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Inhibition of cellular thioredoxin reductase by the anticancer prodrug cloretazine

Tyler R. Schleicher
Colby College

Inhibition of Cellular Thioredoxin Reductase by the Anticancer Prodrug Cloretazine

By Tyler R. Schleicher

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By Tyler R. Schleicher

Approved:

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Mentor. Kevin P. Rice, Assistant Professor of Chemistry

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Reader. Julie Millard, Dorros Professor of Chemistry

_____Date

Vitae

Tyler Ray Schleicher was born June 30, 1986 in Manchester, Connecticut. He was raised by parents Ron and Tracy Schleicher in East Hartford, Connecticut. There he attended and graduated from the public East Hartford High School. After high school, Tyler spent four years at the Colby College to earn his undergraduate degree in Chemistry-Biochemistry. He then entered a Ph.D. program in the Molecular and Cell Biology Department at the University of Connecticut.

Permanent Address

56 Rival Court
East Hartford, CT 06118

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Abstract

Sulfonylhydrazines are a class of DNA alkylating drugs which also produce carbamoylating activity *in situ*. The carbamoylating species is of significance because it has shown to inhibit important thiol-containing enzymes such as glutathione reductase (GR) and thioredoxin reductase (TrxR). TrxR catalyzes dithiol-disulfide exchange reactions on thioredoxin (Trx), which in turn catalyzes other reductive processes such as deoxyribonucleotide biosynthesis. In this study, we demonstrate that TrxR activity is strongly inhibited by the anticancer prodrug Cloretazine both in purified form and in leukemia cell lysates. This inhibition is specific to the carbamoylating activity of methylisocyanate (MiC). In contrast, another important oxidoreductase, glutathione reductase (GR), was inhibited in purified form, but showed little susceptibility to Cloretazine in the cellular context. These results suggest the mode of inhibition against TrxR and GR differs inside of cells. Due to the overexpression of TrxR in cancer cells and its role in DNA metabolism, inhibiting TrxR may be important to the activity of the anticancer agent Cloretazine.

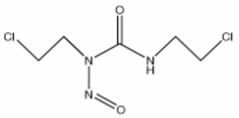
Introduction:

As cancer research evolves, cytotoxic chemotherapy remains one of the most effective options for cancer patients. The use of cytotoxic agents to fight disease began in the 1940's with the discovery of nitrogen mustard [1]. Research involving nitrogen mustard established the principle that drugs could be administered to induce tumor suppression. The creation of national programs, including the National Cancer Chemotherapy Service Center, led to new developments in animal models, cell lines, and transplantable solid tumors [1, 2]. One of the most important advances in chemotherapy was improvements in screening methodology. High throughput screening allowed researchers to identify anticancer agents at a faster pace. Promising drugs became defined as metabolically stable, well adsorbed from oral administration, and containing a favorable toxicity profile [1]. New understandings in cell biology identified cellular activities specific to cancer cells. Cell-cycle proteins, signaling molecules, and growth factors all became new targets for chemotherapy [1, 2]. Unfortunately, most of the anticancer agents designed never make it into clinical use due to lack of efficacy and high toxicities [2, 3]. Understanding how these toxicities arise may help to design more successful agents and to increase the number of viable drugs.

Cancer cells rapidly metabolize and proliferate compared to normal actively dividing cells. The fast proliferation requires continuous DNA replication and metabolism. Thus, tumor cell DNA is a primary target for many cytotoxic agents [4, 5]. DNA alkylating agents are some of the most effective types of chemotherapeutic drugs [5]. These anticancer drugs are responsible for significant increases in survival of many cancer patients [5]. Alkylating agents are able to alkylate a specific position of DNA [4].

Events that directly damage DNA are extremely cytotoxic for cells, as alkylated DNA can inhibit or prevent gene transcription or DNA replication. Although these agents are used to fight cancer, they often prove toxic or carcinogenic. Alkylating drugs are not completely specific to cancer cells; they can cause adverse effects for other actively dividing cells [4, 5].

Nitrosoureas are one class of chemotherapeutic compounds which generate DNA alkylating species. BCNU (1, 3-bis [2-chloroethyl]-2-nitrosourea), a common nitrosourea, is an important multi functional alkylating drug clinically used to treat brain cancers [6]. This anticancer compound generates species with chloroethylating and carbamoylating activity [7, 8]. BCNU also generates species with vinylating, hydroxyethylating, and aminoethylating activity [8]. These extra species add to the toxicity of BCNU and have no therapeutic benefit (Fig. 1) [8].



BCNU

Species	BCNU	Cloretazine	Contribution
Chloroethylating	Yes	Yes	Anticancer
Carbamoylating	Yes	Yes	?
Hydroxyethylating	Yes	No	Toxicity
Vinylating	Yes	No	Toxicity
Aminoethylating	Yes	No	Toxicity

Figure 1: Summary of Reactive Species Produced by BCNU.

The cross-linking activity of BCNU is believed to be the primary cause of its cytotoxicity [9]. Chloroethylation of the O^6 position of guanine initiates the formation of

cross links by the loss of the halide and formation of O^6N^1-ethanoguanine followed by a reaction with a complementary cytosine to make a GC crosslink [7]. The carbamoylating activity is a function of 2-chloroethyl isocyanate (CEiC). Isocyanates capable of carbamoylation are able to react with thiols, such as those on proteins. Specifically, BCNU has been shown to inhibit thiol-containing enzymes such as glutathione reductase, thioredoxin reductase, and ribonucleotide reductase in both purified and cellular forms [8].

Cloretazine, (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine; VNP40101M) is a sulfonylhydrazine anti-cancer compound which has shown broad spectrum antineoplastic activity in preclinical models [8]. Cloretazine is currently being used in clinical trials for blood and brain cancers, notably glioma and acute myeloid leukemia [9, 10]. *In situ* Cloretazine generates two reactive species, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) and methylisocyanate (MiC) (Fig. 2), which are attributed to the Cloretazine's anti-cancer activity [8, 9, 10].

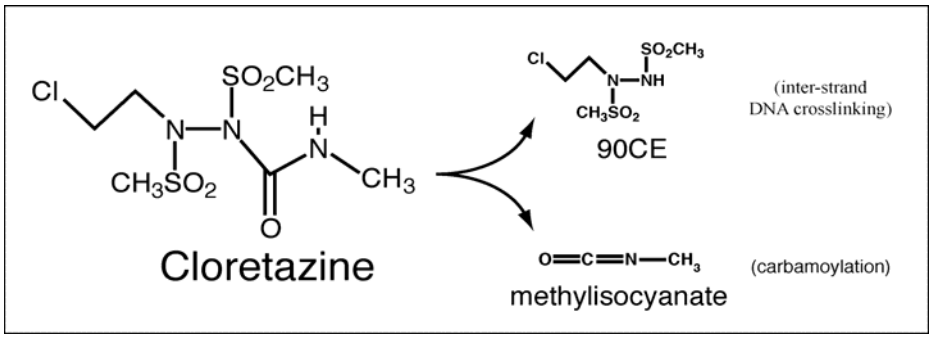


Figure 2: Structure and activation of Cloretazine yielding species with alkylating and carbamoylating activities

90CE is an alkylating species that chloroethylates DNA at the O^6 position of guanine, similar to the chloroethylation by BCNU [9]. This reaction forms a cross-link with the complementary cytosine, resulting in an extremely cytotoxic event for the cell. The other species generated from Cloretazine, methylisocyanate, can carbamoylate sulfhydryl groups, such as those on cysteine residues in proteins [9]. 90CE is also an analog of Cloretazine that chloroethylates, but does not carbamoylate. Similarly, 101MDCE is also an analog of Cloretazine that has the opposite activity (Fig. 3). Cloretazine is especially interesting given the lack of toxic side effects relative to BCNU, as Cloretazine does not generates species with vinylating, hydroxyethylating, and aminoethylating activity (Fig. 1) [9, 10].

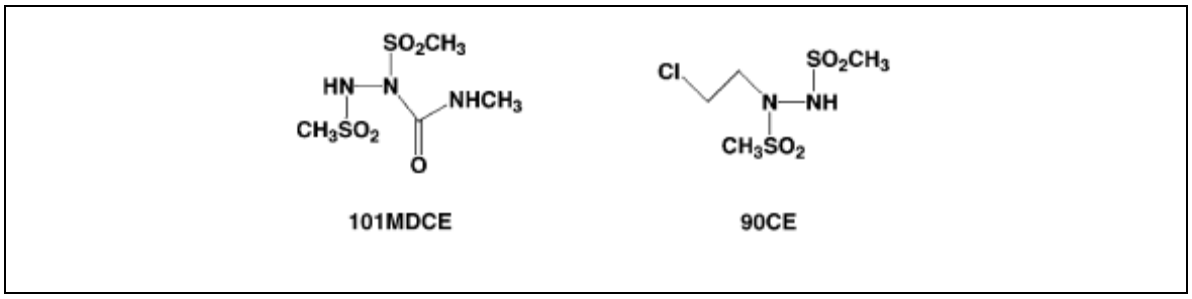


Figure 3: Chemical structure of sulfonylhydrazine prodrugs 101MDCE and 90CE.

The two species produced by Cloretazine, 90CE and methylisocyanate, have a synergism that gives the drug exceptional anticancer activity [9]. The two pathways in which the species work are connected in a way that makes the drug more effective then either species alone. The anticancer activity of Cloretazine is mostly due to cytotoxic cross-linking of DNA. Cloretazine has been shown in cell-free systems to yield more cross-links than the similar agent BCNU [10]. The synergism is perhaps explained by

effects of carbamoylation on the enzymes of DNA repair and metabolism. A study involving Cloretazine and the direct repair protein O^6- alkylguanine-DNA-alkyltransferase (AGT) also showed the synergism between 90CE and methyl iscocyanate [8]. AGT is responsible for restoring the damaged O^6 guanine monoaducts to its native state [8]. For experiments conducted without AGT present, 90CE and Cloretazine generated the same number of DNA cross links [8]. However, when AGT was present, Cloretazine created a higher number of cross links then 90CE alone, suggesting the synergism of the carbamoylating activity of Cloretazine [8].

In an effort to better understand the cytotoxic mechanism of Cloretazine, it may be useful to examine the effects of the drug on important enzymes of DNA metabolism. Thioredoxin reductase (TrxR) is an enzyme involved in many important cellular processes, including antioxidant defense, redox regulation, and cell growth [11, 12, 13]. TrxR belongs to the class of disulfide oxidoreductases, which work to maintain certain proteins of the cell in reduced states [11]. This class of enzymes may be particularly sensitive to drugs with carbamoylating activity because of the dithiol/disulfide active site. There are three known isoenzymes known that are expressed in different tissues [13]. In this case, TrxR-1 or cytosolic TrxR is of interest. TrxR works by reducing thioredoxin (Trx) with electrons provided by NADPH; reduced Trx then provides the reducing equivalents for other enzyme catalyzed reactions such as that of ribonucleotide reductase (Fig. 4) [12].

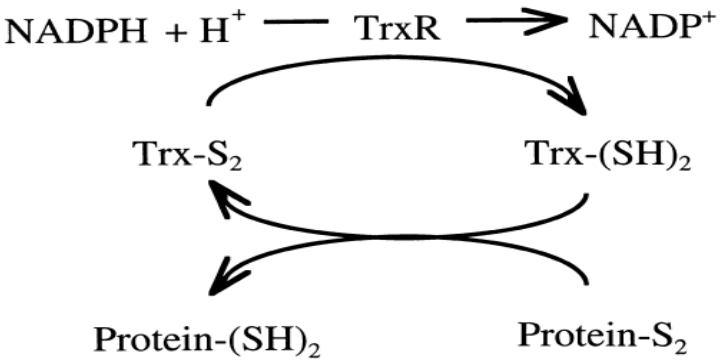


Figure 4: Function of TrxR/Trx system in the cell.

Given their high metabolic levels, malignant cells tend to overexpress TrxR [13]. Previous work in our laboratory has shown that agents with carbamoylating activity inhibit purified TrxR from rat liver (Fig. 5) (unpublished data).

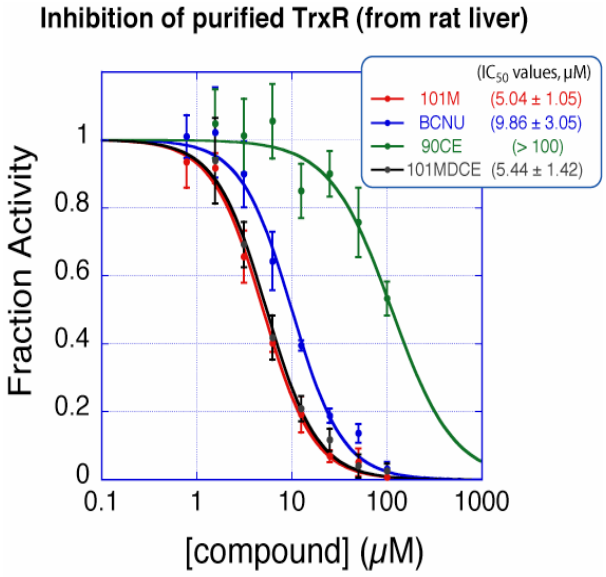


Figure 5: Inhibition of Purified TrxR by agents with carbamoylating activity (unpublished data).

Of all the enzymes thus far exposed to Cloretazine, TrxR is the most sensitive to the drug (unpublished observation). Inhibition concentrations (IC_{50} values) of carbamoylating

agents against TrxR were in micromolar range (Fig. 5). Most other enzymes tested have had markedly higher IC_{50} values (unpublished observation). Typically enzyme inhibitors are only considered viable pharmaceutical agents with nanomolar IC_{50} values. However, micromolar concentrations are clinically relevant for Cloretazine, because up to 100 μM Cloretazine has been measured in patients [7]. Interestingly, one study reported that a similar oxidoreductase, glutathione reductase, is also inhibited by Cloretazine in purified form, but further work showed that the drug was unable to inhibit the enzyme in a cellular environment [9]. This provides the basis of the research examining Cloretazine's inhibition of cellular Trx-R.

Using mammalian cell culture and a TrxR enzyme assay, the activity of TrxR was measured under varying concentrations of the studied agents. L1210 murine leukemia cells treated with the drugs Cloretazine, BCNU, 90CE, and 101MDCE were harvested, lysed and clarified. Due to TrxR's wide substrate potential, the enzyme can reduce 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), which is colorless, but turns yellow upon reduction to 2-nitro-5-mercaptobenzoic acid. Thus, the enzymatic activity of cellular TrxR from L1210 lysate can be measured spectrophotometrically.

Materials and Methods

Enzymes and Biochemicals

Cloretazine, 101MDCE, and 90CE were synthesized, purified, characterized as described elsewhere and provided by Prof. Alan Sartorelli of Yale University [14]. BCNU was purchased from Sigma (St. Louis, MO). Stock solutions of drugs were prepared by dissolving them in dry DMSO to concentrations of 200 mM and storing them at -20°C.

Dilutions of the respective drugs were also prepared in dry DMSO. Purified TrxR from rat liver was purchased from Sigma (St. Louis, MO). Bovine Serum Albumin (BSA) was purchased from Fischer Scientific (Suwanee, GA). Protease Arrest of the protease inhibitor cocktail was purchased from G Biosciences (St. Louis, MO). ß-nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma (St. Louis, MO). NADPH was prepared by dissolving it in 10 mM Tris-Cl pH 7.4 to a concentration measured spectrophotometrically at 340 nm using the extinction coefficient 6.22 μM^{-1} cm^{-1}.

Cell Culture

L1210 murine leukemia cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, in an atmosphere of 5% CO_2 at 37°C. The cells were maintained at densities between 10^4 to 10^6 cells/mL.

Preparation of L1210 Cell Lysates

L1210 cells were harvested from cultures containing greater then 10^8 total cells by centrifugation (Du Pont Sorvall TC centrifuge) at 1,300 rpm for 5 minutes at room temperature. The supernatant was removed by aspiration and the cells were then resuspended in fresh RPMI 1640 medium pre-warmed to 37 °C to give a cell density of 5 x 10^6cells/mL. 15 mL Falcon tubes containing 3.5 x 10^7 cells were treated with a given agent (Cloretazine, 101MDCE, 90CE, BCNU, or DMSO) at a final concentration of 200 μM or 50 μM with 0.1% v/v DMSO. Control cells were also treated with 0.1% v/v

DMSO. The samples were incubated at 37° C in 5 % CO_2 environment for three hours with the cap on loosely to allow for gas exchange. The samples were mixed halfway through the incubation by several gentle inversions. Post-incubation, the cells were harvested by centrifugation at 1,300 rpm for 5 min as before. Each cell pellet was washed in 1 mL of 1x phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH [7.4]) and centrifuged again at 1,300 rpm for 5 min at room temperature as before. The samples were resuspended in 500 µL of lysis buffer (50 mM Tris-Cl pH 7.4, 50 mM EDTA, [1x] Protease Arrest) and then lysed either by sonication (five cycles at 60% power with a Fisher sonic dismembrator model 300) or three consecutive freeze/thaw cycles (-70°C/37°C). The lysates were clarified by high speed centrifugation at 14,000 rpm in a Thermo IEC MicroCL 21 centrifuge for five minutes at room temperature. The supernatant was removed by aspiration, retained, and kept on ice.

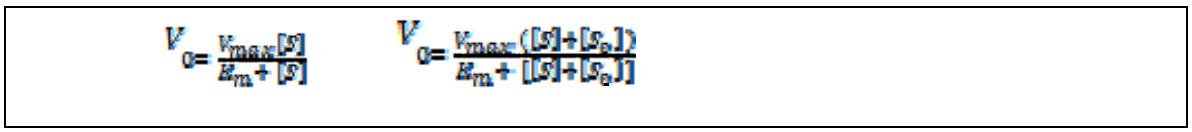
Cellular TrxR Assay

Lysates were analyzed for TrxR activity with a TrxR assay. 70 µL of TrxR cocktail (100 mM potassium phosphate pH 7.4, 1 mM EDTA pH 8, 5 mM 5,5-dithiobis-(2-nitrobenzoic acid (DTNB), 0.3 mM reduced ß-nicotinamide adenine dinucleotide phosphate (NADPH)) was added to 30 µL of lysate in a 96-well plate. All reactions were carried out in triplicate. For the negative control, the TrxR cocktail was the same except for the NADPH was absent and 10 mM Tris-Cl pH 7.4 was added in place. The reaction progress was monitored by the change in absorbance at 412 nm by the reduction of

DTNB with a Molecular Devices Spectramax M2 spectrophotomer for 10 minutes at 25°C. Readings were taken every 34 seconds for a total of 18 data points per experiment. Data were transferred to a Microsoft Excel spreadsheet where the change in absorbance versus time was calculated for the most linear portion of data. These slope values were adjusted using the extinction coefficient of DTNB (14150 M^{-1} cm^{-1}). Enzymatic activity was normalized to the protein concentration in the lysate sample measured by a Bio-Rad Protein assay according to a manufacturer's protocol against a BSA standard curve [15]. Activities were background corrected by subtracting out the negative control activity and were reported as a fraction of the positive control activity (no agent).

Variable NADPH Assay

L1210 cell lysates harvested without drug exposure were analyzed for TrxR activity with varying NADPH concentrations in a TrxR cocktail. Five, two-fold serial dilutions of NADPH were prepared starting at 200 μM (100 μM, 50 μM, 25 μM, 12.5 μM) plus a sixth well with only buffer (10 mM Tris pH 7.4). 60 μL of TrxR cocktail (100 mM potassium phosphate pH 7.4, 1 mM EDTA pH 8, 5 mM DTNB) and 10 μL of the respective NADPH dilutions were added to 30 μL of lysate (in duplicate) to a 96-well plate. The reaction progress was monitored by the change in absorbance at 412 nm by the reduction of DTNB with a Molecular Devices Spectramax M2 spectrophotomer for 10 minutes at 25 °C. Readings were taken every 34 seconds for a total of 18 data points. V_{max} points correlating to TrxR activities were analyzed in Excel using Michaelis-Menten kinetics (Fig. 6) to find the best correlation of substrate concentration and rate.



$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \qquad V_0 = \frac{V_{max}([S]+[S_0])}{K_m + ([S]+[S_0])}$$

Figure 6: Michaelis-Menten equation and adjust equation used to fit the data obtain from the varying NADPH experiment. * see results section

Results

TrxR Activity

The inhibition of TrxR by Cloretazine, BCNU, 101MDCE, and 90CE was analyzed by incubating L1210 cells with drug for 3 hours at 37 °C in 5 % CO_2. TrxR activity is dependent on availability of NADPH [8]. Fraction activity of TrxR was calculated by subtracting out the negative control and taking that as a fraction of the difference of the positive and negative controls. Cloretazine, BCNU, and 101MDCE inhibited the activity of cellular TrxR (Fig. 7). 90CE did not inhibit cellular TrxR, showing no effect on the activity of the enzyme. Lysates from cells treated with 200 μM concentrations of Cloretazine retained only 8.67 % of the control activity ± 9.8%. Lysates from cells treated with 200 μM BCNU retained 7.83 % activity ± 13.3 % and 200 μM 101MDCE retained 34.6 % activity ± 12.4 %. These activities were significantly less than the activity of cell lysates treated with 200 μM concentrations of 90CE which resulted in activities of 94.8 % ± 12.0 %. Cell lysates treated with 50 μM concentrations of Cloretazine retained 49.8 % of the control activity ± 9.62 %. Lysates treated with 50 μM concentrations of BCNU retained 44.4 % activity ± 7.22 % and lysates treated with 50 μM concentrations of 101MDCE resulted in 57.5 % of the control activity ± 13.4 %.

These 50 µM concentrations of agents also show significantly lower activities compared to 90CE which remained unaffected with 86.8 % of the control activity ± 13.8 %. These results suggest drugs with carbamoylating activity are able to inhibit TrxR in the cellular context. Cloretazine and BCNU had similar inhibitory effects at both 50 and 200 µM concentrations, showing no major differences between the two (Fig. 7). 101MDCE, while able to inhibit TrxR, differed from the other carbamoylating agents in ability to inhibit TrxR at the 200 µM concentration, but showed similar effects to BCNU and Cloretazine at 50 µM (Fig. 7). 90CE, a DNA alkylating agent, lacking carbamoylating activity, showed no ability to inhibit TrxR. Activities around 90 % suggest that the enzyme is still functional and carrying out biochemical reductions.

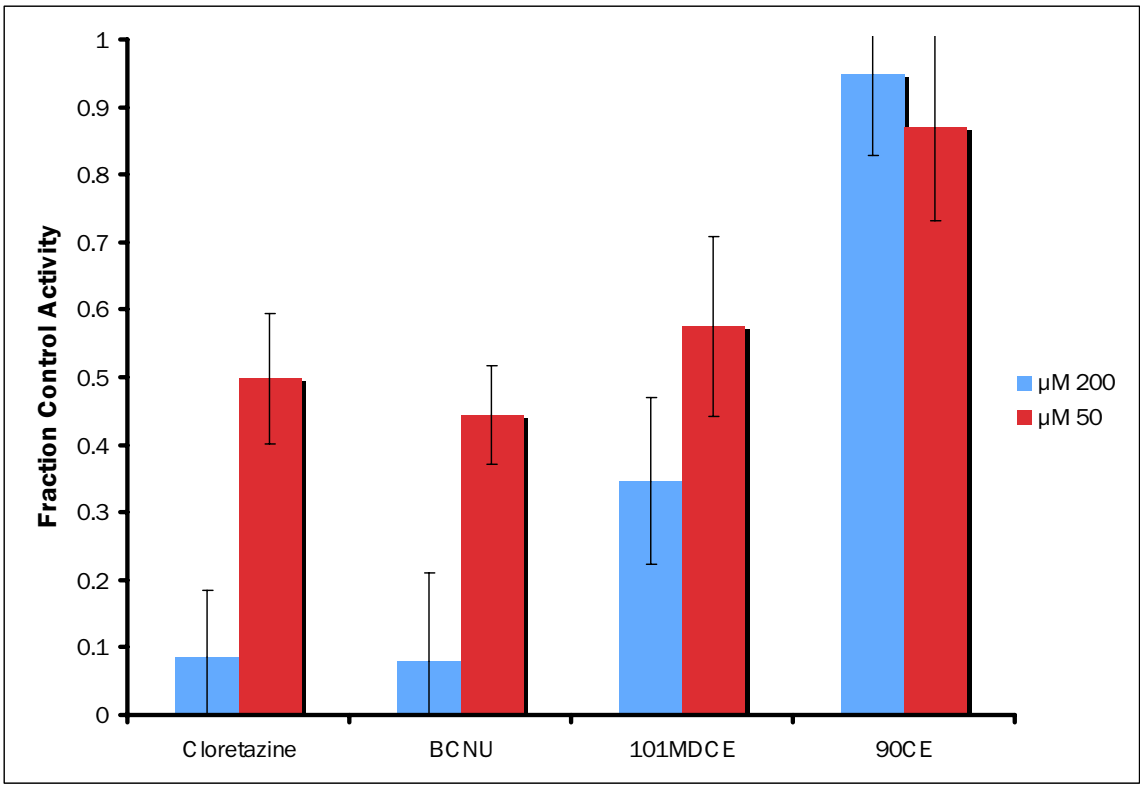


Figure 7: Inhibition of cellular thioredoxin reductase by agents with carbamoylating activities.

Variable NADPH

By varying the NADPH in experiments measuring the activity of TrxR from cell lysate, the initial enzyme velocity can be plotted as a function of the concentration of substrate according to the Michaelis-Menten equation. In the absence of added NADPH, TrxR activity was still measured. The endogenous concentration of NADPH was extracted using Michaelis-Menten kinetic principals. Adjusting for the Michaelis-Menten equation to account for an initial level of NADPH present in the cell, we could solve for the endogenous concentration of NADPH.

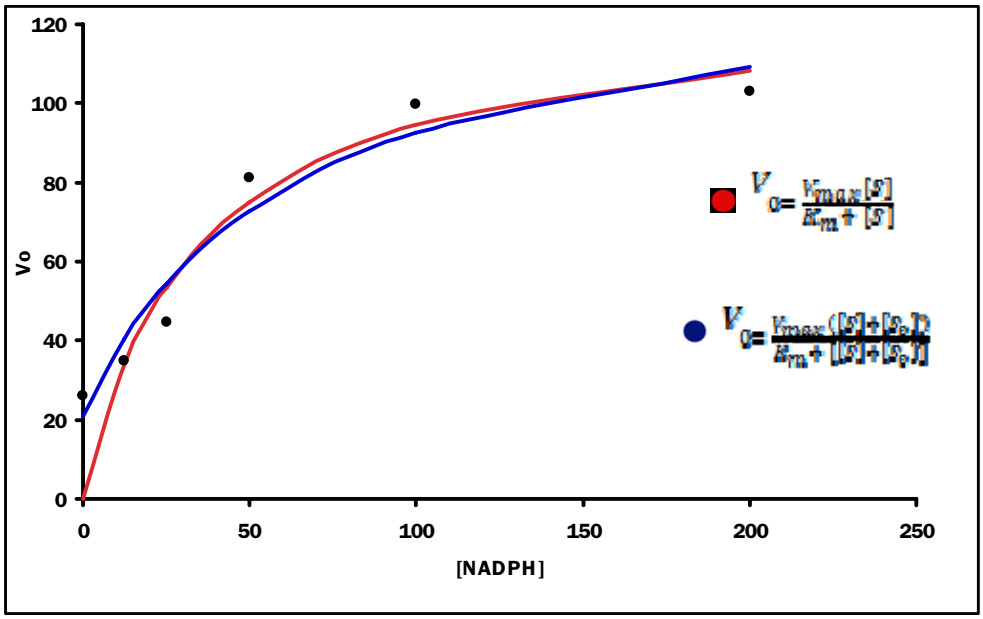


Figure 8: Michaeis- Menten Plot to calculate the endogenous concentration of NADPH in L1210 cell lysates

The red curve (Fig. 8) represents the theoretical curve for the rate of TrxR at a given concentration of NADPH. The blue curve represents the actual measured data. The data were fit using the solver tool in Excel. $[S]_0$ was extracted using a least squares fit to the hyperbolic function. The NADPH concentration was measured to be 9.4 μM. Due to the dilution of the cells in lysis buffer, it is approximated that this endogenous concentration of NADPH is closer to 45 μM, which is comparable to literature values of human erythrocytes [16].

Discussion

A relatively new class of chematherapetuic agents, sulfonylhydrazines has provided promising results in clinical and preclinical trials. Cloretazine is an anticancer agent that has shown broad anti-tumor activity in preclinical models [10]. Currently, Cloretazine is in clinical trials for several cancers, showing significant activity against acute myeloid leukemia. Single agent trials of Cloretazine have produced remissions in patients suffering from leukemia [10]. The lack of toxicity makes Cloretazine favorable for combinatory treatments with other agents [5]. Although the mechanism of the drug is extremely complex and yet to be fully understood, the chemistry of Cloretazine is relatively simple. The drug decomposes after base activation to yield two species with alkylating and carbamoylating activities respectively. Cloretazine owes its anticancer activity to its ability to cross link DNA by its alkylating species. However, it is believed that the carbamoylating activity enhances the cytotoxicity of the cross linking through synergistic mechanisms.

Along with Cloretazine, nitrosoureas represent a class of cytotoxic agents used in chemotherapy that generate alkylating and carbamoylating activities. BCNU, also known as Carmustine, has already proven its clinical usefulness, as it has been used for years to treat lymphomas and brain tumors [5]. One major difference between Cloretazine and BCNU is that BCNU decomposes to produce hydroxyethylating, vinylating and aminoethylating species in addition to the chloroethylating and carbamoylating ones, which Cloretazine does not. These extra reactive species increase the toxicity of BCNU and have no therapeutic benefit as they are known to cause carcinogenic and mutagenic events [5]. These associated toxicities of BCNU promote the search for better DNA alkylating agents with similar activities.

Another important difference between Cloretazine and BCNU is of the isocyanates produced by each compound. Cloretazine generates MiC, while BCNU forms Chloroethyl isocyanate (CEiC). Both are efficient in carbamoylating activity, but CEiC can be hydrolyzed to form 2-chloroethylamine, which can damage DNA [7]. This deleterious effect is not found with MiC. The therapeutic superiority of Cloretazine over BCNU, besides forming more cross links, may also be due to the differences in isocyanates. As we have discussed, DNA alkylation by chloroethylating has been studied extensively. However, the activity of isocyanates remains elusive, although recent evidence suggests a significant role for carbamoylating activity. Further research is necessary to fully understand the therapeutic benefit and clinical significance of isocyanates.

The nature of isocyanates with carbamoylating activities provides a class of target proteins that contain reactive thiols. TrxR is an oxidoreductase containing reactive thiols

in the form of cysteine residues, with the main function to provide reducing equivalents for other biochemical reactions. TrxR is also involved in maintaining redox equilibrium in the cell and plays a role in apoptotic pathways. Interestingly, this important enzyme is elevated in tumors and malignant tissue, where it supports cell growth and proliferation [11, 13]. The importance of the TrxR/Trx system in the cell will ultimately lead to serious effects if TrxR is inhibited. Inhibition of TrxR leads to reduced amounts of Trx to be used as reducing equivalents. Ribonucleotide reductase, which is dependent on these reducing equivalents, then loses function, and lower levels of deoxyribonucleotides are produced. As 90CE alkylates and damages DNA, the cell is then unable to repair the damaged DNA due to the lack of nucleotides present. It is also critical for the cell to replicate the genetic material in order to maintain the rapid division. With the decreased concentration of deoxyribonucletides, the cancer cells will be unable to carry out division and replication, making Cloretazine effective at blocking tumor growth. Inhibiting TrxR can lead to an increase in reactive oxygen species (ROS), apoptosis, and a decrease in tumor growth among other things [13]. The elevated levels of TrxR and the variety of cytotoxic events provided by inhibiting TrxR make the enzyme a favorable anticancer target.

In previous work, purified TrxR was shown to be inhibited by Cloretazine in a cell free environment. Here we have shown that under cellular conditions Cloretazine was also effective in inhibiting TrxR. Not only does Cloretazine inhibit the activity of TrxR, but it acts to a similar extent as the clinically viable agent BCNU. Surprisingly, a similar oxidoreductase, glutathione reductase (GR), was shown to be inhibited in purified form, but not under the cellular context by Cloretazine [9]. BCNU was able to inhibit GR in both the purified and cellular form. This selective inhibition may be explained by the

differences in isocyanates generated by the two drugs. Chloroethyl isocyanate (CEiC) was able to inhibit both TrxR and GR, while MiC inhibited only TrxR. GR could have a stronger affinity for CEiC than MiC, and is therefore more efficient in a cellular environment in inhibiting the enzyme. However, the inability of MiC to inhibit GR is therapeutically beneficial. GR is an extremely important protein involved in antioxidant defense, primarily in the pulmonary tissues, where reactive oxygen species are constantly evolved [9]. Inhibition of this enzyme leaves these cells and tissues more sensitive to toxic and deleterious effects of ROS damage [9]. The therapeutic superiority of Cloretazine over BCNU is evident from these results. BCNU inhibits both GR and TrxR, but leads to toxic side effects; here we show that Cloretazine has a similar *in vivo* inhibition of TrxR and lacks the toxicity of BCNU.

As an attempt to explain the high background during the experiments, the activity of TrxR was measured using varying NADPH. The activity at zero added NADPH could arise from two possibilities. First, there could be a source of cellular NADPH. Second, another enzyme or molecule could be reducing DTNB. Endogenous NADPH is reasonable explanation since we used whole cell extracts to analyze TrxR. Normally cells have pools of NADPH available for reactions, or in this case enough for activity of TrxR to still be measurable. In order to determine the endogenous level of NADPH, activities at various concentrations of NADPH were measured. Then using an adjusted Michaelis-Menten equation, the initial substrate concentration could be calculated. The levels of NADPH are slightly elevated, but still comparable to literature values of normal erythrocytes [16]. This is understandable as cancer cells are rapidly metabolizing compared to normal proliferating cells and may require more NADPH than usual.

Another method to test the high background and prove that TrxR is being measured properly would be to utilize a known inhibitor of TrxR. Using a known inhibitor should completely knock out the activity of TrxR reducing DTNB at any concentration of NADPH. This would let us know that we are correctly assuming that TrxR is reducing DTNB and not something else.

In order to better understand the mechanism of anticancer activity of Cloretazine, we investigated the inhibitory effects of carbamoylation on the enzyme TrxR in a cellular environment. The therapeutic benefit of isocyanates still requires further research, but as studies progress they appear to be important to the cytotoxicity of the respective drugs. As for Cloretazine, it is more evident that the carbamoylating activity of MiC synergizes with the cross linking of 90CE through effects of DNA metabolism, ultimately increasing the cytotoxicity of this prodrug. In this study Cloretazine was able to inhibit cellular TrxR in almost equal amounts as BCNU, yet Cloretazine lacks the toxic side effects of BCNU, including inhibition of GR. This could suggest that Cloretazine could eventually replace BCNU in chemotherapies and expand the therapeutic benefit to the patient.

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