 h compared to 1 h in our study). The reasons for this fundamental difference are not known but may have been due to the redox activity of the iron-Dp44mT complex [4] expressed over the much longer treatment time in the study by Rao *et al.* [13]. Prototypical topoisomerase II poisons such as etoposide rapidly induce cleavable complexes (Figs. 3 and 4). It is known that topoisomerase II α -mediated DNA damage is greatly stimulated by abasic, oxidized and alkylated DNA lesions [35,36]. Thus, the longer 6 – 24 h treatment times [13] may have produced iron-Dp44mT complex-induced oxidative DNA damage that indirectly stimulated the topoisomerase II α -DNA complexes that were observed.

Finally, our cell cycle results (Fig. 5) on synchronized cells showed the lack of a G₂/M block, a result again consistent with the conclusion that Dp44mT exerted its effect by a mechanism distinct from topoisomerase II α poisoning. These results show that these two thiosemicarbazones do not target DNA topoisomerase II α , clarifying previous literature that may be misleading in terms of the cellular targeting of these new agents. Because there is interest in clinical development of these thiosemicarbazones, our results may contribute to further development of these agents by avoiding study of a target that does not play an important role in their mechanism(s) of action. Although this study did not identify which

target or targets are primarily responsible for the growth inhibitory effects of Dp44mT in human leukemia K562 cells, taken together, our results using a variety of assays and strategies, indicate that topoisomerase II α is not likely an immediate and direct target for this class of metal chelators. Subsequent investigations of thiosemicarbazones should proceed with a focus on other more robustly validated targets such as ribonucleotide reductase [11] and/or MAPK pathway effectors [12].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Canadian Institutes of Health Research, the Canada Research Chairs Program, and a Canada Research Chair in Drug Development for B.B.H. and by a grant to J.C.Y. from the NIH (grant CA090787). We would like to thank Thomas D. Pfister (Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Support Directorate, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD) for providing a protocol of the modified ICE assay prior to publication. The authors thank Andrew J. Bodnar for expert technical assistance.

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Dp44mT

Triapine

Fig. 1.
Structures of the thiosemicarbazones Dp44mT and triapine.

Fig. 2.

Comparison of the growth inhibitory effects of Dp44mT on K562 and K/VP.5 cells with reduced levels of topoisomerase II α . K562 (○,△) and K/VP.5 (●,▲) cells were treated with Dp44mT (○, ●) or triapine (△,▲) for 48 h prior to the assessment of growth inhibition. The curved lines are calculated from non-linear least squares fits to 2-parameter logistic equations for K562 (solid line) and K/VP.5 (broken line) cells and yield IC_{50} -values of 48 ± 9 nM and 60 ± 12 nM, for Dp44mT, and 476 ± 39 nM and 661 ± 69 nM for triapine, respectively. Results for each Dp44mT concentration are the mean $\pm$ SEM from 5 – 17 observations obtained from a total of 17 experiments performed on separate days. Results for each triapine concentration are the mean $\pm$ SEM from 3 observations from three experiments performed on separate days.

Fig. 3.

(A) Effect of triapine, Dp44mT and dexrazoxane on the topoisomerase II α -mediated decatenation activity of kDNA. This fluorescent image of the ethidium bromide containing gel shows that topoisomerase II α (Topo II α) decatenated kDNA to its open circular (OC) form (lane 9). Topoisomerase II α was present in the reaction mixture for all lanes but lane 8. ORI is the gel origin. The positive control topoisomerase II catalytic inhibitor dexrazoxane (lanes 17 and 18) reduced the amount of decatenated kDNA and increased the amount of kDNA remaining at the origin. (B) Effect of Dp44mT, triapine and etoposide on the topoisomerase II α -mediated cleavage of supercoiled pBR322 plasmid DNA. This fluorescent image of the ethidium bromide-stained gel shows that topoisomerase II α (Topo II α) converted supercoiled (SC) pBR322 DNA (lane 1) to relaxed (RLX) DNA (lane 2). In this assay the relaxed DNA runs slightly ahead of the supercoiled DNA because the gel was run in the presence of ethidium bromide. Topoisomerase II α was present in the reaction mixture in all lanes but 1. As shown in lane 3, etoposide treatment produced significant amounts of linear DNA (LIN). A small amount of nicked circular (NC) is normally present in the pBR322 DNA. A densitometric analysis of the linear DNA bands showed that etoposide increased DNA cleavage 12-fold compared to the control (+Topo II α). By comparison, Dp44mT and triapine showed at most a 1.2-fold increase in linearized DNA. Results shown are representative of 4 similar experiments performed on separate days.

Fig. 4.

Effect of Dp44mT on cellular topoisomerase II covalent complexes and topoisomerase II α band depletion in cells. (A) Chemiluminescent image of a Western slot blot determination of cellular covalent topoisomerase II α (Topo II α) and topoisomerase II β (Topo II β) DNA cleavage complexes produced in K562 cells determined using an ICE assay. In these experiments K562 cells were treated with DMSO vehicle (lane 1), with the positive control etoposide (lanes 2 and 3), or with Dp44mT (lanes 4 and 5) for 1 h. (B) Effect on formation of topoisomerase II α -DNA covalent complexes from a 1 h treatment of K562 and K/VP.5 cells with either etoposide or Dp44mT. Treatment with etoposide produced far more topoisomerase II-DNA cleavage complex in K562 cells than in topoisomerase II α -deficient

K/VP.5 cells. Dp44mT produced no detectable cleavage complexes. The results are an average of 2 determinations and the error bars are average deviations. (C) Topoisomerase II α band depletion assays of the effect of a 3 h treatment of K562 cells with either DMSO (vehicle control), etoposide (50 μ M), triapine (100 μ M) or Dp44mT (100 μ M). β -Actin loading controls are shown for comparison and were used to normalize results. While treatment with etoposide significantly decreased topoisomerase II α levels (bottom figure, *** $p = 0.005$, Wilcoxon Signed-Rank test), treatment with triapine or Dp44mT had no significant effect. The results measured relative to vehicle-treated control values are the mean $\pm$ SEM from 4 – 5 experiments performed on separate days.

Fig. 5.

Cell cycle effects of Dp44mT treatment of synchronized CHO cells. CHO cells that had been synchronized in G_0/G_1 through serum starvation were replated with serum and were untreated (○), or treated with the DMSO vehicle (●) with 0.1 μ M Dp44mT directly after replating and allowed to grow for the times indicated, after which they were subjected to cell cycle analysis of their propidium iodide-stained DNA. In (A) the cell counts are displayed on the vertical axis and the DNA content is plotted on the horizontal axis. In (B) the percentage of the cells in G_0/G_1 (top), S (middle) and G_2/M (bottom) phases is plotted as a function of time. The solid lines were a least-squares calculated spline fit to the data. As shown in the top plot a very high percentage of the serum starved cells were initially present

in G₀/G₁. After serum repletion the percentage of control cells in each phase varied periodically as the cells progressed through several cell cycles.