Published in final edited form as:

Biochem Pharmacol. 2014 October 1; 91(3): 312–322. doi:10.1016/j.bcp.2014.08.006.

Distinct mechanisms of cell-kill by triapine and its terminally dimethylated derivative Dp44mT due to a loss or gain of activity of their copper(II) complexes

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Abstract

Triapine, currently being evaluated as an antitumor agent in phase II clinical trials, and its terminally dimethylated derivative Dp44mT share the α -pyridyl thiosemicarbazone backbone that functions as ligands for transition metal ions. Yet, Dp44mT is approximately 100-fold more potent than triapine in cytotoxicity assays. The aims of this study were to elucidate the mechanisms underlying their potency disparity and to determine their kinetics of cell-kill in culture to aid in the formulation of their clinical dosing schedules. The addition of Cu^{2+} inactivated triapine in a 1:1 stoichiometric fashion, while it potentiated the cytotoxicity of Dp44mT. Clonogenic assays after finite-time drug-exposure revealed that triapine produced cell-kill in two phases, one completed within 20 min that caused limited cell-kill, and the other occurring after 16 h of exposure that produced extensive cell-kill. The ribonucleotide reductase inhibitor triapine at 0.4 μM caused immediate complete arrest of DNA synthesis, whereas Dp44mT at this concentration did not appreciably inhibit DNA synthesis. The inhibition of DNA synthesis by triapine was reversible upon its removal from the medium. Cell death after 16 h exposure to triapine paralleled the appearance of phospho-(γ)H2AX, a marker of DNA double-strand breaks induced by collapse of DNA replication forks after prolonged replication arrest. In contrast to triapine, Dp44mT produced robust cell-kill within 1 h in a concentration-dependent manner. The short-term action of both agents was prevented by thiols, indicative of the involvement of reactive oxygen species. The time dependency in the production of cell-kill by triapine should be considered in treatment regimens.

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interests were disclosed.

Keywords

Triapine (3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone); Dp44mT (di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone); Metal coordination; DNA replication stress; DNA double-strand breaks; Reactive oxygen species

1. Introduction

The search for novel compounds that selectively cause tumor cell-kill is continuing, since current chemotherapy remains ineffective in eradicating tumors at advanced stages. The hallmark of combination chemotherapy is an exploitation of multiple and discrete tumor sites of vulnerability to achieve a higher probability of cure and to avert emergence of drug resistance. Essential transition metal ions such as iron (Fe) and copper (Cu) play pivotal roles in cellular metabolism, including cell proliferation, angiogenesis and metastasis. Because of their elevated levels in a variety of malignancies, development of novel Fe and Cu chelators has become a promising anticancer strategy [1–3].

The iron chelator, triapine (3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone), designed and synthesized in our laboratory [4,5], was chosen for clinical development because of its broad-spectrum antitumor activity alone or in combination with cisplatin, doxorubicin, or etoposide in preclinical models [6,7]. Triapine, as shown with a number of α -(*N*)-heterocyclic thiosemicarbazones that we have synthesized [8,9], is an inhibitor of ribonucleotide reductase (RNR) [10]. Mammalian RNR is a heteromeric enzyme consisting of R1 and either R2 or p53R2 subunits, catalyzing the reductive conversion of RNA building blocks to DNA building blocks for DNA replication and repair [11,12]. RNR, as such, is critical to all living cells, controlling cell proliferation and maintaining genomic stability [11]. Since tumor cells generally proliferate at a faster rate than their normal counterparts, RNR appears to be a legitimate target for cancer chemotherapy [13,14]. Because triapine is 100 to 1000 times more potent than the clinically used RNR inhibitor hydroxyurea [6,7], the pursuit of the clinical applicability of triapine appears to be worthwhile.

Recent notable development of the class of α -pyridyl thiosemicarbazones involves the introduction of terminally dimethylated derivatives and the impact of this modification on biological activity. Thus, the terminally dimethylated analog Dp44mT (di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone) has emerged as a “super-triapine” with elevated cytotoxic potency by two orders of magnitude [15–18]. Both the addition of two methyl groups to the terminal position and the removal of the 3-amino group from the pyridine ring of triapine, which lead to pyridine-2-carboxaldehyde 4,4-dimethyl-3-thiosemicarbazone (P44mT), contribute to potentiation [19]. The non-methylated counterpart of P44mT is the prototypic pyridine-2-carboxaldehyde thiosemicarbazone (PT). The discovery of the antileukemic activity of PT reported nearly 6 decades ago [20] ignited the subsequent research. These four α -pyridyl thiosemicarbazones with (Dp44mT and P44mT) or without (triapine and PT) terminal dimethylation were employed in this study (see Fig. 1A for their structures).

In cytotoxicity assays utilized to obtain IC₅₀ values for triapine and Dp44mT in previous studies [7,15–18], cells were continuously exposed to test agents until the end of assays. Because this setting does not reflect the transient nature of tumor-drug contacts occurring *in vivo*, we have employed clonogenic survival assays where test agents were removed after a defined period of exposure to measure the kinetics of cell-kill (see Fig. 1B for various cytotoxicity assays used in this study). This approach uncovered the long (16 h) and short (1 h) exposure times necessary to produce effective cell-kill by triapine and Dp44mT, respectively.

Although triapine and Dp44mT are well-known chelators of transition metal ions such as iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) [1], the exact metal ion partners relevant to their biological activities are yet to be identified. Fe and Cu complexes have long been predicted as biologically active forms due to intracellular redox cycling potential, and the efforts of synthesizing preformed complexes with Fe, Cu or other metal ions have been pursued in an attempt to improve therapeutic efficacy [5,21–23]. In this study, we have discovered that the simple addition of Cu²⁺ to the culture medium abrogates the cytotoxicity of triapine and PT in a 1:1 stoichiometric manner. In contrast, the terminally dimethylated derivatives Dp44mT and P44mT are not only resistant to inactivation by Cu²⁺, but their cytotoxicity is potentiated by this metal ion. To our knowledge, this is the first demonstration that triapine and Dp44mT differ in metal ion dependency.

Although the most notable biochemical change detectable in triapine treated cells is the immediate complete cessation of DNA synthesis [10] caused by imbalance in dNTP pools [24], the precise mechanisms by which inhibition of DNA synthesis leads to cell death are unknown. Recently, the sequential events in which disruption of DNA replication forks is ultimately conveyed to DNA double-strand breaks following prolonged replication stress have been analyzed using the RNR inhibitor hydroxyurea [25]. In this report, we have confirmed that triapine produces DNA double-strand breaks in a manner analogous to hydroxyurea and the implications of the time dependency of cell-kill by triapine in formulating an *in vivo* treatment schedule are discussed.

2. Materials and methods

2.1. Reagents

Triapine, PT, and P44mT were synthesized in our laboratory as previously described [4, 16]. Dp44mT was from Santa Cruz Biotechnology (Dallas, TX). FeCl₃•6H₂O, FeSO₄•7H₂O, MnCl₂•4H₂O, CoCl₂•4H₂O, NiSO₄•6H₂O, CuCl₂•2H₂O, ZnSO₄•7H₂O, N-acetyl-L-cysteine, α-monothioglycerol, hydroxyurea, chloroquine, cisplatin and catalase (C-1345), were from Sigma-Aldrich (St. Louis, MO). Deferoxamine mesylate and bleomycin were from Cayman Chemical (Ann Arbor, MI). Superoxide dismutase (574594) was from EMD Millipore (Billerica, MA). Dithiothreitol was from Bio-Rad (Hercules, CA).

Triapine and PT were dissolved in anhydrous DMSO at 200 mM. Dp44mT and P44mT were dissolved in DMSO at 10 mM. The stock solutions were further diluted with DMSO and added to cell cultures with the final DMSO concentration being less than 0.05%. Cisplatin, which undergoes relatively slow solvolysis in DMSO [26], was dissolved in DMSO at 20

mM, immediately aliquoted and stored at -70°C . Thawed aliquots were used one time. Bleomycin was freshly prepared in H_2O at 10 mM.

2.2. Cell lines

Human myeloid leukemia HL-60 cells [27] were obtained from Dr. Robert C. Gallo in 1980 and authenticated by ATCC in February 2014. Purported human ovarian carcinoma BG-1 cells were obtained from Dr. Joanne B. Weidhaas in 2012; STR analysis performed by ATCC in April 2014, revealed that BG-1 was genotypically identical to human breast carcinoma MCF-7 cells. Identification of BG-1 as MCF-7 independently reported by Korch *et al.* [28] suggests that mislabeling might have occurred in the laboratory of original distributors. MCF-7 cells acquired in this laboratory differed from MCF-7 cells (HTB-22) available from ATCC in morphology in that the former was spindle-shaped, while the latter formed dorm-like structures. The cell lines were maintained in DMEM/F12 medium supplemented with 10% FBS, 50 units/ml of penicillin, and 50 $\mu\text{g}/\text{ml}$ of streptomycin.

2.3. Growth inhibition assays and clonogenic survival assays

Growth inhibition assays based on cell counts using HL-60 cells continuously exposed to various agents for 3 days were previously described [29]. Clonogenic survival assays using MCF-7 cells were carried out as described [30]. The predetermined number of cells that yielded 20 to 150 colonies/well was allowed to adhere overnight before treatment. For limited-time exposure, the medium was aspirated after treatment and cells were washed once with fresh medium. Washing was omitted for continuous exposure. In both settings, cells were incubated for a total of 7 days for colony propagation. Colony forming efficiencies of untreated MCF-7 cells were approximately 0.25.

2.4. Analyses of phospho-(γ)H2AX

After HL-60 cells were treated, histones were extracted from 3×10^6 cells, subjected to 15% PAGE, and analyzed by western blots as described previously [31]. Chemiluminescent images were captured using G:Box iChemi XR (Syngene). Mouse monoclonal anti-phospho-histone H2AX antibody (clone JBW301) was from EMD Millipore.

2.5. Measurement of DNA synthesis

HL-60 cells (2×10^6 cells/ml) were labeled with 1 $\mu\text{Ci}/\text{ml}$ of [methyl- ^3H]thymidine (MT 6036, Moravek Biochemicals, Inc., Brea, CA) for 1 h and radioactivity in the acid-insoluble fraction from 100 μl of the cell suspension in triplicate was determined as previously described [32].

2.6. Alkaline single-cell gel electrophoresis (Comet) assays

HL-60 cells following various treatments were subjected to alkaline comet assays using Trevigen CometAssay Kit (4250-050-K, Trevigen, Gaithersburg, MD) according to instructions provided by the manufacturer. Comets were stained with SYBR Gold nucleic acid gel stain solution (Life Technologies, Carlsbad, CA) and photographed under a Zeiss Axiovert 200 fluorescence microscope equipped with an AxioCam MRm camera.

2.7. Statistical and mathematical analyses

Growth inhibition assays, clonogenic assays, and measurements of DNA synthesis were repeated at least three times and values of $I(E)C_{50}$ (the mean) $\pm$ standard deviations are presented. $I(E)C_{50}$ values were derived from logistic 3-parameter regression analyses using KaleidaGraph software (Synergy Software, Reading, PA).

3. Results

3.1. The IC_{50} values for triapine and its derivatives determined by growth inhibition assays

The structures of triapine, Dp44mT, P44mT, and PT are shown in Fig. 1A. The cytotoxic activity of these agents was determined using growth inhibition assays (Fig. 1B) where HL-60 human leukemia cells were continuously exposed to these chelators for 3 days. Consistent with previous observations [4,15–19], terminally di-methylated Dp44mT and P44mT were 50 to 100 fold more potent than triapine, while triapine was three fold more potent than PT (Fig. 1C).

The cytotoxic action of triapine was not unique to HL-60 cells; three other human leukemia cell lines (NB4, U937 and TF-1) and four murine leukemia cell lines (L1210, P388, WEHI-3D⁺, Friend MEL) exhibited indistinguishable sensitivity to triapine with IC_{50} values ranging from 0.30 to 0.38 μ M (data not shown).

3.2. Kinetics of cell-kill by triapine and Dp44mT measured by clonogenic survival assays

The growth inhibition assays described above did not reveal either the clonogenic potential of the treated cells or the time course of the cytotoxic actions. Thus, the kinetics of cell kill by triapine and Dp44mT were examined in MCF-7 human breast carcinoma cells using clonogenic assays where cells were exposed to these chelators for a finite time period (Fig. 1B). These agents exhibited distinct kinetics of cell-kill (Fig. 2). Triapine at 5 μ M elicited a biphasic mode of cell-kill; it produced approximately 50% cell-kill within 20 min and over 99% cell-kill after a 16 h exposure (Fig. 2A). An increase in triapine concentration up to 80 μ M did not result in further cell-kill upon 1 h exposure (Fig. 2B). In contrast, Dp44mT at 0.05 μ M, a 100 times lower concentration used for triapine, produced a marked cell-kill within 60 min (Fig. 2D). Unlike triapine (Fig. 2B), cell-kill by Dp44mT was concentration-dependent upon 1 h exposure (Fig. 2E). Upon continuous exposure for 7 days in MCF-7 cells, the LC_{90} values for triapine and Dp44mT were 0.32 and 0.001 μ M, respectively (Fig. 2C and 2F).

Cell-killing activity of triapine was not unique to MCF-7 cells; triapine caused a 25–66% cell-kill within 1 h and the LC_{50} values upon continuous exposure ranged from 0.23 to 0.43 μ M in three other human carcinoma cell lines IGROV-1, A549 and HCT-116 (data not shown).

3.3. Effects of transition metal ions on the cytotoxic activity of triapine and Dp44mT examined by growth inhibition assays

The presence of an N*-N*-S* tridentate coordination system in the α -pyridyl thiosemicarbazone (see Fig. 1A) allows chelation with transition metal ions [1, 21]. In an

attempt to identify chelation partners of potential biological importance, HL-60 cells were exposed to a fixed concentration of triapine or Dp44mT in the presence of varying concentrations of Mn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , and Zn^{2+} for 3 days.

Cu^{2+} elicited opposite effects in that this metal ion abrogated the cytotoxicity of the non-methylated analogs, triapine and PT (Fig. 3A), while potentiating that of the dimethylated analogs, Dp44mT and P44mT (Fig. 3B). The potentiating effect of Cu^{2+} on Dp44mT was consistent with previous findings that the Dp44mT-copper complex possessed pronounced redox activity [33]. The inactivation of triapine by Cu^{2+} is addressed in the Discussion section. The EC_{50} values (1.2 and 2.6 μM) for Cu^{2+} against 0.8 μM triapine and 3.2 μM PT, respectively, were suggestive of a 1:1 binding stoichiometry between triapine/PT and Cu^{2+} . Metal ions such as Fe^{2+} , Co^{2+} , Ni^{2+} , and Zn^{2+} were variably inhibitory to both triapine and Dp44mT (Fig. 3C to 3F). Co^{2+} was the most potent inhibitor, with an EC_{50} value of 0.46 μM against 0.8 μM triapine, suggestive of a 2:1 triapine/ Co^{2+} binding stoichiometry (Fig. 3D). The order of inhibitory potency was $\text{Co}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+} > \text{Zn}^{2+}$ (Fig. 3C to 3F). Mn^{2+} and Fe^{3+} up to 100 μM neither enhanced nor inhibited the cytotoxic activity of triapine or Dp44mT. The metal ions alone at the tested concentrations were not toxic to HL-60 cells (data not shown).

3.4. Reversal of the short-term action of triapine and Dp44mT by thiols

Triapine and Dp44mT at 5 and 0.05 μM reduced cell survival by approximately 50 and 90%, respectively, within 1 h (Fig. 2B and 2E). Dp44mT has been reported to accumulate in lysosomes and induce oxidative stress as copper complexes [33–35]. In addition, N-acetyl-L-cysteine (NAC) has been reported to partially reverse the cytotoxicity of Dp44mT [36]. To clarify the mode of the rapid phase of cell-kill, MCF-7 cells were exposed to triapine or Dp44mT for 1 h in the presence of various potential modulators (Fig. 4A to 4D).

Desferoxamine mesylate (DFO), chloroquine (CQ), and superoxide dismutase (SOD) plus catalase (CAT), at the concentrations indicated, did not interfere with the short-term action of triapine (Fig. 4A) and Dp44mT (Fig. 4B). In contrast, thiols such as NAC, α -monothioglycerol (MTG) and dithiothreitol (DTT) at the concentrations indicated, partially or completely prevented the short-term cell-kill by triapine and Dp44mT. Even though MTG alone caused approximately 20% cell-kill, both the thiol toxicity and the chelator (triapine or Dp44mT) toxicity disappeared in their co-presence (Fig. 4A and 4B).

The metal ions that inhibited the action of triapine (Cu^{2+} , Co^{2+} , Ni^{2+} and Fe^{2+}) and the metal ions that inhibited the action of Dp44mT (Co^{2+} , Ni^{2+} , Fe^{2+} and Zn^{2+}) in growth inhibition assays (Fig. 3), also prevented the short-term cell-kill by these agents (Fig. 4C and 4D).

NAC, MTG and DTT at the concentrations indicated substantially reversed the long-term cytotoxicity of Dp44mT in growth inhibition assays in HL-60 cells (Fig. 4F), while these thiols showed little effect on that of triapine (Fig. 4E). IC_{50} values for NAC, MTG and DTT alone were predetermined (>10 mM, ~ 3.5 mM and 0.52 ± 0.2 mM, respectively) and minimally cytotoxic concentrations were employed in these assays.

3.5. Relationship between inhibition of DNA synthesis and formation of γ H2AX

We previously reported that triapine at 1 μ M decreased dATP and dGTP pools to less than 10% of the control values within 1 h and that the reduction in the dATP pool was sustained in the presence of triapine [24]. The rapid decrease in dNTP pools was consistent with the immediate and selective inhibition of DNA synthesis in triapine-treated cells [10]. Furthermore, a collapse of DNA replication forks after prolonged replication stress caused by the RNR inhibitor hydroxyurea (HU) has been shown to result in induction of DNA double-strand breaks [25].

To determine whether triapine and Dp44mT possessed the capacity to cause DNA double-strand breaks, formation of γ H2AX was analyzed in HL-60 cells treated with triapine, Dp44mT, cisplatin (negative control agent) and bleomycin (positive control agent) for 16 h (Fig. 5A). The concentrations of triapine (0.4 μ M) and the control agents used were approximately 1.5 times higher than their respective IC_{50} values (Table 1). Triapine induced γ H2AX accumulation to an extent equivalent to that of bleomycin, while Dp44mT at 10 nM, approximately 4 times higher than the IC_{50} value (2.7 nM), was much less effective (Fig. 5A). Time-course studies revealed that γ H2AX accumulation by triapine peaked at 16 hours, while that by Dp44mT was much less intense and delayed (Fig. 5B). HU at 0.12 mM (~ 1.5 times higher than IC_{50} , Table 1) caused γ H2AX accumulation with kinetics similar to that of triapine (Fig. 5B).

To examine the relationship between γ H2AX accumulation and inhibition of DNA synthesis, DNA synthesis in HL-60 cells treated with triapine, Dp44mT and HU was measured by incorporation of 3 H-thymidine into an acid-insoluble fraction. Complete arrest of DNA synthesis occurred in HL-60 cells treated with 0.4 μ M triapine or 0.1 mM HU for 1 h, while Dp44mT at the concentrations (0.025 to 0.4 μ M) used for triapine did not significantly affect DNA synthesis (Fig. 5C). Inhibition of DNA synthesis caused by 0.4 μ M triapine and 0.12 mM HU after 1 h exposure was reversible upon removal of the inhibitors from the medium with the rate of DNA synthesis returning above 75% of the control level within 8 h (Fig. 5D).

3.6. DNA single-strand breaks in HL-60 cells treated with triapine, Dp44mT and control agents

Whether triapine or Dp44mT possessed the capacity to cause DNA single-strand breaks was examined using alkaline comet assays. HL-60 cells were treated with triapine, bleomycin, or cisplatin, at an approximately 1.5-fold greater concentration than their respective IC_{50} values for 16 h. The dose of X-rays employed (3 Gy) was also approximately 1.5 times greater than the ID_{50} value (Table 1). Because Dp44mT at 0.2 μ M caused ~98% cell-kill in MCF-7 cells in 1 h (Fig. 2E), HL-60 cells were treated with this agent at this concentration for 1 h. Bleomycin (Fig. 6C) and X-ray irradiation (Fig. 6E) produced extensive comet tailing, while cisplatin (Fig. 6D) produced nuclear swelling, but no comet tailing. Relative to the positive control treatment (bleomycin and X-rays), the effects of both triapine (Fig. 6A) and Dp44mT (Fig. 6F) were minor, excluding DNA as their primary site of action.

3.7. Length of exposure-time necessary for progressive cell-kill by triapine

To determine the optimum exposure-time for cell-kill, MCF-7 cells were exposed to varying concentrations of triapine for 8, 16, or 24 h and clonogenic survival was recorded (Fig. 7A). The 8 h exposure was insufficient for cell-kill even at exceedingly high concentrations (40 to 120 μM) (Fig. 7A). In contrast, the concentrations required for cell-kill dramatically decreased to 0.4 to 1.2 μM with the 16 h and 24 h exposures (Fig. 7A). These findings indicate that prolonged (16 h) DNA replication arrest is required for DNA double-strand breaks (Fig. 5A and 5B) and extensive cell-kill by triapine.

3.8. Interval necessary for restoring sensitivity to a second challenge of triapine

Triapine at 5 μM produced 50% cell-kill within 20 min (rapid phase of cell-kill, Fig. 2A); higher concentrations of up to 120 μM or a longer exposure time up to 8 h (Fig. 7A) did not cause further cell-kill, suggesting the existence of a limiting cellular factor(s). In an attempt to characterize this factor, MCF-7 cells, first treated with 5 μM triapine for 1 h, were washed and secondly challenged with 5 μM triapine for 1 h after 0 to 4 h interval (Fig. 7B). The sensitivity to the rapid phase of cell-kill by triapine was restored during a 2 h interval.

3.9. Mechanisms of the rapid and slow phases of cell-kill by triapine and Dp44mT

The difference in metal ion dependency of triapine and Dp44mT, resulting in differences in the kinetics and predominant mode of cell-kill by these chelators shown in this report, is summarized in Fig. 7C (slow phase) and Fig. 7D (rapid phase).

4. Discussion

The major findings in this report employing triapine and its terminally dimethylated derivative Dp44mT are as follows. (A) A 1:1 stoichiometric concentration of Cu^{2+} inactivates triapine, while the cytotoxic activity of Dp44mT is enhanced by this metal ion. (B) Dp44mT has the capacity to produce robust cell-kill within 1 h (the rapid phase of cell-kill, Fig. 7D), while triapine is limited in this activity. (C) Triapine produces progressive cell-kill via inhibition of RNR and arrest of DNA replication. This mode of cell-kill by triapine requires a long (16 h) exposure time (the slow phase of cell-kill, Fig. 7C). In contrast to triapine, Dp44mT does not appreciably inhibit DNA replication.

We examined the effects of the addition of transition metal ions, including Mn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , and Zn^{2+} to the medium on the cytotoxic activity of triapine and Dp44mT using growth inhibition assays. Our observation that Cu^{2+} potentiates the cytotoxic action of Dp44mT is consistent with the recent report showing a similar phenomenon [37]. Our observation that triapine is inactivated by Cu^{2+} is in agreement with the report indicating an indirect evidence for desulfurization of a thiosemicarbazonecopper(II) complex in aqueous basic medium [38]. The reaction scheme for the triapine/ $\text{Cu}(\text{II})$ complex undergoing base-catalyzed loss of sulfur and ultimately forming of a nitrile has been proposed [1]. The insolubility of CuS (solubility product constant, 4×10^{-36}) [39] is the plausible driving force in this desulfurization reaction [1]. This reaction requires the presence of at least one hydrogen atom at the terminal amino group, while terminal dimethylation blocks this reaction. In accord with this scheme, the cytotoxic activity of

Dp4mT, the terminally monomethylated analog of Dp44mT, is similar to that of triapine (the reported IC_{50} value for Dp4mT, 0.3 μ M [18]). Therefore, the inactivation of triapine by Cu^{2+} , the complete resistance of Dp44mT to inactivation by Cu^{2+} , and the potentiation of the cytotoxic activity of Dp44mT by Cu^{2+} , collectively account for the distinct modes of cell-kill as well as the potency disparity of the two agents.

Our and other laboratories have reported the synthesis of triapine precomplexed with copper ($[Cu(Triapine)Cl_2] \cdot H_2O$ and $Cu(OAc)-Triapine$) in methanol and an increase or decrease in their cytotoxic or RNR inhibitory activity relative to metal-free triapine [6,21,22]. In the present study, the cytotoxic activity of triapine was abrogated upon simple addition of Cu^{2+} to the culture medium in both long-term (3 days, Fig. 3A) and short-term (1 h, Fig. 4C) assays in a 1:1 stoichiometric manner (Fig. 3A). These conflicting observations suggest distinct ways in which triapine reacts with Cu^{2+} in alkaline aqueous medium and in acidic organic solvent. Solution equilibrium studies of triapine and copper(II) in a water/DMSO mixture reported by Enyedy *et al.* [40] did not indicate a desulfurization reaction.

We have also found that the addition of Co^{2+} , Ni^{2+} , Fe^{2+} , or Zn^{2+} in the medium variably inhibited the cytotoxic activity of both triapine and Dp44mT. Redox potentials of Dp44mT complexes with Fe and Cu lie within a range accessible to cellular oxidants and reductants [21]. These findings collectively pinpoint the triapine/Fe complex, and the Dp44mT/Cu and Dp44mT/Fe complexes as biologically active forms. The inhibitory activity of Fe^{2+} suggests that the redox-activity of the iron complexes is important for cytotoxic action.

The immediate biochemical change detectable in triapine-treated intact cells is selective inhibition of DNA synthesis [10]. We confirmed the immediate inhibition of DNA synthesis following treatment with the RNR inhibitors, triapine and HU. Triapine was 200- to 300-fold more potent than HU in inhibition of DNA synthesis (IC_{50} , 0.06 versus 13 μ M) and in inhibition of cell growth (IC_{50} , 0.29 versus 87 μ M, Table 1). In contrast to triapine and HU, Dp44mT at the same concentrations used for triapine (0.025 to 0.4 μ M) did not appreciably inhibit DNA synthesis, suggesting that Dp44mT had a weaker affinity for RNR. Yu *et al.* [36] have reported that Dp44mT possesses the capacity to inactivate RNR through perturbation of the thiol systems essential for the enzyme. In their study, RNR activity was measured by monitoring the tyrosyl radical by EPR in cells treated with Dp44mT at 25 μ M for 24 h. In our study, Dp44mT at 0.05 to 0.2 μ M produced 90 to 98% cell-kill after only 1 h exposure (Fig. 2E). The discrepancy in the concentration and exposure time in these studies suggests a weak cause-and-effect relationship between inhibition of RNR and cell-kill by Dp44mT.

The complete shutdown of DNA synthesis following treatment with triapine for 1 h did not lead to rapid cell death. Contrarily, a long lag period (16 h) during which cells needed to be exposed to triapine, existed before cell-kill occurred. Imbalance in dNTP pools causes widespread stalling of DNA replication forks in a form structurally vulnerable to incurring DNA damage, forcing cells to enter a state referred to as “replicative stress”, resulting in the activation of the checkpoint network. If these stress-activating obstacles are not removed in timely fashion, the replication machinery dissociates from the DNA and DNA double-strand breaks are induced by the DNA structure-selective endonuclease MUS81 in a FBH1

helicase-dependent manner [25]. These sequences were characterized using HU [25]. We have assumed that triapine causes cell-kill in an analogous manner, since both HU and triapine cause immediate inhibition of DNA synthesis and require 16 to 24 h exposure periods to produce extensive cell-kill [this study and 25].

RNR is an oxygen-dependent radical tetrameric enzyme composed of two subunits, R1 (α_2 homodimer) and R2 (β_2 homodimer). Each α_2 polypeptide harbors the active site, allosteric sites and redox-active disulfides, while each β_2 polypeptide harnesses an oxygen-linked diferric iron center and a stable tyrosyl radical essential for enzymatic activity [11,12]. Since HU is a potent reductant, it is likely to inactivate RNR by directly reducing the tyrosyl radical [22]. Unlike HU, however, neither metal-free triapine nor its metal complexes are potent reductants [22]. Studies on the mechanism of inhibition of RNR by triapine using recombinant RNR proteins in the presence or absence of a reducing agent, generated several models, including inactivation of the enzyme by ROS generated from redox cycling of the triapine/Fe complex, direct reduction of the tyrosyl radical by triapine/Fe²⁺, and mobilization of Fe from the di-iron center by metal-free triapine [22,41,42]. In our study, FeCl₃ at 100 μ M in the medium exerted no effect on the cytotoxic activity of triapine (Fig. 3), suggesting that the preformed triapine/Fe³⁺ complex possessed the capacity to inhibit RNR in cells.

The short-term cell-kill by Dp44mT and triapine was partially or completely prevented by thiols such as NAC, MTG and DTT, which alone were variably cytotoxic to cultured cells. Many thiols are known to produce a biphasic cytotoxicity [43]. Thiol toxicity is mediated via metal catalyzed production of H₂O₂ and is enhanced by copper, either free or derived from ceruloplasmin in serum [43]. Thus, the reversal of the Dp44mT/triapine toxicity by thiols observed in our study could be due to combination of the capacity of thiols to keep Dp44mT/triapine metal complexes in reduced forms to prevent redox cycling and to scavenge toxic ROS. Cellular copper levels are elevated in a variety of malignancies and targeting the stress response to ROS can result in preferential cell-kill in cancer cells [3]. A copper chelate of thiosemicarbazone NSC 689534 inhibits tumor growth via oxidative/ER stress [44]. The small chemical piperlongumine targeting the stress response to ROS causes selective killing of cancer cells [45]. The small molecule erastin selectively produces 'ferroptosis' in human cell lines engineered to express oncogenic HRAS by generating ROS in an iron-dependent manner [46].

In this report, we have presented an intriguing example in which closely related chelators, Dp44mT and triapine with or without terminal dimethylation, respectively, exert antineoplastic activity by distinct mechanisms due to apparent differences in metal ion dependency. These differences are reflected in host toxicity, in that triapine causes hematological toxicities including anemia and methemoglobinemia in humans [47, 48], while Dp44mT produces cardiac fibrosis in mice [17], the toxicity likely attributable to robust ROS production by Dp44mT/Cu^{I/II} complexes. While the ability of Dp44mT to produce rapid cell-kill within 1 h in cultured cells predicts its efficacy during finite tumor-drug contacts *in vivo*, the time dependency for cell-kill by triapine warrants consideration in formulation of a different treatment schedule. The results of phase I and II trials for triapine have been described in 18 publications from 2002 through 2014; only a few studies yielded

encouraging results. The majority of studies employed a 2 h i.v. infusion of triapine per day at doses up to 120 mg/m² (C_{max}, 5 μM), the regimens determined from phase I trials as tolerable doses [49]. The elimination half-life (t_{1/2}) for triapine ranged 30 to 120 min [49]. Our finding that cell-kill by triapine requires prolonged replication stress *in vitro*, suggests an optimum regimen consisting of frequent administrations or 16 h infusion of triapine to maintain plasma concentrations at 0.4 to 1 μM to sustain DNA replication stress. Furthermore, low but sustained concentrations of triapine are likely to reduce host toxicity such as methemoglobinemia and hypoxia. The efficacy of triapine with these regimens is currently being tested using preclinical models in our laboratory.

Acknowledgments

This work was supported in part by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Grant K12HD047018 (E.S.R) and a grant from the National Foundation for Cancer Research (A.C.S). We are grateful to Drs. Dawit Kidane and Joann Sweasy for the use and instruction of fluorescent microscopy, to Paul Bongiorno for the permission and instruction in employing the X-ray irradiation apparatus, and to Dr. Bret Halpern for his helpful discussions.

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Fig. 1.

The structures of triapine and its derivatives (A), the diagram of various cytotoxicity assays employed in this study (B), and the IC₅₀ values for the four chelators determined by growth inhibition assays (C). Panel A, the N,N,S (pyridyl nitrogen, azomethine nitrogen, and thione sulfur) tridentate metal coordination system is marked by asterisks in triapine. The numbering of the thiosemicarbazone is indicated in Dp44mT. Panel B, limited-time versus continuous exposure, and assays for clonogenic survival versus growth inhibition are

diagramed. Panel C, HL-60 cells were continuously exposed to various agents for 3 days and the percent growth inhibition was calculated using the log of the cell number.

Fig. 2.

Kinetics of cell-kill by triapine and Dp44mT examined by clonogenic assays in MCF-7 cells. Panels A and D, MCF-7 cells were exposed to 5 μ M triapine (A) or 0.05 μ M Dp44mT (D) for the indicated periods, washed and further incubated for 7 days for colony propagation. Panels B and E, MCF-7 cells were treated with various concentrations of triapine (B) or Dp44mT (E) for 1 h, washed and further incubated for 7 days. Panels C and F, cells were continuously exposed to various concentrations of triapine (C) or Dp44mT (F) for 7 days. Note that the scale of the y-axis in (B) is linear and the scale of the x-axis in (F) is in nM.

Fig. 3.

Effects of transition metal ions on the cytotoxic activity of triapine and Dp44mT measured using growth inhibition assays. Panels A to F, HL-60 cell were continuously exposed to a fixed concentration of triapine or Dp44mT in the presence of various concentrations of metal ions for 3 days. To measure counteracting action of metal ions, the maximum inhibitory concentrations (0.8, 3.2, and 0.01 μM) of triapine, PT and Dp44mT, respectively, were employed. To measure potentiating action of metal ions, the minimum inhibitory concentrations (0.001 and 0.002 μM) of Dp44mT and P44mT, respectively, were employed. Each metal ion, freshly prepared in H_2O at 100 mM, was added to medium prior to chelator.

Standard deviation (SD) bars in the figures are omitted for clarity, as values of IC₅₀ (the mean) $\pm$ SD are provided in the tables.

Fig. 4.

Effects of thiols and metal ions on the rapid or slow phase of cytotoxicity of triapine and Dp44mT. Panels A and B, MCF-7 cells were exposed to 5 μ M triapine (A) or 0.05 μ M Dp44mT (B) in the presence of various potential modulators for 1 h, washed and further incubated for 7 days. The concentrations of SOD and CAT were 10 and 20 μ g/ml, respectively. Panels C and D, MCF-7 cells were exposed to 5 μ M triapine (C) or 0.05 μ M Dp44mT (D) in the presence of various metal ions for 1 h, washed and further incubated for 7 days. Panels E and F, HL-60 cells were continuously exposed to triapine (E) or Dp44mT

(F) in the presence of various thiols for 3 days. For the IC_{50} values, the standard deviations were less than 20% of the averages in all measurements and not shown. In all experiments, each test agent was added to medium prior to chelator.

Fig. 5.

Relationship between inhibition of DNA synthesis and accumulation of γ H2AX. Panels A and B, HL-60 cells (4×10^5 cells/ml) were treated with various agents for 16 h (A) or for the times specified (B). Histones were extracted from 3×10^6 cells and subjected to western analyses using an anti-phospho-histone H2AX antibody. For a loading control, duplicate gels were stained with 0.05% Coomassie Brilliant Blue (A) or GelCode Blue Safe Protein Stain (Thermo Scientific, Lockford, IL) (B). Panel C, HL-60 cells (2×10^6 cells/ml) were treated with various agents for 1 h and labeled with 3 H-thymidine for an additional 1 h. Radioactivity in the acid-insoluble fraction was determined. Panel D, HL-60 cells (4×10^5 cells/ml) were pretreated with 0.4 μ M of triapine or 0.2 mM of HU for 1 h, washed and resuspended in fresh medium. At 0, 4, 8, and 16 h, cells were condensed to 2×10^6 cells/ml and labeled with 3 H-thymidine for 1 h, and radioactivity in the acid-insoluble fraction was determined.

Fig. 6. DNA single-strand breaks in HL-60 cells treated with triapine, Dp44mT and control agents (bleomycin, cisplatin, and X-rays). Panels A to F, HL-60 cells (4×10^5 cells/ml) untreated or treated as indicated, were subjected to alkaline comet assays. Immediately after X-ray irradiation, cells were chilled on ice to prevent DNA repair.

Fig. 7.

Exposure-time dependent cell-kill by triapine (A), the interval necessary to restore the sensitivity to the second challenge of triapine (B), and the proposed mechanisms for the slow (C) and rapid (D) phases of cell-kill by triapine and Dp44mT. Panels A, MCF-7 cells were exposed to various concentrations of triapine for 8, 16 or 24 h, and clonogenic survival was determined. Panel B, MCF-7 cells were first challenged with 5 μM triapine for 1 h and washed. At 0, 1, 2 and 4 h thereafter, cells were again challenged with 5 μM triapine for 1 h and washed, and clonogenic survival was determined. Panel C, the sequences of the slow

phase of cell-kill by triapine and Dp44mT are summarized. RPA denotes replication protein A. Panel D, the sequences of the rapid phase of cell-kill by triapine and Dp44mT are summarized.

Table 1IC₅₀ values for various agents in growth inhibition assays and in measurements of DNA synthesis

Agent	Inhibition of cell growth ^a IC(D) ₅₀ (μM)	Inhibition of DNA synthesis ^b IC ₅₀ (μM)
Triapine	0.29±0.07	0.06±0.02
Dp44mT	0.0027±0.0007	
Hydroxyurea	87±12	13±4
Cisplatin	1.4±0.2	
Bleomycin	6.7±0.9 (Gy)	
X-rays	2.5±0.3	

^aGrowth inhibition was measured in HL-60 cells treated with various agents after incubation for 3 days.

^bDNA synthesis was measured in HL-60 cells treated with various agents for 1 h followed by labeling with ³H-thymidine for 1 h.