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Patterns of Apoptotic Poly(ADP-ribose) Polymerase Cleavage Induced by Laromustine and its Analogs

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Patterns of Apoptotic Poly(ADP-ribose) Polymerase Cleavage Induced by Laromustine and its Analogs

By Adam N. Paine

A Thesis Presented to the Department of Chemistry, Colby College, Waterville, ME
In Partial Fulfillment of the Requirements for Graduation With Honors in Chemistry

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Patterns of Apoptotic Poly(ADP-ribose) Polymerase Cleavage Induced by Laromustine and its Analogs

By Adam N. Paine

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Curriculum Vitae

Adam Nicholas Paine was born September 8, 1987 in Lewiston, Maine to parents Carl G. and Dianne S. Paine. Growing up in Bowdoin, Maine, Adam graduated from Mt. Ararat High School and matriculated at Colby College in Waterville, Maine. At Colby Adam worked under Assistant Professor of Chemistry Kevin P. Rice and received a Bachelor of Arts in Chemistry – Biochemistry. Following graduation Adam accepted a temporary position working under Dr. Fabio Candotti at the Human Genome Research Institute in Bethesda, Maryland.

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitae</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>Discussion</td>
<td>21</td>
</tr>
<tr>
<td>References</td>
<td>26</td>
</tr>
</tbody>
</table>
Abstract

The anticancer prodrug Laromustine (VNP40101M) has produced promising remission rates in clinical trials among leukemic patients relative to currently available chemotherapeutics. Such improvements demand that the agent’s mechanism of action be elucidated. This study aimed to determine the role of poly(ADP-ribose) polymerase (PARP) in Laromustine-induced cell death. Previous studies indicated that the methyl isocyanate produced upon activation of Laromustine is largely responsible for its effective induction of apoptosis. Results reported herein strongly support the activation of a PARP-dependent apoptotic pathway by Laromustine’s carbamoylating and chlorethylating subspecies. Furthermore, it is evident that the Laromustine-induced PARP-dependent apoptosis is primarily attributable to methyl isocyanate. As PARP has been implicated in numerous apoptotic pathways, these results advance the ongoing characterization of Laromustine’s cellular death mechanism and begin the process of sequencing apoptotic events.
**Introduction**

Although our understanding of the various forms of cancer has drastically improved over the last few decades, cancer remains the second leading cause of death in the United States.¹ A detailed picture of a variety of abnormally functioning mechanisms has led to the development of effective treatments for some types of cancer. However, other forms have proved too heterogeneous to produce common effective treatments. Leukemia, among the most diverse forms of cancer, is generally described as the development of abnormalities in blood and bone marrow cells. Among adults, the most common form of acute leukemia is acute myeloid leukemia (AML). AML is characterized by the presence of proliferative leukemic stem cells accompanied by the accumulation of myeloid stem cells that have developed into immature white blood cells called myeloid blasts rather than healthy granulocytes or monocytes.²⁻³ The lack of properly functioning white blood cells leads to immunosuppression and the accumulation of these cells leaves little room for the development of other healthy blood cells in the bone marrow.³

Of all types of leukemia, AML is generally accepted as the most fatal. Death is certain for patients receiving no treatment and there is a 4.3% five-year survival rate among patients over 65, years of age who receive treatment.⁴ Although the median age of AML incidence is 65 it exists as one of the leading causes of death due to cancer in younger patients.⁴ Risk factors for AML are not comprehensively understood, with over 100 known genetic predispositions and contributing oncogenes including the commonly
studied RAS, FLT3, and TP53 genes.\textsuperscript{5,6} AML incidence related to activation of these oncogenes has been correlated to occupational chemical and radiation exposure.\textsuperscript{6}

With such heterogeneity, AML has proven a difficult disease to treat. In current clinical practice, the most common chemotherapeutic approach includes a combination of the nucleoside analog cytarabine (Ara-C) and daunorubicin, a DNA intercalator.\textsuperscript{7,8} The remission rates for this treatment are quite variable depending on age and elected dosing regimen and are not very promising for older patients who represent the bulk of AML cases.\textsuperscript{9} Both Ara-C and daunorubicin have been used for over a decade now and no new treatment that significantly improves relapse statistics has come to the market. Given this lack of progress, several new approaches are being currently investigated including enzyme inhibitors, antisense DNA, purine analogues, immunological therapy, and alkylating agents.\textsuperscript{5}

A promising new treatment for AML, currently in Phase III of FDA trials, is Laromustine (VNP40101M, Cloretazine in previous literature), developed by Vion Pharmaceuticals (New Haven, CT). Laromustine, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine, is a sulfonylhydrazine prodrug that results in two dominant chemical species after intravenous injection (Figure 1).\textsuperscript{10} Following abstraction of the amide proton in the bloodstream, destabilization leads to electron rearrangement and an eventual split into two distinct electrophilic species, 90CE (2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine) and methylisocyanate.\textsuperscript{11} 90CE is a chloroethylating compound that alkylates guanine bases at the O\textsuperscript{6} position. Following alkylation, an interstrand crosslink is formed when a complementary cytosine attacks an intermediary ring formed on the guanine.\textsuperscript{12} Interstrand cross links are commonly formed
by many antineoplastic compounds and can inhibit DNA replication, eventually leading to cellular death.\textsuperscript{13} The crosslink produced by 90CE is exceptionally effective as it does not disrupt the architecture of the DNA double-helix as significantly as other crosslinks, making it difficult for the cell to detect and repair.\textsuperscript{14} Before the crosslink is formed, the cell can repair the guanine alkylation with the enzyme alkylguanine-DNA alkyltransferase (AGT). Many cancer cells have lower levels of AGT relative to non-cancer cells, which can render Laromustine more cytotoxic to tumor cells than healthy cells. Clinical trials have supported this theory as patients with decreased AGT levels have had higher remission rates.\textsuperscript{15} Due to its efficacy as a crosslinker, and the observed dependence on AGT expression, 90CE is thought to be the primary therapeutic subspecies of Laromustine.\textsuperscript{14}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The novel sulfonylhydrazine prodrug Laromustine, which results in the two active species shown, 90CE and methyl isocyanate, upon abstraction of the amide hydrogen. Inset species, 101MDCE, is the prodrug analog that provides methyl isocyanate \textit{in vitro}.}
\end{figure}

The other species produced upon metabolization of Laromustine, methyl isocyanate, is a carbamoylating compound exhibiting preference for thiols such as those found in cysteine residues.\textsuperscript{10} Carbamoylation is known to have a variety of effects in the cell, and has been shown to potentiate the therapeutic properties of Laromustine. Possible mechanisms for potentiation include the partial inhibition of AGT and other proteins that
would assist in repairing the DNA damage induced by 90CE. As such, the two subspecies of Laromustine are believed to work synergistically. In order to study the effects of the two species individually, a prodrug of methyl isocyanate and an analog of Laromustine, 101MDCE (1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]-hydrazine), is used to assess the contributions of the carbamoylating species.

Through clinical trials, Laromustine has proven to be an effective drug for AML patients as well as other cancers such as high-risk myelodysplastic syndrome (MDS). Induced remission rates appear to be comparable to or better than those seen with traditional treatments. Toxicity levels are comparable or less than those found with other clinically used drugs with myelosuppression being the dose-limiting factor. In several clinical trials remission rates have been effectively potentiated through combination therapy with other drugs, including Ara-C. In addition to treatment for both de novo and relapse AML patients, Laromustine has been suggested for use in patients with brain tumors as it effectively crosses the blood-brain barrier. In preliminary clinical trials, testing efficacy on brain tumors, Laromustine was moderately effective, but not to the extent seen in AML studies, largely due to increased toxicity observed with younger patients and in the brain. The latest AML phase III clinical trial produced impressive remission results when combined with Ara-C, however, the rates of myelosuppression were higher than desired and thus a dose rescheduling has been suggested and will need to be tested in another phase III trial before the drug is approved. In addition to its clinical success, Laromustine has shown promising results in a variety of cell lines commonly used in leukemia research. In searching for effective drugs for the treatment of cancer, it is advantageous to use compounds that
induce apoptotic or programmed cell death rather than compounds that induce necrotic
death. Necrosis results in cellular waste and potential toxins being expelled into the
interstitial fluid leading to inflammation and other undesirable effects, including death of
nearby cells. Apoptosis on the other hand is a more confined cell death regulated by
numerous signaling pathways. Many leukemia cell lines have been shown to undergo
apoptotic cell death following treatment with various chemotherapeutics. There are
multitudes of apoptotic signaling pathways known, many of which are far from being
fully elucidated. Repeated studies have shown that the specific apoptotic pathway that a
cell chooses depends on the origin of stimuli.23 From the outside of the cell the most
notable apoptotic receptors are the tumor necrosis factor (TNF) receptors, including the
widely studied Fas receptor which is activated by the ligand FasL, leading to an apoptotic
signaling cascade within the cell.24 From within the cell, apoptosis can be induced by a
currently unknown number of both inhibitory and excitatory signals. The release of
cytochrome c from the mitochondria is required for the majority of known apoptotic
pathways. Cytochrome c activates a series of cysteine proteases called caspases which
subsequently trigger a cascade of protein cleavages committing the cell to apoptotic death
(Figure 2).25 Additional apoptotic signaling pathways are continuously being identified,
many of which are not ubiquitous across different cells lines.26
There is currently a lack of consensus on the mechanism of cellular death induced by Laromustine in commonly used cell lines. Morris, et al. report limited apoptosis in human immortalized promyelocytic leukemia (HL60) cells following treatment with pharmacologically relevant concentrations of Laromustine. However, unpublished data
from Praggastis and Rice show significant apoptosis in the HL60 cell line induced by incubation with Laromustine.\textsuperscript{22} Apoptosis in the HL60 line was shown to be specifically induced by the 101MDCE carbamoylating species analog of Laromustine, thus offering possible insight into the drug’s increased efficacy.\textsuperscript{22} Although other drugs such as BCNU (1,3-bis[2-chloroethyl]-2-nitrosourea) result in chloroethylating and carbamoylating species upon activation, the activity of the carbamoylating species has been shown to behave distinctly.\textsuperscript{10} This dissimilar behavior of the carbamoylating species could partially explain why Laromustine outperforms these otherwise similar drugs.

In recent years the enzyme poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) has come to the forefront of cellular apoptotic mechanism analysis. PARP is a nuclear protein which poly-ADP-ribosylates surrounding proteins, including itself, and has many roles in the nuclear environment. One of the major functions of PARP is the recognition of lesions in DNA, such as single-strand DNA nicks, and the subsequent recruitment of other base excision repair (BER) proteins such as XRCCI, APE 1, and DNA ligase III.\textsuperscript{27} The role of PARP in base excision repair has been extensively studied and has led to the characterization of its domains based on how they are involved in BER (Figure 3). Other pathways in which PARP is involved include transcription, replication, and most importantly to this study, apoptosis.
Figure 3. PARP activity segmentation with location of apoptotic caspase-3 cleavage highlighted. Significant discrepancies exist in the literature about the size of full-length PARP (113 kDa – 122 kDa) and its active segments; however, it is widely accepted that the apoptotic fragments are 89 and 24 kDa.

PARP uses nicotinamide adenine dinucleotide (NAD$^+$) as a substrate for poly-ADP-ribosylation, a branched polymer, releasing nicotinamide as a leaving group at each addition. Because NAD$^+$ is the substrate, increased activity of PARP diminishes the cellular pool of NAD$^+$ and leads to a decrease in NADH reducing equivalents necessary for ATP production. Such a decrease in availability of cellular energy resources leads to a necrotic cell death in the case of PARP over-activation (Figure 4). Apoptosis is an active process that requires ATP. As such, apoptosis cannot occur if a significant amount of the cell’s energy source is depleted. Since fragmentation of DNA occurs during apoptosis, PARP would be over-stimulated if in active form during late-stage apoptosis.

The majority of studies focusing on PARP have shown that the normal poly-ADP-ribosylating activity of PARP is inhibited during apoptosis via proteolytic cleavage. PARP inhibitors have been shown to induce apoptosis, supporting the theory that PARP is cleaved in order to avoid depletion of the cell’s energy sources which are necessary for apoptosis. Regardless of the reason for cleavage, what is evident is that the majority of
apoptotic pathways studied to date result in cleavage of PARP.

**Figure 4.** The role of PARP in DNA base excision repair and cellular-death mechanisms.

Numerous studies have shown that caspase-3 is the upstream protease which cleaves PARP between Asp\(^{214}\) and Gly\(^{215}\) residues, resulting in 89kDa and 24kDa fragments which have become characteristic markers of apoptosis (Figures 2 and 4).\(^{28}\) Caspase-3 is indirectly activated by the release of cytochrome c from the mitochondria as well as extracellular stimuli.\(^{25}\) It is believed that the majority of apoptotic pathways involve the activation of caspase-3. In some cell lines, apoptosis is dependent on caspase-3 activation. However, some cell lines undergo apoptosis without the presence of active caspase-3.\(^{32}\) The cleavage of PARP, following caspase-3 activation, is one of the earliest detectable signals that the cell has committed to an apoptotic death as many of the upstream signals are difficult to monitor and are not committed steps.\(^{33}\) Many types of apoptosis inducing compounds, including etoposide
and camptothecin, produce significant PARP cleavage.\textsuperscript{34} Apoptosis induced by TNF receptor activation has shown an age dependent PARP cleavage pattern with cells from older patients leading to more significant cleavage of PARP.\textsuperscript{35} From its evident role in induced apoptosis it follows that PARP has become important to the study of antineoplastic mechanisms.

PARP cleavage in necrotic cell death has also been observed. This either occurred after PARP over-activation, seen in some necrotic death pathways, or in one of the many other mechanisms of necrotic cell death. Treatment of HL60 cells with necrosis-inducing cytochalasin B produced a variety of other fragments in addition to a relatively small amount of the 89kDa fragment.\textsuperscript{36} Drugs that specifically target and inhibit PARP have been developed to be used in conjunction with DNA damaging chemotherapeutics in attempts to potentiate their effects.\textsuperscript{37} The idea behind these drugs is that inhibition of PARP leads to inhibition of BER, which can repair much of the DNA damage caused by chemotherapeutics. Other PARP inhibiting drugs have been developed in an attempt to prevent over-consumption of available cellular energy and subsequent cell death.\textsuperscript{38}

In an effort to further understand the role of PARP in anticancer treatments this study investigates the role of PARP in the treatment of HL60 cells with Laromustine. The results presented herein continue previous experiments that indicated the induction of apoptosis in HL60 cells exposed to Laromustine.\textsuperscript{22} In addition to Laromustine, its analogs, 90CE and 101MDCE, are individually tested in order to determine if one or both of the active compounds of Laromustine is responsible for any observable PARP modification. Western blot detection of the 89 kDa apoptotic cleavage fragment revealed that Laromustine, 90CE, and 101MDCE induce the cleavage of PARP. Furthermore, it is
evident that the majority of Laromustine-induced apoptotic PARP cleavage is a result of its carbamoylating activity.

**Materials & Methods**

*Cell Culture and Drug Incubations*

Human promyelocytic leukemia cells (HL60) were purchased from the American Type Culture Collection and cultured in RPMI 1640 media (supplemented with 10% fetal bovine serum and 50mg/ml gentamicin) at 37°C, 5% CO₂, and ~85% humidity. Cells were kept in a concentration range of 1 x 10⁵ – 1 x 10⁶ cells/ml and harvested for experimentation at a concentration of ≈ 5 x 10⁵ cells/ml. Cell viability was monitored with a trypan blue exclusion assay using an automated cell counter (Nexcelom Bioscience, Cellometer Auto T4). Prior to treatment with compounds, cells were pelleted at 500 x g for 10 min and brought up in fresh media to obtain a concentration of 1 x 10⁶ cells/ml. Laromustine, 90CE and, 101MDCE were synthesized and generously donated by the lab of Professor Alan Sartorelli at Yale University. Agents were prepared in 200 μM stock solutions in dry DMSO and stored desiccated at -20°C. A stock solution of 68 mM etoposide (Sigma-Aldrich, St. Louis) in dry DMSO was stored at 4°C. Cells were treated with 50, 100, and 200 μM Laromustine, 90CE, or 101MDCE for varying periods of time at 37°C with gentle agitation on an orbital shaker. Etoposide apoptotic control cells were treated with 68 μM etoposide under identical conditions as other compounds. All trials included a control incubation with 0.1% (v/v) DMSO.
Cell Lysis and Protein Extraction

Following treatment, cells were harvested from culture at 500 x g for 10 min and the supernatant was aspirated. The cell pellet was then washed twice with 4°C phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ 2mM KH₂PO₄) , each wash centrifuged as above. Whole-cell lysis samples were prepared using Mammalian Protein Extraction Reagent (M-PER®, Thermo Scientific) with 10 μl/ml Protease Arrest™ and 10 μl/ml EDTA (G-Biosciences) according to the manufacturer’s protocol. Total extracted protein concentration was determined using a Bradford Assay (Bio-Rad) with a Molecular Devices, Spectra M2 spectrophotometer (SoftMax® Pro Software version 5.2 revision C). Bovine serum albumin (BSA) in M-PER® was used for standard curve generation. Total protein concentration of all samples was brought to ~1600 μg/ml using M-PER, with 10 μl/ml Protease Arrest™ and 10 μl/ml EDTA to dilute samples of higher concentration.

Electrophoresis and Western blotting

SDS-bromophenol blue loading buffer was added to all cell lysate samples in a 1:1 ratio bringing the concentration of total protein to 800 μg/mL. Samples were then denatured in a heating block at 100°C for 3 min. 25 μL of each sample (~20 μg total protein) were run on Pierce 10% Precise™ 12-well Tris-Hepes-SDS protein gels for ~1 hr at 120 V in Hoefer SE 250 mini-vertical gel electrophoresis unit. All gels were run with Precision Plus Protein™ Standard Kaleidoscope™ 10-250 kDa protein ladder (Bio-Rad) and a mouse or rabbit IgG secondary antibody control (Calbiochem). The proteins in the gels were then transferred to a nitrocellulose membrane using a Hoefer™ TE 22 tank transfer
unit with 20% methanol SDS-Tris glycine (25mM Tris, 192mM glycine, 3.5mM SDS) transfer solution at 400 mA for 1 hr. Following transfer, the membranes were cut into two pieces at the 50 kDa marker and blocked using 0.5% non-fat milk in Tris-buffered saline (50mM Tris-HCl, 135mM NaCl, pH 7.4) with 0.1% v/v Tween-20 (TBS-T) using SNAP i.d. Western blotting unit (Millipore). The membrane sections containing proteins larger than 50 kDa were incubated with one of the following antibodies diluted in 0.5% non-fat milk in TBS-T overnight at 4°C with gentle agitation: full-length anti-PARP polyclonal antibody developed in rabbit, 1:10,000 dilution (Bethyl Laboratories Inc.); anti-cleaved PARP (Asp214) polyclonal antibody developed in rabbit, 1:1,000 dilution (Cell Signaling); or anti-PARP C2-10 monoclonal antibody developed in mouse, 1:2000 dilution (Trevigen). The membrane sections containing proteins < 50 kDa were incubated overnight at 4°C with an anti-actin polyclonal antibody developed in rabbit at a 1:400 dilution (Sigma-Aldrich, St. Louis). Following overnight incubations membranes were incubated with their respective anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP) linked secondary antibody (GE Healthcare) for 1 hr at room temperature at a dilution of 1:10,000. After all incubations, membranes were washed three times with TBS-T in the SNAP i.d unit. Following the final TBS-T wash, each membrane was incubated at room temperature with ECL Plus western blotting detection reagents (GE Healthcare) for 5 min. Excess detection reagent was poured off and membranes were developed using a XRS+ ChemiDoc molecular imager (Bio-Rad). All bands were quantified relative to β-actin using Bio-Rad Quantity One Software (Version 4.6.9). All 89 kDa bands resulting from treatment with antineoplastic candidates were measured relative to DMSO treated samples. In preliminary trials leading to this study membranes
were developed on Kodak X-Ray film (~10 second exposure) and corresponding fixer and developer (Kodak).

**Results**

*Three-hour post-treatment Western blot troubleshooting*

Following the results of Praggastis and Rice, which indicated maximal induction of apoptosis within the first hour following exposure to Laromustine and 101MDCE, initial time trials probing for PARP cleavage were limited to 3 hours following exposure to all compounds. Except for the etoposide apoptotic control, no significant accumulation of the 89 kDa PARP fragment relative to DMSO treatment was observed in the initial 3 hours using the anti-cleaved PARP (Asp214) polyclonal antibody. Western blots using the anti-full-length PARP polyclonal antibody indicated that the quantity of full-length PARP remained consistent over the first 3 hours of exposure to Laromustine, 90CE, and 101MDCE while decreasing minimally at 3 hours following exposure to etoposide. To account for any non-apoptotic, or post-apoptotic fragmentation of PARP, we used the C2-10 monoclonal antibody, which reacts with full-length PARP as well as at least four characterized PARP fragments. Again, there was no significant cleavage of PARP relative to DMSO following treatment with Laromustine and its two analogues. Concentrations of lysed cells were increased and decreased compared to previous protocols in several 3-hour iterations, again yielding no PARP cleavage. Drug concentrations were decreased in order to account for the possibility that we were exposing cells to concentrations that induced PARP cleavage too rapidly to detect with our methods; however, PARP fragmentation was again undetected.
Laromustine, 90CE, and 101MDCE induce PARP cleavage

Trials probing for PARP cleavage were expanded to 6 hours, resulting in the observation of significant accumulation of the 89 kDa cleavage fragment relative to DMSO at 6 hours. Understanding that the signature apoptotic fragment is degraded in late-stage apoptosis, we expanded the time trials to 24 hours. Control experiments showed no significant accumulation of PARP fragments in DMSO treated cells over 24 hours (Figure 5). Twenty-four hour time-course results show significant PARP cleavage resulting from incubation with Laromustine, 90CE, 101MDCE and the etoposide apoptotic control. 90CE and etoposide displayed maximum induction of apoptotic PARP cleavage at 6 hours post-treatment while Laromustine and 101MDCE showed the most significant accumulation of the 89 kDa fragment at 12 hours following treatment (Figures 5 & 6).

![Figure 5](image.png)

**Figure 5.** Western blot visualization of 89kDa apoptotic PARP cleavage fragment accumulation with anti-cleaved PARP (Asp214) antibody (1,1000 dilution) following treatment with 68μM etoposide apoptotic control, 200μM Laromustine, 90CE, and 101MDCE. All bands were quantified relative to corresponding β-actin band intensity (not shown in figure) detected with anti-actin polyclonal antibody (1:400 dilution).
The most significant cleavage resulting from any of the three compounds tested at 200 μM was observed at 12 hours with Laromustine. However, trials run in triplicate indicate that 101MDCE can lead to the same levels of PARP cleavage at 12 hours as Laromustine (Figure 6). Over time, both Laromustine and 101MDCE induced significantly more apoptotic cleavage of PARP than 90CE. At 6 hours post-treatment Laromustine, 90CE, and 101MDCE had statistically comparable levels of 89 kDa fragment accumulation. Although only produced from a single trial, it appears that 68 μM etoposide results in more accumulation of the 89 kDa PARP cleavage product than any of the three compounds tested at 200 μM.
Figure 6. Twenty-four hour time dependent accumulation of 89 kDa apoptotic PARP fragment following treatment with 68 μM etoposide apoptotic control, 200 μM Laromustine, 90CE, and 101MDCE. Except for etoposide, all data points represent duplicate or triplicate trials.

Apoptotic PARP cleavage is invariable from 50-200 μM

Because Laromustine-induced 89 kDa fragment accumulation reached a maximum at 12-hours post-treatment, we chose this time point to test the concentration dependence of PARP cleavage. HL60 cells were incubated with 50, 100, and 200 μM Laromustine, 90CE, and 101MDCE and subsequent PARP cleavage was visualized with the anti-cleaved PARP (Asp214) polyclonal antibody (Figure 7).
Figure 7. Western blot visualization of 89 kDa PARP cleavage fragment accumulation with anti-cleaved PARP (Asp214) antibody (1:1,000 dilution) 12-hours following treatment with 200, 100, and 50 μM Laromustine, 90CE, and 101MDCE. Beta-actin (~42 kDa) was detected with anti-actin antibody (1:400 dilution), quantified, and used to equilibrate concentration of protein loaded on SDS-polyacrylamide gels.

The 12-hour post-treatment cleavage pattern observed in initial trials, where Laromustine and 101MDCE resulted in significantly more cleavage than 90CE, was consistent across the three concentrations tested. No concentration dependence was observed, as cleaved fragment accumulation was consistent across the three concentrations tested (Figure 8).
Discussion

Repeated studies have indicated that the methyl isocyanate produced by activation of Laromustine is largely responsible for its effective induction of apoptosis.\textsuperscript{16,22} Results reported herein strongly support induction of a PARP-dependent apoptotic mechanism by Laromustine’s carbamoylating and chloroethylating metabolites, see Figure 1. Furthermore, our results suggest that the PARP-dependent apoptosis resulting from Laromustine exposure is more attributable to its carbamoylating rather than chloroethylating activity. It is evident that 90CE and 101MDCE produce significantly different patterns of apoptotic PARP cleavage as 90CE induced cleavage reached a maximum at 6 hours and was an abrupt spike whereas 101MDCE-induced cleavage reached a maximum at 12 hours and was more gradual than 90CE.
Although at dissimilar magnitudes, the 90CE pattern of PARP cleavage appears to
closely resemble that of etoposide-induced PARP cleavage. Etoposide has been used
extensively as a chemotherapeutic agent, acting as an inhibitor of topoisomerase II
similar to another commonly used antineoplastic agent, doxorubicin.\textsuperscript{41} Topoisomerase II
is an enzyme with many DNA-related functions including the formation of double-
stranded breaks followed by recombination. Etoposide inhibits topoisomerase II after it
has formed a double-strand break intermediate, subsequently leading to the accumulation
of DNA fragments. Although the exact pathway leading to PARP cleavage from
etoposide exposure is not fully understood, it is known that the associated accumulation
of DNA fragments leads to activation of a caspase-dependent apoptotic pathway.\textsuperscript{42} The
cross-links formed by 90CE similarly lead to accumulation of DNA that is effectively
useless to the cell as it cannot be replicated or transcribed. Our results suggest that the
90CE cross-links lead to activation of a caspase-dependent apoptotic pathway, which
leads to the cleavage of PARP at a similar time-point as etoposide. It cannot be stated
from our results that the activated pathways are identical as a significant number of
apoptotic pathways result in the cleavage of PARP. However, it can be stated that the
PARP-dependent pathways activated by etoposide and 90CE are more similar to one
another than to the pathways activated by Laromustine and 101MDCE.

The effect of methyl isocyanate carbamoylation within the cell has been of
continued interest. The results of this study indicate that carbamoylation leads to
apoptotic cleavage of PARP. The direct or indirect nature of this effect cannot be inferred
from these results, as there are numerous upstream proteins that could be activated and/or
deactivated by carbamoylation. It is evident that the PARP-dependent apoptotic pathway
activated by Laromustine closely resembles the pathway activated by 101MDCE, both of which result in a greater effect than 90CE. These results allow the inference that the PARP-dependent apoptotic pathway activated by Laromustine is primarily a result of methyl isocyanate activity with less significant contribution from 90CE.

In previous work, methyl isocyanate and 90CE have displayed synergistic activity at levels not evident in this report. Although both 90CE and 101MDCE lead to PARP cleavage, it does not appear that one potentiates the others’ effect in Laromustine treatment. In fact, our results indicate that the PARP cleavage resulting from methyl isocyanate and 90CE are not even completely cumulative. This finding has a number of possible explanations and warrants further investigation. One possibility is that methyl isocyanate and 90CE activate separate converging pathways that cannot run simultaneously. Our results suggest that if this explanation were correct, that the methyl isocyanate-activated pathway predominates over the 90CE pathway.

Of note, a recent study showed that co-incubation of tumor cells with a PARP inhibitor and a DNA-damaging agent significantly potentiated chemotherapeutic efficacy. It is logical to apply this finding to Laromustine and our study where methyl isocyanate leads to significant cleavage of PARP and 90CE leads to DNA damage. It is important to emphasize that our results do not indicate a lack of synergism between methyl isocyanate and 90CE as shown in previous work. Methyl isocyanate likely does potentiate the effects of 90CE and our results suggest that the cellular-death mechanism related to this potentiation does not include the cleavage of PARP. Although PARP cleavage by caspase 3 is involved in many well-characterized cellular death pathways, there are also a very significant number of PARP-independent pathways. Additionally,
several PARP-dependent pathways have been proposed that involve the activation of PARP rather than cleavage, which would not have been observable in this study (Figure 2).\textsuperscript{44}

The PARP-dependent pathways evident in this study did not show concentration dependence in the 50-200 \( \mu \text{M} \) range. Determining clinically relevant concentrations at which to conduct \textit{ex vivo} trials is a difficult task. Studies have attempted to determine the concentration of Laromustine and its metabolites in the blood following the clinically developed bolus injection of 600 mg/m\(^2\), reporting \(~35 \mu \text{M} \) Laromustine and \(~0.9 \mu \text{M} \) 90CE in the plasma with higher bolus doses leading to Laromustine reaching nearly 100\( \mu \text{M} \).\textsuperscript{45} The relevance of these values is a point of contention as many factors must be accounted for, including the relatively short half-lives of 90CE and methyl isocyanate and the unknown rate of cellular uptake and target binding.\textsuperscript{12} Given these additional parameters to consider, it is likely that the actual concentrations that cells are exposed to is significantly higher than those reported in the plasma following injection. Previous \textit{ex vivo} studies with Laromustine and its analogues set the precedent for the concentrations elected for this study.\textsuperscript{22} Future studies should test a range of lower concentrations in order to observe any changes in the timing and/or magnitude of PARP cleavage and associated apoptotic pathways.

The fact that the time dependent PARP cleavage reported herein appears to contradict the timeline of Laromustine-induced apoptosis previously reported by Praggastis and Rice must be addressed.\textsuperscript{22} Their data, indicating maximal induction of apoptosis at 3 hours following treatment with Laromustine and 101MDCE, were obtained from trials where HL60 cells were stained with annexin V and propidium iodide (PI)
followed by flow cytometry analysis. As annexin V detects the externalization of phosphatidylserine (PS) into the outer leaflet of the plasma membrane, our results could align if PS externalization preceded PARP cleavage. Although the order of apoptotic events within the cell is far from completely elucidated in any cell line, studies have indicated that PS externalization precedes most nuclear-based apoptotic events.\textsuperscript{46} However, this cannot completely explain the order of events as a significant portion of the apoptotic cells at $\geq$ 3 hours following treatment were stained with PI which indicates loss of membrane integrity in late apoptotic or necrotic cells.

It is not unreasonable that a chemotherapeutic compound results in a 6-hour delayed PARP cleavage event as initiation of PARP cleavage has been observed as late as 8 hours post-treatment with other known antineoplastic.\textsuperscript{47} Again, it is possible and likely that Laromustine is activating several apoptotic pathways, which could include PARP-independent pathways early on followed by PARP-dependent pathways. As convenient as it would be for these data sets to align, it would be irresponsible to force them into congruence. Current studies are looking into the possibility that mutation rates in high split-count HL60 cells are more rapid than previously accepted. This study may determine that the two data sets cannot be compared. In combining these results there is evident need for standardization as well as a method that can visualize apoptotic events in real time or at least in less than 3-hour increments.

Although only a small portion of the bigger picture, this study builds on our developing comprehension of the cell-death mechanism induced by the promising antineoplastic candidate Laromustine. From its success in clinical trials it is evident that an understanding of the cell-death mechanism may not be necessary for it to be adapted
into the clinician’s arsenal. However, studies of this nature frequently lead to development of more effective drugs and identify new targets for future chemotherapeutics. Results of this study indicate that Laromustine induces both PARP-dependent and independent apoptotic pathways via carbamoylating and chlorethylating activity. A significant portion of the Laromustine mechanism remains to be characterized in future work with Laromustine and its metabolites, much of which will focus upstream of PARP.

References

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