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Thioredoxin reductase is inhibited by the carbamoylating activity of the anticancer sulfonylhydrazine drug Laromustine

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Abstract

The thioredoxin system facilitates proliferative processes in cells and is upregulated in many cancers. The activities of both thioredoxin (Trx) and its reductase (TrxR) are mediated by oxidation/reduction reactions among cysteine residues. A common target in preclinical anticancer research, TrxR is reported here to be significantly inhibited by the anticancer agent Laromustine. This agent, which has been in clinical trials for acute myelogenous leukemia and glioblastoma multiforme, is understood to be cytotoxic principally via interstrand DNA crosslinking that originates from a 2-chloroethylating species generated upon activation *in situ*. The spontaneous decomposition of Laromustine also yields methyl isocyanate, which readily carbamoylates thiols and primary amines. Purified rat liver TrxR was inhibited by Laromustine with a clinically relevant IC₅₀ value of 4.65 μ M. A derivative of Laromustine that lacks carbamoylating activity did not appreciably inhibit TrxR while another derivative, lacking only the 2-chloroethylating activity, retained its inhibitory potency. Furthermore, in assays measuring TrxR activity in murine cell lysates, a similar pattern of inhibition among these compounds was observed. These data contrast with previous studies demonstrating that glutathione reductase, another enzyme that relies on cysteine-mediated redox chemistry, was not inhibited by methylcarbamoylating agents when measured in cell lysates. Mass spectrometry of Laromustine-treated enzyme revealed significant carbamoylation of TrxR, albeit not on known catalytically active residues. However, there was no evidence of 2-chloroethylation anywhere on the protein. The inhibition of TrxR is likely to contribute to the cytotoxic, anticancer mechanism of action for Laromustine.

Keywords

thioredoxin reductase; alkylating agents; methyl isocyanate; Laromustine

Introduction

The preclinical prodrug Laromustine [Cloretazine; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine] yields two reactive electrophiles, methyl isocyanate and 90CE (Fig. 1), upon base-catalyzed activation *in situ* which carbamoylate and 2-chloroethylate, respectively, nucleophilic functional groups in the cell [1–3]. The 2-

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chloroethylation of the O6 position of guanine in DNA is believed to be the major cytotoxic lesion and results in a particularly toxic interstrand DNA crosslink between the guanine and its complementary cytosine [4, 5]. Methyl isocyanate easily modifies sulfhydryl groups under physiological conditions, resulting in a somewhat labile sulfur-linked carbamoyl adduct. Carbamoylated amines are more thermodynamically stable, but are likely not as kinetically favored in a cellular context, given that primary amines will be largely protonated at physiological pH. 101MDCE, an analog of Laromustine that lacks 2-chloroethylating activity, while retaining carbamoylating activity, can itself be acutely cytotoxic in cultured cells, and can also amplify the cytotoxicity of 90CE *in vitro* [1, 4, 6]. Possible therapeutically relevant targets of the carbamoylating activity of Laromustine *in vivo* include O⁶-alkylguanine-DNA alkyltransferase [6] and DNA polymerase β [7], both of which are involved in DNA repair. The complete mechanism of action by which methyl isocyanate contributes to the antineoplastic activity of Laromustine is not yet fully understood.

Another DNA-alkylating agent, Carmustine [BCNU; 1,3-bis(2-chloroethyl)-1-nitrosourea], generates 2-chloroethyl isocyanate, rather than methyl isocyanate, upon aqueous decomposition [8, 9]. The pharmacological merits of the isocyanates generated by nitrosoureas have been controversial [9–12], but there is evidence for a positive contribution from methyl isocyanate cogenerated by many anticancer agents [6, 13–15]. Although methyl and 2-chloroethyl isocyanates are similarly reactive electrophiles, there are significant functional differences between these species in cultured cells. One such example is the comparative inhibition of the enzyme glutathione reductase (GR), which functions in systems primarily responsible for clearance of reactive oxygen species [16, 17]. Carmustine inhibits cellular GR by up to 90% at clinical doses, a phenomenon implicated as a cause of the pulmonary toxicity often seen in carmustine-treated animals and human cancer patients [18]. Laromustine does not produce similar inhibition of cellular GR activity, either in human erythrocytes or L1210 murine leukemia cells, despite Carmustine and Laromustine being equally potent inhibitors of the purified enzyme [13]. Given the significance of methyl isocyanate towards the therapeutic efficacy of Laromustine, it is likely that the critical target(s) of methyl isocyanate has not been fully revealed.

The thioredoxin system, which involves thioredoxin (Trx), Trx reductase (TrxR), and NADPH, is another endogenous antioxidant system [19–21]. In its reduced form, Trx can provide reducing equivalents for several biochemical transformations via its oxidation to a disulfide species. TrxR regenerates reduced Trx via the oxidation of the soluble electron carrier NADPH. The thioredoxin system has multiple functions in the cell, including redox homeostasis and the regulation of apoptosis and cell growth [22, 23]. Trx can prevent cellular apoptosis by scavenging reactive oxygen species, thereby providing protection from oxidative stress. It also acts anti-apoptotically by regulating the activities of transcription factors such as NF- κ B and AP-1, and by directly binding and inhibiting the activity of the pro-apoptotic protein apoptosis signal-regulating kinase 1 [24, 25]. TrxR also delivers reducing equivalents to ribonucleotide reductase, which is necessary for the synthesis of deoxyribonucleotides towards the synthesis of DNA [22]. With so many cell proliferative consequences of a robust thioredoxin system, TrxR is increasingly viewed as an attractive target for anticancer agents [26–28]. Both Trx and TrxR are upregulated in malignant cells and their activities are reported to be essential for tumor progression [29]. TrxR knockout mice have an embryonic lethal phenotype [30] while disruptions of TrxR in a variety of human cell types either decreases tumorigenicity [31] or increases likelihood of oxidative cell death [32–34].

The functional form of the 55 kDa TrxR polypeptide is a homodimer. The active site residues are within a C-terminal region that also incorporates a flavin adenine dinucleotide

cofactor. Crystal structures and biochemical analyses have demonstrated the direct involvement of a selenocysteine residue very near the carboxy-terminus in the catalytic mechanism of TrxR [35, 36]. There are at least three other cysteine residues required for catalysis and a total of 15 cysteine residues in the rat TrxR homolog. In addition, there are 39 lysine residues in the primary sequence of TrxR. Any, or all, of these nucleophilic groups could be targets for methyl isocyanate's carbamoylating activity. The inhibition of TrxR activity has been reported using several reactive electrophiles [37, 38], including many existing antitumor drugs [28, 39]. Reported here is the potent inhibition of TrxR by the methyl isocyanate-bearing anticancer agent Laromustine. Using synthetic analogs of the drug, this inhibition was directly attributed to its carbamoylating activity, rather than to its 2-chloroethylating activity. The concentrations of Laromustine at which inhibition was measured were within clinically relevant dose ranges, and the inhibition was observed against both purified enzyme and enzyme from lysates of drug-treated cells. Furthermore, methyl carbamoylated residues of TrxR were directly observed using tandem mass spectrometry. These data further explicate the mechanism of action for this potentially clinically useful anticancer agent.

Materials and Methods

Small molecules

The syntheses of Laromustine and 90CE were carried out using the methods of Shyam, *et al.* [40, 41] using reagents from Sigma Aldrich (St. Louis, MO), resulting in material with spectroscopic signatures identical to those published in the literature. 101MDCE was kindly provided by Alan Sartorelli of the Yale University School of Medicine. Carmustine was purchased from Sigma. All compounds were dissolved in dry DMSO to concentrations of 200 mM. Stock solutions were stored desiccated at -20°C . Dilutions were also prepared in dry DMSO.

Cells and biochemicals

L1210 murine leukemia cells (American Type Culture Collection #CCL-219) were cultured in RPMI-1640 media supplemented with 10% bovine serum albumin and 1% penicillin/streptomycin at a density between 5.0×10^4 cells/mL and 1.0×10^6 cells/mL at 37°C and 5% CO_2 . Cell culture media and supplements were purchased from Lonza (Basel, Switzerland). Purified rat liver thioredoxin reductase 1 (TrxR) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma. ProteaseArrest protease inhibitor cocktail was purchased from G-Biosciences (Maryland Heights, MO). The Bio-Rad Protein Assay was purchased from Bio-Rad (Hercules, CA). ProtoBlue Safe colloidal Coomassie stain was purchased from National Diagnostics (Atlanta, GA). All other purchased reagents were of the highest available quality.

In vitro assay using purified TrxR

TrxR activity was determined by monitoring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as measured by the increase in absorbance at 412 nm. Five u/mL TrxR were pre-incubated with 0–200 μM Laromustine, Carmustine, 90CE, or 101MDCE in 40 mM Tris-HCl (pH 8.0), 100 μM NADPH, and 10% DMSO at room temperature. For the pre-incubation time course experiments, TrxR was treated with 7.4 μM Laromustine for the indicated times. For the dose-dependence experiments, enzyme reactions were initiated after 2 hr of preincubation with drugs. The pre-incubated enzyme was added to a reaction of the following final composition: 0.5 unit/mL TrxR, 5 mM DTNB, 300 μM NADPH, 100 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 1% DMSO. Reactions were carried out in wells of a UV-transparent, flat-bottomed 96-well plate in a total volume of 100 μL and allowed to react at 25°C while measuring absorbance of 412 nm light in a SpectraMax M2

plate reader (Molecular Devices) every 8 sec. Activity was measured as the rate of absorbance change during the first 1 min of reaction progress. All reactions were performed in triplicate. Data were normalized to the positive control (TrxR pre-incubated with DMSO) and the negative control (without TrxR) and expressed as fraction activity. IC₅₀ values were calculated by fitting the fraction activity data to the following hyperbolic equation: $1/(1+(x/a)^b)$, where 'x' refers to drug concentration and 'a' is solved as the IC₅₀ value.

In vitro TrxR assay using cell lysates

L1210 cells were harvested and resuspended in 5 mL fresh media to a density of 7.0×10^7 cells/mL and treated with 50 μ M or 200 μ M Laromustine, 101MDCE, 90CE, or Carmustine, each delivered in DMSO (final [DMSO] = 0.1%) for 3 hr at 37°C. Control cells were treated with an equivalent volume of DMSO. Cell samples were gently mixed periodically during the incubation. Treated and control cells were collected using a clinical centrifuge at 500 $\times g$ for 5 min. Supernatants were aspirated, and the cell pellets were washed with 1 mL phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ 2 mM KH₂PO₄ pH 7.4) and once again centrifuged at 500 $\times g$ for 5 min. Each cell sample was resuspended in 500 μ L of lysis buffer (50 mM Tris-Cl pH 7.4, 50 mM EDTA, 1x Protease Arrest) and then lysed by sonication three times for 30 sec at 60% power, interspersed with 5 min incubations on ice, using a Model 300 Fisher Sonic Dismembrator. Lysates were clarified by microcentrifugation at 14,000 rpm, 4°C and normalized according to total protein concentration using a Bradford protein assay. Thirty μ L of each lysate sample were transferred to separate wells of an ultraviolet-transparent 96-well plate. The reaction was initiated by transferring 70 μ L of TrxR cocktail (100 mM potassium phosphate, pH 7.4; 1 mM EDTA; 5 mM DTNB; 0.3 mM NADPH) to the wells containing the lysate. All reactions were carried out in triplicate. The negative control TrxR cocktail lacked NADPH. Reaction progress was monitored at 412 nm for 10 min at 25°C using a Molecular Devices Spectramax M2 spectrophotometer. Readings were taken every 34 sec for a total of 18 data points per experiment. Enzymatic activity was determined based on the change in absorbance of light versus time. Data are expressed as activity beyond the negative control activity as a fraction of that of the positive control.

Mass spectrometry analysis of drug-treated TrxR

Purified rat liver TrxR (2.2 μ g) was treated with 1.0 or 0.1 mM Laromustine or 101MDCE in 100 mM Tris-HCl (pH 8.0), 100 μ M NADPH, and 10% DMSO (drug vehicle) for 1 hr at room temperature in a total volume of 11 μ L. Control protein was exposed to the same conditions without drug. Samples were separated on 10% denaturing polyacrylamide gels using Laemmli loading buffer lacking reducing agent. Gels were stained using a colloidal Coomassie stain according to vendor protocol. The single TrxR bands were excised from the gel and the gel slices were destained in 50% acetonitrile / water and desiccated. The samples were then digested with proteinase-K for 1 hr at room temperature. Subsequently, peptides were extracted, dried, desalted on an OASIS C₁₈ HLB micro-SPE plate and analyzed by nanoscale microcapillary LC-MS/MS as described [42, 43]. Samples were loaded using a FAMOS autosampler (LC Packings/Dionex, Sunnyvale CA) onto a custom manufactured reverse-phase, Reprosil-Pur 3 μ m, 200 Å C₁₈-AQ particle filled (Dr. Maisch GMBH, Ammerbuch-Entringen, Germany) fritless-tip microcapillary column (0.125 $\times$ 180 mm, 7 μ m tip) (Polymicro Technologies, Phoenix, AZ, and Sutter Instruments, Novato, CA) using an HPLC-driven (Agilent 1200 capLC) split-flow system designed to maintain 1200–1500 psi at the head of the analytical column. The resultant peptide eluate was directed into an LTQ-Orbitrap high performance mass spectrometer operating in a data-dependent sequencing acquisition mode across a 60 min reverse-phase gradient (5% acetonitrile / 0.1% formic acid to 30% acetonitrile / 0.1% formic acid). The collected tandem mass spectra were data-searched using the SEQUEST algorithm [44, 45] with a single FASTA file corresponding to

the rat TrxR protein sequence (www.uniprot.org; O89049). Variable modifications for methyl carbamylation (56.02146 Da) and 2-chloroethylation (61.99233 Da) were allowed for Cys and Lys residues. Data were required to pass a ± 2.5 ppm mass accuracy filter.

Results

Purified TrxR activity is inhibited by isocyanate bearing compounds

TrxR, the only enzyme known to reduce the oxidized cytosolic form of Trx [20], is a known target of the nitrosourea class of antineoplastic alkylating agents such as Carmustine [46]. TrxR shares significant homology with glutathione reductase, which, when purified, is inhibited by both Carmustine and Laromustine *in vitro* [13]. To determine the effects of Laromustine, Carmustine, 90CE, and 101MDCE on TrxR, we performed enzymatic assays using enzyme that had been exposed to various concentrations of the agents. All agents were exposed to the purified enzyme for 2 hours at pH 8 to allow for drug activation. Laromustine, 101MDCE, and Carmustine inhibited TrxR with IC_{50} values of 4.65 ± 0.37 , 5.37 ± 0.58 , and 7.63 ± 0.72 μ M, respectively (Fig. 2). These IC_{50} values are approximately an order of magnitude lower than those observed with glutathione reductase using very similar experiments [13]. The extent of Carmustine inhibition reported here was more substantial than reported elsewhere [39, 46]. The inhibition of TrxR by 90CE, which is devoid of carbamoylating activity, was considerably, with an IC_{50} value in excess of 100 μ M.

The activity of Laromustine-treated TrxR recovers with time

Given the dependence of TrxR's catalytic activity on cysteine thiols and the thiophilic nature of Laromustine's isocyanate subspecies, the likely cause of the TrxR inhibition by Laromustine is carbamoyl adduction on one or more of the active site cysteine residues. However, such a modification is likely to be hydrolytically reversible. To assess this possibility, 5 units/mL (31.5 μ g/mL) rat liver TrxR was treated with 7.4 μ M Laromustine, a concentration approximately 50% higher than its IC_{50} value, at pH 8 and room temperature for 8 hours. At the indicated times, aliquots of the pre-incubated enzyme were removed and added to a reaction cocktail containing substrates. Activity of Laromustine-treated TrxR fell to 33% of the control activity after 2 hours. However, over the next 6 hours enzyme activity steadily recovered to nearly 50% of control activity (Fig. 3). These data suggest a transient inhibitory phenomenon, most easily explained by the formation of at least one methyl carbamoyl adduct in the TrxR active site, which may have been slowly hydrolyzed back to a sulfhydryl moiety.

TrxR activity in L1210 cell lysates is inhibited by both Laromustine and Carmustine

Enzyme activity in clarified lysates from cells treated with the agents was also measured by monitoring the reduction of DTNB at 412 nm. Cells were treated for 3 hours with Laromustine, 101MDCE, 90CE, and Carmustine. In each experiment, TrxR activity was corrected by subtracting the activity of the negative control, which lacked NADPH, and reported as a fraction of the positive control activity. While Laromustine, 101MDCE, and Carmustine strongly inhibited TrxR activity, 90CE was not a strong inhibitor of the enzyme (Fig. 4). Lysates from cells treated with 200 μ M Laromustine, 101MDCE, 90CE, and Carmustine retained only $8.16\% \pm 8.84\%$, $7.79\% \pm 5.59\%$, $73.3\% \pm 13.6\%$, and $6.43\% \pm 9.36\%$ of the control activity, respectively. Lysates treated with 50 μ M Laromustine, 101MDCE, 90CE, and Carmustine retained $49.2\% \pm 13.5\%$, $33.8\% \pm 12.1\%$, $78.9\% \pm 8.72\%$, and $63.0\% \pm 14.6\%$ of the control activity, respectively. Therefore, treating cells with agents that generate isocyanates resulted in an almost complete inhibition of TrxR at 200 μ M and approximately 50% inhibition at 50 μ M. Neither concentration of 90CE yielded appreciable inhibition of cellular TrxR activity. That purified enzyme was inhibited to the

same degree by methyl carbamoylating activity as enzyme in cell lysates stands in contrast to similar experiments using glutathione reductase, in which only purified enzyme was inhibited [13].

Several residues on purified TrxR were carbamoylated, but not 2-chloroethylated, upon exposure to Laromustine

Rat liver TrxR was treated with 1 mM or 100 μ M Laromustine and 101MDCE at pH 8.0, as was done for the enzyme assay. After SDS-PAGE separation, the TrxR samples were digested with the non-specific protease proteinase-K and subjected to high mass accuracy LC-MS/MS analysis. On average, we observed ~70% protein coverage across the different sample types. Among the protein samples treated with either agent, there were 16 lysine residues with a demonstrable methyl carbamoyl adduct (Table 1). These lysine adducts, representing nearly half of the lysine residues in the TrxR polypeptide, were observed in a concentration-dependent manner. One cysteine residue, C475, also showed evidence of methyl carbamoylation, albeit very weakly and most evident in the protein sequence treated with the higher concentration of 101MDCE (Fig. 5). However, upon extraction of the precursor peptide mass $\pm$ 2.5 ppm from the other drug-treated samples, a modest methyl carbamoyl signature on C475 was seen in the lower concentration of 101MDCE and the Laromustine samples (data not shown). There were no adducts seen on any of these nucleophilic residues when the protein was treated only with DMSO. In the Laromustine-treated samples, there was no 2-chloroethylation observed on any of the lysines or cysteines.

Discussion

The disruption of the thioredoxin system is an intriguing avenue for chemotherapeutic action. The general mission of any cytotoxic anticancer agent is to disrupt processes that promote cell growth and proliferation. While Trx and TrxR are not unique carcinogenic gene products, their upregulation in cancer cells provides an opportunity for an antineoplastic strategy. The thioredoxin system contributes to several proliferative phenomena and is considered to be required for tumor progression [29, 31]. There have been many reports demonstrating that novel and existing compounds, including Carmustine, can act as TrxR inhibitors [28, 37–39]. Investigators have been also been researching the possible biochemical and cellular outcomes of TrxR inhibition, such as the induction of apoptotic pathways upon disruption of TrxR activity [47–49]. The potent inhibition of TrxR by the relatively recently developed pre-clinical anticancer drug Laromustine offers further details as to its mechanism of action and clinical potential.

The inhibition of TrxR, an oxidoreductase dependent on active site cysteine residues, by Laromustine was not unexpected. Laromustine co-generates the thiophilic electrophile methyl isocyanate in aqueous solution. However, it is more significant that this inhibition was as evident using enzyme from cell lysates as it was using purified enzyme. Previous work demonstrated the potent inhibition of purified glutathione reductase by methyl carbamoylating agents, but this inhibitory activity disappeared when measured in a cellular context [13]. Indeed, given the high concentration of nucleophilic functional groups inside of cells, it is conceptually challenging to expect the highly reactive electrophile methyl isocyanate to significantly modify any particular cellular target enough to affect its activity. However, Laromustine and its carbamoylating analog, 101MDCE, have been shown to inhibit cellular DNA synthesis using both *in vitro* [7] and *ex vivo* [3] methods. Inhibition of TrxR activity from Laromustine-treated cells suggests a clinical relevance in this pathway.

The most plausible explanation for the inhibition of TrxR by Laromustine and 101MDCE reported here is a covalent adduction of one or more active site cysteine thiols. As such, the nucleophilic amino acid functional groups of TrxR were investigated after exposure to these

agents by mass spectrometry. The data showed extensive methyl carbamoylation on TrxR using both Laromustine and 101MDCE and no evidence of 2-chloroethylation on any of the nucleophilic side chains. These observations seemingly support the enzyme assay data that showed significant inhibition of TrxR activity by Laromustine and 101MDCE, but not by 90CE, which lacks carbamoylating activity. However, the overwhelming majority of the carbamoyl adducts were on lysine, rather than cysteine residues. The only modified cysteine was not one of the residues thought to be involved in catalysis. While it is possible that the lysine adducts are responsible for the decrease in the activity of TrxR, there is reason to continue to suspect carbamoylation of the active site cysteines as the inhibitory mechanism of Laromustine's inhibition of TrxR. It is likely that a carbamoyl adduct on a sulfur would be significantly more labile than the corresponding adduct on a lysine's nitrogen. The requisite lag time and chemical manipulations necessary to process samples for mass spectrometry may allow sufficient opportunity for abstraction of the cysteines' carbamoyl modifications. To address this possibility, TrxR enzyme activity was measured as a function of the time in which it was exposed to Laromustine. While there was a precipitous two-hour decline in enzymatic activity, corresponding to the drug's activation and release of methyl isocyanate in aqueous solution [13], enzyme activity recovered over the subsequent six hours. It is very likely that this phenomenon maps to the labile nature of the carbamoylated cysteine residues.

While the pursuit of drugs that target biochemical mechanisms unique to cancer cells continues to be a robust effort, there remains ample cause to remain invested in general cytotoxic agents. In this era of rapid whole genome sequencing, the paradigms of classification and therapeutic regimens for cancer are being rethought. The recognition that a given tumor, classified by its morphology and location in the body, can be the consequence of a number of different genetic origins, and that a given genetic cause can correspond to a number of canonically defined tumors, is changing the clinical approach to cancer treatment [50]. The outcomes of this transformative use of cancer genomics, as well as new approaches to disease modeling and biomarker screening, include new indications for drugs that have long been in existence. For example, new data point to the use of 5-fluoruracil against certain pediatric ependymomas [51] and the potential utility of the anti-diabetic agent metformin against pancreatic cancer stem cells [52]. Clearly, there is still much to discover as scientists and clinicians work towards the ultimate goal of true personalized medicine [53].

With the renewed interest in pre-clinical screening of both novel and established anticancer agents, thorough analyses of compounds' molecular mechanism of action is as important as ever. Laromustine originated as a relatively straightforward prodrug for 2-chloroethylating activity, which is also associated with Carmustine, a nitrosourea indicated for some brain and lymphatic tumors [54]. However, while Carmustine treatment can result in significant toxicity due to non-therapeutic vinylating, hydroxyethylating, and aminoethylating activities [55], Laromustine's clinical toxicity appears relatively limited to the bone marrow [56]. While there is evidence to suggest that the DNA crosslinking resulting from Laromustine's 2-chloroethylating activity is more efficient than that of Carmustine [5], the activity of this agent's co-generated isocyanate is also relevant to its clinical potential. The carbamoylating activity of Laromustine is thought to synergize with its 2-chloroethylating activity towards the cytotoxicity of cancer cells [1, 6]. There are likely to be numerous targets of the promiscuous reactivity of methyl isocyanate that may help explain that synergism, including the repair-specific DNA polymerase beta [7]. It is unclear whether the inhibition of TrxR reported here could augment the benefits of the DNA crosslinking, 2-chloroethylating species, but the antineoplastic potential of TrxR inhibition is nevertheless evident.

Laromustine has been the subject of several clinical trials indicated against acute myelogenous leukemia (AML) and glioblastoma multiforme. A New Drug Application was filed with the Food and Drug Administration in early 2009 seeking approval of Laromustine, in combination with AraC, another general cytotoxic agent, for elderly AML patients. Though the application was rejected because of insufficient data, strong interest remains in this drug due to its unique structure, manageable toxicity profile, and indication against AML, a particularly devastating cancer. Recently, there has been renewed interest in using the DNA methylating agent Temozolomide to sensitize patients to Laromustine treatment [57]. In the meantime, the more thoroughly the mechanism of action is understood, the more likely clinicians will be to apply this agent to the appropriate patients.

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Fig. 1.
Chemical structures for Laromustine, 101MDCE, 90CE, and Carmustine. The activation of Laromustine in aqueous solution yields 90CE and methyl isocyanate

Fig. 2. Inhibition of purified rat liver TrxR by Laromustine (blue), 101MDCE (red), 90CE (purple), and Carmustine (purple). Enzyme was pre-incubated with drugs for 2 hr at pH 8.0 before introducing DTNB and NADPH substrates. Activity was determined by monitoring the reduction of DTNB at 412 nm as a function of time. Data are reported as a fraction of the positive control (DMSO vehicle) activity and fit to a hyperbolic function as described in Materials and Methods

Fig. 3.

Time course of purified TrxR pre-incubation with 7.4 μ M Laromustine at pH 8.0. At the indicated times, Laromustine-treated TrxR was added to DTNB and NADPH substrates and enzyme activity was measured using the DTNB reduction assay. Data are reported as a fraction of the positive control activity, which was measured using TrxR pre-incubated with an equivalent volume of DMSO at identical time intervals

Fig. 4. Inhibition of TrxR activity from L1210 cell lysates by 50 μ M (blue) or 200 μ M (red) Laromustine, 101MDCE, 90CE, and Carmustine. Cells in culture were treated with drugs for 3 hr before harvesting and lysing cells. TrxR activity from clarified cell lysates was measured using the DTNB reduction assay and reported as a fraction of the positive control (DMSO vehicle) activity

Fig. 5. Tandem mass spectrum of methyl carbamoyl-adducted Cys 475. Low-energy collision-induced dissociation (CID) spectrum of the doubly-charged ($M+H^{2+}$)²⁺ precursor ion corresponding to the mass of the TrxR peptide IGIHPVC* -1.9 ppm, where * = methyl carbamoyl. A complete y-ion series is observed, including an intense, proline-directed y₃ fragment ion

Table 1

Methyl carbamoyl adducts on purified TrxR observed via mass spectrometry

	Laromustine		101MDCE	
	1 mM	100 μM	1 mM	100 μM
C475			✓	
K5	✓		✓	✓
K37			✓	
K81	✓	✓	✓	
K95	✓		✓	
K100	✓		✓	
K123	✓		✓	
K124	✓		✓	
K146	✓		✓	
K148	✓		✓	
K235			✓	
K255	✓	✓	✓	
K308			✓	
K312	✓	✓	✓	✓
K389	✓		✓	
K393	✓	✓	✓	
K430	✓		✓	✓