2008

Effects of the anticancer agent Cloretazine on human apurinic endonuclease-1 activity

Jennifer L. Bushee
Colby College

Follow this and additional works at: https://digitalcommons.colby.edu/honorstheses

Part of the Chemistry Commons

Colby College theses are protected by copyright. They may be viewed or downloaded from this site for the purposes of research and scholarship. Reproduction or distribution for commercial purposes is prohibited without written permission of the author.

Recommended Citation
https://digitalcommons.colby.edu/honorstheses/43

This Honors Thesis (Open Access) is brought to you for free and open access by the Student Research at Digital Commons @ Colby. It has been accepted for inclusion in Honors Theses by an authorized administrator of Digital Commons @ Colby. For more information, please contact mfkelly@colby.edu.
Effects of the Anticancer Agent Cloretazine on Human Apurinic Endonuclease-1 Activity

By Jennifer L. Bushee

A Thesis Presented to the Department of Chemistry
Colby College, Waterville, ME 04901
In Partial Fulfillment of the Requirements for Graduation
With Honors in Chemistry

Submitted May, 2008
VITAE

Jennifer Lyn Bushee was born in Beverly, Massachusetts on October 9, 1986, the daughter of Michael F. Bushee and Ann Marie Bushee. After completing her work at Masconomet Regional High School, Boxford, MA in 2000, she entered Colby College in Waterville, Maine. She will receive the degree of Bachelors of Arts in Chemistry-Biochemistry with a concentration in Cell and Molecular Biology/Biochemistry from Colby College in May, 2008.

Permanent Address: 13 Ross Lane
Middleton, MA 01949
ACKNOWLEDGEMENTS

First and foremost, I thank my research advisor and mentor, Professor Kevin Rice; this thesis would not have been possible without his help, guidance, and input throughout my research. Not only did Professor Rice stay involved with the project when it deviated from the initial course, he also was able to provide further insight to the troubleshooting and new methods of approaching the project. Thank you very much for inviting me into your lab, as well as for your encouragement and support.

I would also like to thank Professor Julie Millard, for being my thesis reader, sharing the use of her laboratory throughout the process, and for her advice. Also, the extensive material and hard work that was advocated in the Biochemistry labs and lectures continued to build upon the strong backbone of laboratory skills and knowledge that was needed to pursue independent research.

For his insight in creating a plausible mechanism for why abasic DNA is spontaneously cleaved by Mg$^{2+}$, I must thank Professor Jeffrey Katz. His help facilitated a full assessment of the cofactor of interest and its role in the APE/Ref-1 mechanism.

My growing interest in chemistry and the sciences is due to the support and positive influence of each of my professors. For everything that I have learned from them and many laboratory successes and failures, I thank the Colby College Chemistry professors and faculty for their dedication to teaching and cultivating students like myself. I would also like to thank the members of the Rice Research lab for their enthusiasm inside and out of the lab, which provided an inviting and positive research area.

Most importantly, this thesis would not have been possible without the support of my friends and family. I cannot thank each of them enough for the love and encouragement that
they have given me throughout my four years at Colby College. To my friends at Colby who have seen me both excited and frustrated from successful experiments to continued troubleshooting, I thank you for keeping me sane throughout the process. I cannot begin to explain the value of your friendship. To my parents, Michael and Ann Marie, and my sisters, Jessica and Andrea, thank you for always believing in me and never allowing me to give up on any of my dreams.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitae</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>14</td>
</tr>
<tr>
<td>Results</td>
<td>17</td>
</tr>
<tr>
<td>Discussion</td>
<td>23</td>
</tr>
<tr>
<td>References</td>
<td>26</td>
</tr>
</tbody>
</table>
ABSTRACT

The carbamolyating activity of Cloretazine, a novel anticancer prodrug, modifies protein function and acts synergistically with the chloroethylating species, which alkylates DNA. The reactive subspecies responsible for carbamolyating activity is methyl isocyanate, which reacts with sulfhydryl groups and amines. DNA repair enzymes have been identified as potential targets for the modification by methyl isocyanate; in particular, base excision repair enzymes. Enzymes in DNA base excision repair include DNA polymerase beta (Pol β) and apurinic/apyrimidic endonuclease-1 (APE/Ref-1). APE/Ref-1 hydrolyzes the 5'-phosphodiester DNA backbone from an abasic DNA template, where the base was excised by a DNA glycosylase. The entire nucleotide is then re-inserted by the nucleotidyl transferase activity of Pol β, which also cleaves the 5'-deoxyribose phosphate via lyase activity. Previous research in our laboratory investigated the inhibition of Pol β's AP lyase and nucleoside transferase activity. Cloretazine's carbamoylating activity inhibits the nucleotidyl transferase activity of Pol-β, but not its lyase activity. An efficient enzymatic assay was developed and used to determine the anticancer drug's effect on APE/Ref-1's hydrolytic activity. We found that the endonucleolytic activity of APE-1 is not significantly inhibited by Cloretazine.
INTRODUCTION

Cancer Chemotherapy

The initial efforts to combat cancer date back to the early 1940s with the use of nitrogen mustard, previously used for chemical warfare, as a chemotherapeutic agent. The treatment was designed as a result of mutagenic characteristics found in many deceased soldiers during World War I from exposure to mustard gas (1,2). Side effects of the gas included underdeveloped and decreased cell counts in vital organs and decreased bone marrow density. The agent was used to treat non-Hodgkin's lymphoma, where an active ethyleneimmonium ring, an alkylating intermediate, forms and reacts with the electron donating sites on DNA bases to create a covalent bond (1). The alkylating damage leads to DNA crosstieks and apoptosis. In the 1960s clinical assays were developed to better understand the cytotoxic effects of cancer treatments. Some methods use high cytotoxic treatments to inhibit cell division, while other agents can physically damage DNA via alkylation. Because of genetic difference between oncogenes and normal genomes in combination with advanced molecular biology techniques, additional chemotherapeutic compounds focus on metabolically targeted therapies.

Advancements in biochemistry and molecular and cell biology prompted the development of targeted therapies that have revolutionized anticancer treatments (1,3). Targeted therapies focus on cancer from a genetic and mechanistic level. These therapies analyze the human genome and the recognition of genomic sequences that encode specific cancer types. These treatments are designed to activate or deregulate tumor cellular processing components and pathways that include signaling proteins, growth factors and kinase cascades (4). These anticancer agents possess increased clinical antitumor efficacy, lower toxicities, and lower rates of drug resistance because of tumor instability (2). One field of research involves the epidermal
growth factor receptor (EGFR) family. Studies have shown overexpression of HER-2/neu proteins in approximately 25% of human breast cancer patients (5). HER-2/neu encodes a membrane transport protein that is homologous to EGFR (6). Herceptin (Trastuzumab) was developed to deregulate tumor cells that had uncontrolled HER-2/neu production. The drug inhibits the formation of a HER-2/neu ligand required for apoptosis (2,5). Recent studies documented that Herceptin could also affect vascular endothelial growth factor and angiopoietin-I regulation, and inhibit DNA repair mechanisms (6,7). A combination treatment of Herceptin and Taxol (Paclitaxel), another cancer treatment, showed a 60-70% success rate of anticancer breast cancer patient treatments (4). Pathway-specific chemotherapy suggests high clinical potential because the therapy targets the signaling pathways critical to tumor growth coupled with decreased toxic side effects from the treatment. These targeted therapeutic agents will be based on a mechanistic and a genetic understanding of cancer.

Targeted therapies are being used against a disease that is characterized by undivided cell division caused by genomic mutations. Molecular targets cannot take into account all sequence variability and the variability between patients in different populations (4). Patients must match the targeted genomic sequences, test positive or negative for specific receptors, and meet various other clinical parameters for which the study focused on developing a treatment. Additional complications for targeted therapies include mutations in promoter sequences or within an oncogene’s coding region (2,4). The future advancement of molecular biology techniques will enhance the potential efficacy of targeted treatments. The treatments can also be used in combination with cytotoxic chemotherapeutic agents, which increase tumor cell toxicity.

Modern chemotherapeutic agents target specific mechanisms to prevent tumor cell growth, repair or replication to increase cytotoxicity. For example, antifolates block the function
of enzymes requiring a folate substrate. Similar to nitrogen mustards, antifolates result in bone marrow toxicity; however, the cytotoxic effects of antifolates are reversible due to brief remissions and the agents maintaining their potent anticancer properties (1). Another class of chemotherapeutic agents is the alkaloids, a natural product with nitrogen containing rings (8). Vinca alkaloids inhibit mitosis by preventing the formation of microtubules (1,8). Other alkaloids include taxanes, such as Taxol, which promote microtubule assembly and are used as an ovarian cancer treatment (2,8). Furthermore, a camptothecin analogue, irinotecan, inhibits topoisomerase I enzymes that nick and unwind the supercoiled DNA downstream of the replication fork during DNA replication (1). Finally, DNA purine analogs, such as 6-mercaptopurine, are used to inhibit the DNA synthesis pathway (1,9). These analogs are classified as anti-metabolites that increase cytotoxicity as their anticancer properties.

DNA alkylating agents are also common in cytotoxic anticancer treatments that physically damage DNA. Preclinical chemotherapy studies continue to include agents designed to effectively alkylate DNA (10). Alkylating agents act as electrophiles on a nucleophilic site on a DNA base. "Soft electrophiles" prefer to react with soft nucleophiles, like the N7 and N3 positions of guanine in DNA. Soft electrophiles that attack the N7 position of a guanine residue result in a slow depurination and the hydrolysis of the DNA backbone to create a break in the DNA strand (11). Hard electrophiles, however, react more efficiently with hard nucleophiles, like the O6 position of guanine or the oxygen-phosphodiester bond in the DNA backbone (12). Alkylating agents can produce a mixture of alkylated products. For example, N-methyl-N-nitrosourea alkylates 68% at the N7 position and 7.5% at the O6 position (12). Clinically useful alkylating anticancer agents in the chemethylnitrosourea (CENU) class promote DNA crosslinks in lymphomas and solid brain tumors (13,14). Interstrand crosslinks inhibit DNA replication and
transcription, often resulting in a programmed cell death. The ability of CENU to crosslink DNA is the primarily recognized mechanism for the drug’s anticancer properties. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a common CENU drug, alkylates DNA in this manner; however, the mutagenic and carcinogenic side effects limit the agent’s utility (14). BCNU is commonly used to treat solid brain tumors and breast cancer, and its most common side effect is a sharp decrease in bone marrow density (15). BCNU generates toxic reactive species that include chloroethylating, hydroethylating, vinylating, and aminoethylating species (14,16). These electrophilic species diminish the effectiveness of BCNU’s carbamoylating activity and decrease BCNU’s therapeutic index because of the added toxic side effects from these species. The chloroethylating species is responsible for the DNA crosslinks, where an ethyl group is added to the O6 position of a guanine residue (15). The chloroethyl group is rearranged to eventually create a crosslink between the N1 position of the guanine residue and the N3 position of the cytosine on the opposite strand (15). However, the aminoethylating species does not have a known clinical effect, and hydroxyethylating species are carcinogenic. The carbamolyating species, chloroethyl-isocyanate, reacts with sulfonyl groups in proteins, and additional effects of other carbamolyating species are under further investigation (14). Researchers continuously seek new drugs that lack the toxic side effects yet retain the efficacy of CENUs.

Cloretazine and other sulfonylhydrazines

The synthesis of sulfonylhyrazines, a cancer chemotherapeutic agent, possesses similar anticancer properties to CENUs. The novel anticancer agent 1,2-bis(methylsulfonyl)-1-2-(methylamino)-carbonyl-hydrazine (Cloretazine) exhibits broad-spectrum anticancer activity comparable to BCNU (12,13,14). Cloretazine is currently in clinical trials for acute myeloid
leukemia and has been shown to inhibit enzymes containing thiols, such as glutathione reductase (18). The prodrug decomposes (activates) into two electrophilic species in the cell, a chloroethylating species and a methyl isocyanate compound, which is a carbamoylating species (Figure 1).

![Chemical Structure of Cloretazine](image)

Figure 1. The decomposition of Cloretazine into two active electrophiles, chloroethylating and carbamoylating electrophiles, and their respective biological targets (12).

The two electrophiles act similarly to the reactive species from BCNU, but they are not structurally identical. The alkylating compound, 90CE, promotes interstrand guanine-cytosine crosslinks by chloroethylating DNA at the O6-guanine position (16,17). The methyl isocyanate, the carbamoylating species, enhances the cytotoxic effects of the chloroethylating activity (16,18). This synergism is fundamental to the success of Cloretazine as an anticancer agent.

Cloretazine’s synergism contributes to the successful anticancer activity found in vivo. Finch *et al.* reported Cloretazine’s anticancer activity against the following murine tumors: P388
and L1210 leukemias, B16 melanoma, M109 lung carcinomas and M5076 reticulum cell sarcoma (14). They also found prominent anticancer activity in human LX-1 lung carcinoma xenographs. The L1210 leukemia mice were treated with a single intraperitoneal dose of Cloretazine, 10, 20, 40, 60 or 80 mg/kg. Comparing the long term survivors of the studies of Cloretazine and BCNU, Cloretazine illustrated better anticancer efficacy and safety margins. *In vivo* results did not show solubility complications or inability of the drug to cross the blood-brain barrier (14).

*In vitro* studies have further investigated the anticancer properties of Cloretazine. Rice *et al.* (18) examined inhibition of cellular glutathione reductase (GR). GR activity from purified enzyme and from cell lysate from L1210 murine leukemia cell lines were treated with 200 μM Cloretazine (half life approximately 1.0 hr, pH 7.4, and 37°C), 90CE (half life approximately 30 s, pH 7.4, and 37°C), and 101MDCE (half life approximately 1.7 min, pH 7.4, and 37°C), and BCNU (Figure 2). Although isocyanates are toxic species at high concentrations, high doses of Cloretazine do not decompose to toxic concentration levels of methylisocyanate. The isocyanate species plays an important role in the synergism between the carbamylating and choroethylating species in the clonogenic assays with Chinese hamster ovary cells. GR inhibition from Cloretazine, 101MDCE, 90CE and BCNU was reported with IC₅₀ values of 54.6 ± 4.3 μM, 32.7 ± 7.7 μM, > 1 mM, and 55.5 ± 2.2 μM, respectively (14). Rice *et al.* hypothesized that an isocyanate metabolite, rather than the isocyanate itself, transfers the carbamoyl group to a nucleophile in the enzyme (18).
The guanine-cytosine DNA crosslinks play an important role in Cloretazine's chemotherapeutic potential. The direct repair DNA mechanism has been previously investigated because of 90CE's chloroethylating selectivity (18). To repair $O^6$-alkylguanine lesions in double stranded DNA, $O^6$-alkylguanine transferase (AGT) transfers the alkyl group to an AGT protein's cystine residue within its active site and deactivates the protein (12,20). Direct repair of damaged nucleotides occurs without breaking double stranded DNA. Quantitatively different AGT expression between some cancerous cell lines and normal cells has increased its potential role in anticancer treatments. Damia and D’Incalci investigated a link between AGT levels and tumor cell sensitivity and alkylating agents (20). AGT’s interaction with Cloreatzine and its activated electrophiles have been previously investigated (16). 90CE and methylisocyanates inactivated the cloned AGT with $IC_{50}$ values of 100 nM and 13 µM, respectively. The synergism between the methylisocyanate and the chloroethylating species was further recognized as potentially relevant to Cloretazine’s clinical anticancer properties. Investigating other repair mechanisms and their respective enzymes would enhance the anticancer properties and the synergism of Cloreatzine.

**DNA Repair Pathway – Base Excision Repair**

Due to various chemical and physical DNA damaging agents, approximately 10,000 abasic sites are generated within a genome per day (21). A decrease in genomic integrity can
lead to complications of a cellular and developmental level such as apoptosis and cancer, respectively, if the damage is not properly repaired (22). Several DNA-repair pathways are employed within a cell to properly correct a damaged nucleotide base or bases, such as direct repair of DNA via AGT, the direct reversal of DNA damage. To further maintain genomic stability, the base excision repair (BER, Figure 3) pathway mends base lesions produced by oxidative and alkylating damage (21,23,24). BER corrects damaged bases in two pathways, either short or long patch repair. Long patch BER enzyme activity is similar to DNA replication, and replaces the damaged nucleotide and a few additional nucleotides around it, typically between 2-14 nucleotides total (22). Short patch BER, the focus of this study, only replaces a single nucleotide. Unlike direct repair, this major repair pathway involves several enzymes to remove the damaged base, perform strand incision, and fill the gap, including a DNA glycosylase, apurinic/apyrimidic endonuclease-1 (APE/Ref-1), and DNA polymerase β (Pol β), respectively. Long patch BER recruits additional enzymes to mend the larger DNA excision by DNA synthesis and ligation process that includes DNA polymerase δ and ε, flap-structure specific endonuclease-1 (FEN1), an endonuclease, and Poly ADP-ribose polymerase (PARP) (22).

Figure 3. Base Excision Repair Pathway. A general model of BER illustrates the single nucleotide replacement caused by a lesion formed on a nucleotide in double stranded DNA, where the lesion is represented by R (e.g., O\(^6\)-alkylguanine).
A DNA glycosylase cleaves the N-glycosidic bond of the damaged base and leaves a deoxyribose while the DNA backbone is left intact (21). APE/Ref-1 then hydrolyzes the sugar-phosphate backbone at the 5' side of the abasic site. The cleavage results in 5'-deoxyribose phosphate and a 3'-hydroxyl group. Pol β inserts a correct nucleotide base pair, and the deoxyribose phosphate then undergoes Pol β’s lyase activity, a β-elimination reaction, to form a 4-hydroxy-2-pentenal-5-phosphate (Figure 4) (25). The β-elimination reaction does not require a cofactor present; however, the deoxyribose phosphate is processed via hydrolytic cleavage when a Mg$^{2+}$ cofactor is present (14, 16). Pol β then inserts a correct nucleotide into the abasic site, and DNA ligase seals the nicked DNA (21, 26). In the presence and absence of a cofactor, the two major enzymes in BER cleave DNA in two different mechanisms.

![Figure 4. AP site mechanisms. The two major enzymes involved in base excision repair, DNA Pol β and APE/Ref-1, cleave the AP site of DNA in two different mechanisms (26).](image-url)
Pol-β cleaves the DNA after the ribose via β-elimination that leaves a 5'-phosphate and a 3'-α,β-unsaturated aldehyde terminus (Figure 4A). Alternatively, APE/Ref-1 cleaves the abasic site at the 5' terminus and produces a 5' 2-deoxyribose-5-phosphate (Figure 4B). The deoxyribose-phosphate is then cleaved into one of two products. In the absence of Mg²⁺, a β-elimination reaction occurs and the reaction produces a 4-hydroxy-2-pentenal-5-phosphate (Figure 4C). Conversely, in the presence of Mg²⁺, 2-deoxyribose-5-phosphate forms via hydrolysis (Figure 4D) (25).

*A purinico/purimic Endonuclease-1*

This study investigates the effects of Cloretazine on purified human APE/Ref-1 activity. APE/Ref-1 is a small, bifunctional enzyme, approximately 37 kDa in size, which possesses excision and reduction-oxidation (redox) activity. The redox and DNA repair activities are located in two different domains, the amino-terminus and the carboxyl-terminus, respectively (27). APE/Ref-1’s excision properties, an alternative pathway to repair O⁶-alkylguanine lesions, are responsible for repairing alkylated DNA. HeLa cells studies have shown that high APE/Ref-1 expression levels decrease the cell’s sensitivity to oxidative stress agents (28). Consequently, increasing the cell’s sensitivity to oxidative or alkylating agents could relate to the cellular levels of APE/Ref-1 and other DNA repair enzymes.

A common side effect of alkylating cancer agents is the myelosuppression, or decrease in bone density. One method to increase their potency is to increase the myeloid growth factors and stem cells to help support the integrity of the cell. Recent studies have developed a chimeric DNA repair protein to increase the levels of DNA repair enzymes within cells exposed to alkylating agents (15). Hansen et al. proposed a form of “protective” gene therapy by linking the BER and direct repair pathway by synthesizing a chimeric protein with both APE/Ref-1 and
AGT activity that would repair a broader range of damaged DNA (15). The study showed that the AGT-APE protein was functional in in vitro assays. HeLa cells, ovarian cancer cell lines, were alkylated by both BCNU and methyl methanesulfonate (MMS) for 7-10 days. The cells infected with the chimeric protein demonstrated a 2-fold survival rate when treated with 75 μM BCNU and a 4-8 fold survival rate increase with a 150 μM BCNU treatment. The 1.0 mM and 2.0 mM MMS-treated HeLa cells illustrated a 10-fold increase in survival rate. Cells that were exposed to 0.5 mM MMS and 75 μM BCNU showed an approximate 10-fold increase in survival rate (15). Hansen et al. demonstrate the possibility to save alkylated DNA in vivo by introducing increased levels of AGT and APE/Ref-1 activity (15).

APE/Ref-1 is a substrate for serine/theronine casein kinase I and II and protein kinase C. The repair activity of APE/Ref-1 is lost by if the enzyme is phosphorylated by protein kinase C. However, the inactivity can be reversed to reactivate the protein via a phosphatase treatment (27). The effects of phosphorylation on the enzyme tie into the redox activity, where APE/Ref-1 has been shown to stimulate various transcription factors, FOS, JUN and p53 (27). A tumor suppressor protein, p53, controls the cell cycle, cell survival, and DNA repair pathways (29). The protein is activated and inactivated by phosphorylation in the correct cellular environment (27,29). Recent studies have shown that APE/Ref-1 also can influence the oxidation of p53 to an activated p53 by the redox activity of APE/Ref-1 (27). Research on p53 and APE/Ref-1 has also revealed that p53 binds to another redox active protein, thioredoxin reductase (TrXR) (29). TrXR is a small enzyme that reduces thioredoxin to TrXR using NADPH (28). Seeman and Hainaut studied the three enzymes together by measuring the expression of APE/Ref-1, p53 and TrXR by RNA interference in cells that express wild-type p53. The study showed that inhibiting TrXR increased the oxidized form of TrXR and increased p53 DNA binding, whereas the
inhibition of APE/Ref-1 decreases p53 DNA binding (29). When both TrXR and APE/Ref-1 were inhibited, p53 binding activity levels remained unaffected (29). Consequently, they proposed a model in which APE/Ref-1 and TrXR are a link between BER and p53. APE/Ref-1 will repair abasic DNA through its endonuclease activity when it is reduced, and when APE/Ref-1 is in its oxidized form, the enzyme activates p53 to stimulate apoptosis. In the model TrXR oxidizes and reduces APE/Ref-1 (29). This model links abasic DNA damage to universal DNA damage which are repaired on a single nucleotide level and as a whole by complete and programmed cell death, respectively. Further investigation of APE/Ref-1 and TrXR will allow a better understanding of the in vivo repair caused by DNA damage.

Previous work in the Rice lab investigated the inhibition of Pol β by Cloretazine (A. Frederick, M. Davis, unpublished work). Pol β studies explored the effects of Cloretazine on the enzyme’s AP lyase and nucleotide transferase activity. Frederick discovered that the carbamoylating activity was responsible for inhibiting Pol β nucleotide transferase activity, but not the AP lyase activity. In this work I developed an APE/Ref-1 specific assay to measure the hydrolysis activity levels of APE/Ref-1 on 32P-labeled oligonucleotides. The method takes into account the requirement of a Mg2+ cofactor that has been shown to spontaneously cleave DNA, and minimizes the effects of the cofactor. I also studied the inhibition of Cloretazine at varying concentrations, (5.0 μM to 1.0 mM) on the excision repair enzyme. APE/Ref-1 is an ideal enzyme for further investigation. The enzyme initiates high efficiency abasic site repair and plays a crucial role in BER.
MATERIALS AND METHODS

Materials

Oligonucleotide strands 1 and 2, TACCGCGGCCCAUCAAGCTTATTGGGTAC and AATGTACCACAATAACGTTGATCGGCGGCCGCGGTA, respectively, were obtained from Operon (Huntsville, AL). [α-32P] dATP (AA0004, 3000Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Cloretazine, 101MDCE, and 90CE were synthesized, purified, and characterized at Yale University (New Haven, CT) as described elsewhere (11,30). BCNU was purchased from Sigma (St. Louis, MO). Drugs were dissolved in dry DMSO to concentrations of 200 mM. Stock solutions were stored desiccated at -20°C for 2 months; all dilutions were also prepared in dry DMSO. Purified human apurinic endonuclease-1 was purchased from Trevigen, Inc. (Helgerman, CT). Klenow exo-1 fragments and uracil DNA glycosylase were purchased from New England Biolabs (Ipswich, MA). All other solvents and biochemicals were obtained from Fisher Scientific (Pittsburg, PA) unless otherwise noted.

3’ DNA end labeling and purification

15 μM strand 1, 15 μM strand 2, and 50 nM NaCl were added in a microcentrifuge tube and the volume was adjusted with distilled water to a total volume of 10 μL. The oligonucleotide mixture was incubated at 90°C for approximately 3 min to ensure complete denaturation, and slowly cooled for 40 min to less than 50°C. The oligonucleotide mixture was then labeled on the 3’ end of the strand by using 80 μCi [α-32P]-dATP, 1.0 unit Klenow (exo-1), 10x New England Biolabs Buffer #2, 2.1 mM MgCl₂, and volume adjusted with distilled water to a total reaction volume of 35 μL per reaction. The mixture was incubated at 37°C for 30 min. 32P-labeled DNA was purified via a QIA Quick Nucleotide Removal kit (Qiagen, Inc., Valencia, CA) according to
manufacturer's instructions, and eluted in 10 mM TrisCl (pH 7.4) and stored at -20°C for up to 1 month.

**DNA Substrate Preparation**

Standard assay conditions included 70 mM HEPES-KOH buffer (pH 7.4), 0.5 mM EDTA, 5% glycerol, 1.0 mM tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), $^{32}$P-labeled oligonucleotide (24 µL added volume, $4.77 \times 10^{-8}$ M final concentration) and 20 units of uracil DNA glycosylase (2.2 µL added volume) in a total volume of 216 µL per reaction. This reaction was incubated at 37°C for 30 min. DNA substrate was stored on ice.

**APE Assay**

Cloretazine, 101MDCE, 90CE, BCNU, or DMSO control was preincubated with 2.5 units/µL of human APE in 100 mM HEPES-KOH (pH 7.4) buffer for 3.0 hr at room temperature. 8 mM MgCl$_2$ and 1.0 unit preincubated APE mixture per reaction were added to 16.0 µL DNA substrate aliquots and incubated at 37°C for 20 min. The APE reaction was quenched with 2.0 M NaBH$_4$. Equal volume formamide (with bromophenol blue and xylene cyanol dye) was added to the reaction mixture and heated for 2-4 min at 90°C and the reaction mixture was cooled on ice. 15% polyacrylamide gels of 0.75 mm thickness were prepared fresh daily via 40% acrylamide solution (19:1, acrylamide: bis-acrylamide, electrophoresis grade), 5x Tris-Borate-EDTA (TBE), and 12% w/v urea while TEMED (15 µL added volume) and 10% APS (200 µL added volume) were added to polymerize the acrylamide, 25 mL total volume. 11 µL aliquots of sample were loaded on the 15% denaturing acrylamide gel and electrophoresed in a 1x TBE buffer for 30 min at 475 V (45-50°C optimal gel temperature) in a Hoefer SE600
chroma standard vertical electrophoresis unit powered by a Fisher Scientific FB1000 power supply with a 1x TBE buffer. The gel was soaked in a 10% methanol, 10% glacial acetic acid fixative on an orbital shaker. Gels were dried for 30 min under vacuum, 1 hr under 80°C heat and vacuum, and then for 30 min under vacuum alone. The dried gels were placed in a phosphorimager cassette overnight at room temperature and analyzed quantitatively by phosphorimaging. Gels were visualized with the Amersham Biosciences Storm840 Phosphorimager with ImageQuant 5.2 software to quantify the data. Data were normalized to the positive and negative controls and expressed as percent activity. APE activity was determined by calculating the ratio of intact oligonucleotide to hydrolyzed oligonucleotide products.
RESULTS

Mg\(^{2+}\) facilitates the spontaneous cleavage of abasic DNA

Spontaneous DNA cleavage by Mg\(^{2+}\) was investigated by incorporating MgCl\(_2\) at various points in the uracil DNA glycosylase (UDG) reaction. Mg\(^{2+}\) cleavage of DNA was measured as a ratio of cleaved DNA product ratio with respect to intact DNA with Mg\(^{2+}\) (Figure 5). The addition of 8 mM MgCl\(_2\) before and after the NaBH\(_4\) quench shows an increase in spontaneous cleavage. The cleaved DNA band, a smaller size than the intact DNA, appears in lanes 3-4 only when MgCl\(_2\) was present in the UDG assay. Additional studies were preformed to examine the effects of no MgCl\(_2\) in the UDG reaction. Because Mg\(^{2+}\) is not a cofactor for UDG, the studies did not illustrate new information (results not shown). The banding pattern result of the UDG reaction alone is equivalent to \(^{32}\)P-labeled DNA oligonucleotide banding pattern. The glycosylase reaction removes the uracil and the enzyme does not affect the phosphodiester backbone, thus the reaction cannot be visualized on a 15% denaturing polyacrylamide gel.

![Figure 5. Uracil Deglycosylase Assay in the presence of 8 mM MgCl\(_2\). Lanes 1 and 5 represent the intact \(^{32}\)P-labeled DNA (used as a radioactive size ladder). 8 mM MgCl\(_2\) was added to the glycosylase reaction buffer and the protocol followed the UDG assay as described in the experimental methods section (Lane 2). 8 mM MgCl\(_2\) was added before (Lane 3) and after (Lane 4) the UGD reaction was stopped with NaBH\(_4\). The positions of the intact DNA and the cleaved DNA are indicated.](image-url)
Decreasing the APE/Ref-1 incubation time from manufacture’s specifications allows clear quantification of enzyme activity.

The UDG reaction with MgCl₂ was expanded to examine the products of APE/Ref-1 and Pol β cleavage (data not shown). Pol β acts as the positive DNA cleavage control. To optimize the APE/Ref-1 specific assay, the enzyme concentrations were varied from 1.0 unit/μL, 0.18 unit/μL, and 0.09 unit/μL. The results showed that the 1.0 unit/μL and the 0.09 unit/μL APE/Ref-1 total assay reactions possessed too much enzyme or too little enzyme concentrations, respectively, for efficient hydrolysis of the labeled oligonucleotide (results not shown). Trevigen defines the unit definition for APE/Ref-1 as one unit of enzyme hydrolyzes 1 pmol of abasic DNA oligonucleotide/hr. The optimal APE/Ref-1 assay was constructed with 0.18 unit/μL APE/Ref-1, 8 mM MgCl₂, and incubated for 40 min (Figure 6). The hydrolysis of DNA over time resulted in a hyperbolic curve that reaches a maximum of 80% of total hydrolysis of the abasic DNA substrate at 40 min.

![Figure 6. Hydrolysis of abasic DNA. Abasic DNA was hydrolyzed via 0.18 unit/μL (2.0 units enzyme total) over a 0-40 min time course (37°C incubation). At times indicated the reaction was quenched with equal volumes 2.0 M NaBH₄. Data standardized to the initial time point 0.0 min.](image-url)
The final incubation time for the APE/Ref-1 hydrolysis reaction is 2 min, which yielded approximately 70% hydrolysis of the DNA substrate, approximately 88% of the total APE/Ref-1 hydrolysis, and the clearest measurable ratio of intact DNA substrate to hydrolyzed product.

Optimization of electrophoresis methodology eliminates single strand oligonucleotides from reannealing.

Complications in quantifying the APE/Ref-1 hydrolysis stemmed from an unknown and unresolved band seen above the intact DNA substrate maker. The molecular weight of the unknown, the intact DNA substrate, and the hydrolyzed DNA product are unknown because a $^{32}$P-labeled DNA ladder was not available. The $^{32}$P-labeled DNA oligonucleotide was run on a 20% native mini gel via standard protocol to determine if there was an additional species present in the labeled oligonucleotides prepared. Due to the high intensity of the radioactive label it could not be determined conclusively whether the labeled oligonucleotide was one or two bands (data not shown). The labeled oligonucleotide was then run on a 15% denaturing polyacrylamide mini gel against a Pol β DNA product to determine if the oligonucleotide was a mixture of single and double stranded DNA, but the gel was too short to distinguish any differences in banding patterns (data not shown). A larger, 20 cm x 20 cm, 15% denaturing gel was rerun and illustrated the presence of only one band in the DNA substrate samples (Figure 7). The denaturing gel illustrated that the oligomer is one intact strand and the unknown band does not stem from the DNA substrate but arises during the UDG or APE/Ref-1 assays. However, it was important to note that the temperature of the mini gel was considerable higher than that in the larger denaturing gels.
Optimal denaturing polyacrylamide gels should be run between 45-50°C. A temperature probe was adhered to one of the outside glass plates used in the vertical gel set up. The temperature of the glass was recorded to measure the approximate temperature of the denaturing gel over a 4.0 hr time course to reach a constant temperature of 45-50°C (results not shown).

The final electrophoresis methodology included preheating the gel for 30 min at 475 V in an empty/unloaded gel and running the gel for 30 min at 475 V to obtain a constant and approximate 45-50°C gel temperature. It is important to note that the plates must be cooled upon completion of electrophoresis for 15-30 min or the plates will crack. If the plates are run at a temperature higher than 50°C, the plates will also crack.

_The inhibition of APE/Ref-1 hydrolysis activity by Cloretazine._

APE/Ref-1 was pre-incubated with 40 μM - 1.0 mM Cloretazine for 3.0 hr at room temperature. Pre-incubation allowed approximately three half-lives of Cloretazine to occur so that the effects of the enzyme were being examined on the enzyme and not the DNA substrate. Cloretazine inhibited the activity of APE/Ref-1 hydrolysis activity; however, the percent control
activity values varied between replicates (Figure 8). The assay volumes were increased by a factor of three to account for possible pipeting error.

![Image of gel showing DNA substrate and hydrolyzed product](image)

**Figure 8.** The inhibition of APE/Ref-1 hydrolysis activity by 5 – 500 μM Cloretazine. Samples were run on a 15% denaturing PAGE, as described in the methods. The controls of the experiment included an intact 32P-labeled DNA substrate (lane 1), a negative hydrolysis control 8 mM MgCl₂ and no APE/Ref-1, and two positive APE/Ref-1 hydrolysis samples without Cloretazine (lanes 3-4). The preincubations were run as follows: 500 μM Cloretazine and duplicate (lanes 5,8 respectively), 50 μM Cloretazine and duplicate (lanes 6, 9, respectively), and 5 μM Cloretazine and duplicate (lanes 7,10 respectively). Intact DNA and hydrolyzed DNA products are labeled.

APE/Ref-1 was pre-incubated with 5 – 500 μM Cloretazine for 3.0 hr at room temperature. Cloretazine at 500 μM, 50 μM, and 5 μM inhibited the activity of APE/Ref-1 with percent control hydrolysis values of 81.8% ± 17, 125% ± 51, 148% ± 45, respectively. Normalizing the hydrolysis to the positive and negative controls illustrates greater than 100% hydrolysis of DNA, due to the decrease in activity of the positive control. Four positive control studies (n = 4 in duplicate) were prepared to determine the activity of the APE/Ref-1. The final average hydrolysis of APE/Ref-1 was 58.6% ± 15. The percent control hydrolysis values of the effects of 500 μM, 50 μM, and 5 μM Cloretazine were normalized for the average control hydrolysis and yielded: 53.7% ± 9, 61.0% ± 9, 82.2% ± 26, respectively (Figure 9). The control hydrolysis is 30% less active than the optimized activity of 70% determined in the kinetics experiment. Consequently, a second kinetics study was performed to reassess the nature of
APE/Ref-1 activity that illustrated a fluxing, hyperbolic curve that reaches a maximum of 70% hydrolysis after a 50 min incubation (Figure 10).

Figure 9. Inhibition of APE/Ref-1 hydrolysis activity by 5-500 μM Cloretazine concentrations for a 30 min UDG reaction incubation followed by a 20 min APE/Ref-1 incubation. Total reaction volumes were quenched with equal volumes 2.0 M NaBr₄ and run on 15% denaturing PAGE at 475 V for 30 min. Samples were visualized via phosphorimagry. Data were reported as quadruplicate average.

Figure 10. Second kinetics assessment of the hydrolysis of abasic DNA. Abasic DNA was hydrolyzed via 0.18 unit/μL total reaction volume (2.0 units enzyme total) over a 0-50 min time course 37°C incubation. At times indicated the reaction was quenched with equal volume 2.0 M NaBH₄. Data were standardized to the initial time point 0.0 min.
DISCUSSION

Due to various chemical and physical DNA damaging agents, abasic sites are generated within the genome. The decrease in genomic stability can lead to complications in cell development and cause apoptosis, cancer, or a variety of negative side effects. Various DNA repair pathways are employed to maintain genomic stability. Understanding these mechanisms can further enhance the treatments available in our chemotherapeutic arsenal. The roles of nitrosoureas as anticancer agents and their reactive subspecies, such as choroethylating and carboxamoylating species, have become increasingly important in the future research to understand the DNA repair mechanisms. Studies have shown that Cloretazine directly inhibits AGT, a predominant enzyme in direct DNA repair \((16)\). Methylisocyanate and the chloroethylating species inhibited AGT with IC\(_{50}\) values of 13 \(\mu\)M and 100 \(\mu\)M, respectively \((16)\). The effects of Cloretazine in AGT\(^-\) versus AGT\(^+\) cell lines also increase the clinical utility of the drug. Cells without AGT are more sensitive to Cloretazine compared to AGT\(^+\) cell lines treated with the same concentration of alkylating agent \((16)\). The research corresponds with clinical data, where acute myelogenous leukemia patients with low AGT levels responded more favorably to Cloretazine treatment than other treatments. Investigating alternative DNA repair mechanisms, such as BER, can further reveal the utility of Cloretazine over nitrosourea compounds.

In the absence of AGT, Pol \(\beta\) may be used to repair alkylated DNA damage via BER \((17, 31)\). Cells deficient in Pol \(\beta\) are also more sensitive to DNA alkylating agents \((31, 32)\). The inhibition of Pol \(\beta\)'s AP lyase and nucleoside transferase activity was investigated to begin the investigation of BER. Cloretazine's carboxamoylating activity inhibits the nucleosylidy transferase activity of Pol-\(\beta\), but not its lyase activity \((A.\ Frederick\ and\ M.\ Davis,\ unpublished\ work)\). The AP lyase activity was also inhibited by concentrations above clinically relevant levels. However,
this study focused on developing an assay specific for APE/Ref-1 hydrolysis and the effects of Cloretazine on another BER enzyme. The enzymatic method that was developed focused on determining the correct concentration of MgCl₂ in the assay to allow complete APE/Ref-1 hydrolysis, but to limit the amount of spontaneous cleavage by the Mg²⁺ cations. The optimal concentration determined was 8.0 mM MgCl₂ which does not show significant cleavage of DNA but promotes APE/Ref-1 hydrolysis.

Additional complications arose due to the electrophoretic method development, where DNA oligonucleotides were reannealing within the 15% denaturing polyacryamide gel and hindering the quantification of the analysis. The optimal gel temperature for complete denaturing of the DNA substrates was between 45-50°C. Overall, the study provided preliminarily data suggesting Cloretazine does not affect the APE/Ref-1 hydrolysis activity at clinically significant concentrations (Figure 9). 500 μM, 50 μM, and 5 μM Cloretazine concentrations were tested on APE/Ref-1 hydrolysis activity, and the results illustrate only 53.7% ± 9, 61.0% ± 9, 82.2% ± 26 percent control activity, respectively. Over three orders of magnitude difference in drug incubations, one would hope to see greater inhibition of APE/Ref-1 activity in order to justify the target.

The investigation of the APE/Ref-1 activity shows a decrease in percent control hydrolysis during the time experiments (Figure 10). The assay was tested to determine the source of limiting enzymatic activity. New APE/Ref-1 was purchased and the ³²P-labeled oligonucleotides were also investigated. These studies did not improve the overall efficacy of the assay. Consequently, the assay may be limited by the uracil-DNA glycosylase activity. If there is not enough enzymatic removal of the uracil in the labeled oligonucleotide, then the hydrolysis of the 5'-phosphodiester backbone cannot occur.
The study has only begun to investigate the importance of Cloretazine in other DNA repair pathways. Inhibition of BER enzymes, in a predominant DNA repair pathway, such as DNA Pol β, APE/Ref-1, FEN1, and DNA Ligase III, could enhance Cloretazine's alkylating agent by specifically targeting cells that overexpress DNA repair enzymes. The initial steps in the investigation of how Cloretazine inhibits the hydrolysis activity of APE/Ref-1 have achieved promising results. Further studies to finalize the *in vitro* APE/Ref-1 specific assay will allow the complete assessment of how Cloretazine, 90CE, 101MDCE and BCNU affect APE/Ref-1 hydrolysis activity. The research would continue to determine other instances where Cloretazine could be a more effective anticancer treatment compared to BCNU. Additional research will illustrate the effects of the Cloretazine’s chloroethylating and carbamoylating moieties compared to the overall effects of BCNU. The effects of APE/Ref-1 redox activity *in vivo* can promote further research on APE/Ref-1 in the presence and absence of p53 transcription factors.
REFERENCES


