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DEVELOPMENT OF A NOVEL CLASS OF CHEMICALS FOR LABELING ABASIC SITES IN CELLULAR DNA AND KILLING CANCER CELLS

by

SHANQIAO WEI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

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MAJOR: CHEMISTRY (Biochemistry)

Approved By:

Advisor Date


DEDICATION

To my husband Biao and daughter Viki
ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

MMR: Mismatch repair
BER: Base excision repair
NER: Nucleotide excision repair
HR: Homologous recombination
PHAs: Polycyclic aromatic hydrocarbons
BCNU: 1, 3-bis (2-chloroethyl)-1-nitrosourea
AP site: Apurinic/apyrimidinic site
TMZ: Temozolomide
APE: AP endonuclease
5’dRP: 5’-deoxyribose-phosphate
Pol β: Polymerase β
PCNA: Proliferating cell nuclear antigen
FEN1: Flap structure-specific endonuclease 1
TLS: Translesion synthesis
NHEJ: Non-homologous end-joining
ARP: Aldehyde reactive probe
MX: Methoxyamine
HPLC: High-performance liquid chromatography
MS/MS: Tandem mass spectrometry
NBHA: O-4-nitrobenzylhydroxylamine
DNase: Deoxyribonuclease
DSBs: DNA double-strand breaks
ATM: Ataxia telangiectasia mutated
5-FU: 5-fluorouracil
AAG: Alkyladenine-DNA glycosylase
MMS: Methyl methanesulfonate
Top I: Topoisomerase I
Top II: Topoisomerase II
B-NHL: B cell non-Hodgkin lymphomas
AID: Activation-induced demainase
SHM: Somatic hypermutation
CSR: Class switch recombination
UNG: Uracil-N glycosylase
TDG: Thymine DNA glycosylase
MBD4: Methyl-binding domain glycosylase 4
Exol: Exonuclease
DLBCL: Diffuse large B cell lymphomas
GC: Germinal center
EBV: Epstein-Barr virus
MM: Multiple myeloma
CHL: Classica Hogkin’s lymphoma
MZL: Marginal zone lymphoma
NSCLC: Non small cell lung cancer
R-CHOP: Rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone

R-CVP: Rituximab, cyclophosphamide, vincristine and prednisone

UDG: Uracil DNA glycosylase

Cy5: Cyanine 5

TE: Tris-EDTA

HRP: Horseradish peroxidase

TBS: Tris-buffered Saline

STV-HRP: Streptavidin-conjugated horseradish peroxidase

DMSO: Dimethyl sulfoxide

FITC: Fluorescein isothiocyanate

PI: Propidium iodide

DPBS: Dulbecco’s Phosphate-Buffered Saline

RT-PCR: Real-time PCR

AA3: O-2-Propynylhydroxylamine hydrochloride

DMF: N, N-dimethylformamide

AA4: O-propylhydroxylamine hydrochloride

AA5: O-(3-butynyl) hydroxylamine hydrochloride

TPP: Triphenylphosphine

THF: Tertahydrofuran

AA6: O-(2-azidoethyl) hydroxylamine hydrochloride
CHAPTER 1: INTRODUCTION

Portions of the text and figures in this chapter were reprinted or adapted from DNA repair journal, Vol 27, Wei, S., Shalhout, S., Ahn, Y., Bhagwat, A.S., A versatile new tool to quantify abasic sites in DNA and inhibit base excision repair, Pages 9-18, Copyright (2015), with permission from Elsevier.

1.1 Abasic (AP) sites in cellular DNA

Deoxyribonucleic acid (DNA) is a biopolymer that carries unique genetic information of an organism. Maintaining the integrity and stability of DNA is essential to the survival, function, and reproduction of an organism. However, DNA integrity is always challenged by endogenous or exogenous agents resulting in DNA damages. If unrepaired, DNA damage could cause mutations and a variety of diseases. Therefore, to preserve genome stability, cells have evolved to correct damages in DNA through several repair systems such as direct repair (DR), nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), homologous recombination (HR), and non-homologous end joining (NHEJ) [1].

DNA damages are defined as alterations to the chemical structure, which could be strand breaks, base modifications, bulky adducts, crosslinks or base loss. For example, ionization radiation can induce single and double-strand breaks in DNA [2]. Reactive oxygen species generated during normal metabolic processes in cells can oxidize guanines into 8-oxoguanines [3]. Bulky adducts in DNA are created by the exposure to carcinogens such as polycyclic aromatic hydrocarbons (PHAs) [4]. Some chemotherapeutic agents like 1, 3-bis(2-chloroethyl)-1-nitrosourea (BCNU) can give to DNA crosslink by methylating guanines [5]. Base
loss arises from several different ways and leads to the formation of abasic (AP) sites [6], which are particular interest to our lab.

Many DNA-damaging chemotherapeutic drugs can induce the formation of AP sites [7]. Thus detection and quantification of AP sites will provide a useful tool for monitoring the effect of drug during the development of chemotherapies. Furthermore, AP sites are created and repaired through BER pathway. Recently, inhibition of BER has been a potential strategy to overcome therapeutic drug resistance and enhance the potency of chemotherapies. As AP sites are involved in this repair pathway, blocking BER by targeting AP sites will provide a novel strategy for cancer therapy [8]. The work presented explores the detection of AP sites in cancers as well as targeting AP sites to improve anti-tumor chemotherapy.

1.1.1 Formation of abasic sites

An AP (apurinic/apyrimidinic) site is considered to be the most commonly generated lesion in DNA, which is derived from a loss of base through the hydrolytic cleavage of \(N\)-glycosidic bonds, leaving a deoxyribose residue in DNA (Figure 1). Numerous endogenous/exogenous agents as well as innate cellular processes can create AP sites in DNA. AP sites are formed in cellular DNA through spontaneously water-mediated depurination and deprimidination[9]. About 10,000 AP sites are generated in this way in a human cell every day[10]. The process of depurination is pH and temperature dependent, and either heat or acidic condition greatly accelerates the rate of depurination. Hence, heat/acid treatment is a common method used for induction of AP sites in DNA[11]. At 70°C and pH 7.4 condition, the rate of depurination of native DNA is \(4 \times 10^{-9}\) sec\(^{-1}\). However, hydrolytic depurination also
spontaneously occurs at a significant rate under physiologic conditions ($k=3 \times 10^{-11}$ sec$^{-1}$) [9]. Hydrolytic depyrimidination happens at a lower rate compared to depurination [12].

![Figure 1 Formation of abasic (AP) sites in DNA through depurination.](image)

AP sites are also created through the action of agents that react with DNA. For example, DNA alkylating agents such as temozolomide (TMZ) transfer an alkyl group to guanine base at 7 nitrogen position. The alkylation of 7-nitrogen of guanine destabilizes the glycosidic linkage by giving a positive charge on the guanine ring and converts guanine into a good leaving group, and increases the rate of depurination. A number of other alkylated bases including $N_3$-methylguanine, $N_3$-methyladenine and $N_7$-methyladenine similarly facilitate the loss of purine residues [12].

Furthermore, many damaged bases are repaired via the BER pathway, which starts with the excision of the damaged base by a DNA glycosylase forming an AP site. Subsequently, AP endonuclease (APE) incises the DNA backbone at the AP site and creates a single-strand break resulting 5'-deoxyribose-phosphate (5’dRP) end, which is further removed by polymerase β (Pol β). Finally, the correct nucleotide is incorporated by Pol β, and the remaining nick is sealed by DNA ligase [13] (Figure3). This BER pathway is called ‘short-patch repair’. BER pathway can also
take place by ‘long-patch repair’, in which more than one nucleotide are inserted by polymerases. In long-patch BER, proliferating cell nuclear antigen (PCNA), flap structure-specific endonuclease 1 (FEN1) and DNA ligase are required to work together and remove the generated flap and seal the nick. Short-patch BER is the dominant pathway. However, when AP sites are reduced or oxidized or when the 5’-dRP intermediate generated by AP endonuclease is refractory to the removal action of pol β, the long-patch BER proceeds to repair DNA [14]. Damaged DNA bases arising from alkylation, oxidation and deamination of normal bases are primarily repaired by BER pathway, producing AP sites as intermediates. Although the glycosylase action is normally coupled with other BER enzymes that process the AP sites, an imbalance in the repair enzymes may cause the AP sites to persist.

![Figure 2. Creation and repair of AP sites in base excision repair pathway](image)

**Figure 2. Creation and repair of AP sites in base excision repair pathway.** AP sites are created through the action of DNA glycosylase. Subsequently, AP endonuclease (APE) cleaves DNA
backbone at AP sites to create a single-strand break. The nick can be processed in either short-patch pathway or long-path pathway. In short-patch BER, polymerase β (Pol β) removes remaining 5'‐deoxyribose‐phosphate (5’dRP) and incorporates the correct complementary deoxynucleotide, and finally the nick is sealed by DNA ligase III. In long-patch BER, polymerases δ/ε insert at least two nucleotides at the single-strand break generated by APE, and FEN1 is responsible for removing the flap. DNA ligase I seals the gap and accomplishes DNA repair. This figure was modified from figures in the references [13-15].

1.1.2 Repair and biological consequence of AP sites

BER is the principal pathway responsible for repairing AP sites lesions in DNA. However, other DNA repair pathway may also be able to remove AP sites. Nucleotide excision repair is known to repair bulky lesions[16]. But, there are some evidences showing that NER pathway acts as a backup for removing AP sites in E. coil cells and yeast, when BER does not function very well [17, 18]. If unrepaired, AP sites potentially cause cell death or mutagenesis by blocking DNA replication and transcription. Replicative DNA polymerases cannot copy AP sites, and the progress of the replication fork is blocked at AP sites causing single-and double-strand breaks. Alternatively, AP sites may be copied by error-prone translesion-synthesis (TLS) polymerases that cause base substitution mutations, but allow replication to continue [19]. The strand breaks resulting from unrepaired AP sites may be repaired using error-free HR, or may be repaired by a NHEJ process that creates small addition/deletion mutations. If unrepaired, the strand breaks lead to gross chromosome alterations such as translocations and cause cell death [17]. Thus the creation of AP sites in the genome and their processing by cellular machinery has profound implications for genome integrity.
Figure 3 Biological consequences of AP sites. AP sites are primarily repaired by BER pathway, and they can be removed by nucleotide excision repair (NER) pathway when base excision repair (BER) is defective. Also, AP sites can be tolerated by translesion synthesis which induces mutations. If unrepaired, AP sites could block DNA replication fork inducing DNA breaks. The resulting breaks might be processed through homologous recombination (HR) or non-homologous end-joining (NHEJ); otherwise, these breaks would lead to cell death.

1.3 Chemistry of AP sites

NMR spectroscopy analysis of AP site in synthetic oligodeoxynucleotides suggested that AP sites might exist in equilibrium between three chemical forms: cyclic hemiacetals, open-chain aldehydes and hydrated aldehydes (Figure 4A). A mixture of α and β cyclic hemiacetal forms (around 1:1 ratio) are predominant, while the aldehyde form only makes up 1–5 % [20]. However, the open-chain aldehydes are most reactive. Aldehydes in the open form of AP sites ligate to alkoxyamines forming stable oximes. Taking the advantage of the reactivity of AP sites with alkoxyamine compounds, many techniques have been developed for the study of AP sites. For example, aldehyde-reactive probe (ARP) and methoxyamine (MX) are two well-known alkoxyamines widely used for labeling AP sites (Figure 4B). Furthermore, AP sites are sensitive to alkaline conditions due to the presence of the aldehyde form. In mild alkaline condition, AP sites undergo a β-elimination reaction yielding α, β-unsaturated sugar residue. A stronger
alkaline condition can induce a second elimination called δ elimination, and the, β-unsaturated aldehyde is further degraded [21]. Also, under neutral heat condition, AP sites are shown to be thermally degraded through β-elimination reaction [22] (Figure 4C).

**Figure 4 Chemistry of AP sites.** (A) Structural forms of an AP site. (B) The reaction between AP sites and alkoxyamines such as methoxyamine and ARP. (C) The reactivity of AP sites under
alkaline and heat condition. The mild alkaline or heat condition promotes a \(\beta\)-elimination reaction resulting in the formation of \(\alpha, \beta\)-unsaturated sugar. Under the strong alkaline condition, AP sites can be further degraded through a \(\delta\) elimination reaction.

1.1.4 Detection and quantification of AP sites in DNA

Utilizing the reactivity of AP sites, many assays for quantification of these DNA lesions have been developed. Most of the techniques are based on the reaction of an aldehyde residue of AP sites with alkoxyamines as previously mentioned. Radioisotope-labeled methoxyamine (\(^{14}\)C-methoxyamine or \(^{11}\)C-methoxyamine) has been used to determine AP sites in DNA treated with peroxynitrite [23] or visualize AP sites in vivo [24]. The number of AP sites are proportional to the radioactivity of methoxyamine in DNA. This quantitative assay shows high sensitivity with a limit of detection of 1 AP site per 1 million nucleotides [23]. With the fast development of chromatographic separation and mass spectrometry technologies, high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) techniques provide a more accurate method to measure AP sites. DNA containing AP sites are labeled with an alkoxyamine called \(O\)-4-nitrobenzylhydroxylamine (NBHA), followed by digestion with a deoxyribonuclease (DNase) into mononucleotides. NBHA-tagged monomeric subunits are isolated and detected by HPLC-MS/MS. This method shows not only high sensitivity with a limit of detection of 3 AP sites per \(10^7\) nucleotides but also can discriminate intact AP sites from cleaved AP sites such as 5’-deoxyribose monophosphate (5’-dRP) [25]. However, it is very difficult to use these techniques with a large number of samples because they either use equipment such as HPLC and MS/MS or use radioisotopes that are incompatible with a clinical setting. In addition, these methods require a large amount of DNA. Another simple quantitative assay for AP sites involves in fluorescently labeled alkoxyamines which allow linking fluorescent tagged to AP sites for
detection, but this method shows low sensitivity with a limit detection of 1 AP site per \(1 \times 10^5\) nucleotides [26].

Consequently, the most commonly used method for the detection and quantification of AP sites is based on the reaction of ARP which reacts with the open form of aldehyde residue in AP sites. An advantage of the use of ARP in labeling AP sites is that multiple samples can be processed in parallel, and the reaction products can be spotted on a membrane to create an ELISA-like assay. ARP has been used to determine AP sites in different mammalian tissues [27], to monitor changes in AP sites during aging [28] and AP sites generated as a result of treatment of cells with carcinogens [29]. It has also been adapted to quantify genomic uracils by excising uracils by uracil-DNA glycosylase to create AP sites followed by ARP treatment [30, 31]. It has been used to determine uracil levels in normal and repair-deficient Escherichia coli cells [31], in normal mammalian tissue and cancer cells [32]. However, ARP-based assays for AP sites suffer from several drawbacks. ARP contains biotin which is also present in a cell and hence fluorescent labeling of AP sites in living or fixed tissues using ARP results in a considerable background. ARP is bulky (MW 331.4), and its reaction with AP sites is likely to be significantly hindered. The presence of biotin within ARP also necessitates the use of protein like streptavidin making the labeling scheme cumbersome and somewhat expensive. Finally, a detailed study of ARP reactivity with AP sites has shown that the reaction creates side products in addition to ARP linked to full-length DNA [33].

1.2 The role of abasic sites in cancer chemotherapy

DNA-damaging agents are a major type of chemotherapeutic drugs for cancer treatment. These agents target DNA in cells and introduce a variety of DNA damages including DNA breaks,
base modifications and strand crosslinks. Most cancer cells often display specific abnormalities in DNA damage response and DNA repair pathways [34]. Due to this property of cancers, DNA-damaging agents have been widely developed to kill cancer [35]. However, the use of many DNA-damaging drugs is primarily limited by the development of drug resistance. In many cases of failure of treatment with DNA-damaging agents, the resistance arises from a change in repair proteins expression and increased DNA repair capacity [36-38]. To strengthen DNA-damaging agents and overcome drug resistance, disruption of DNA repair pathway is a rational approach [34, 39]. As BER is the major pathway responsible for repairing most DNA lesions introduced by DNA-damaging agents [40-42], a number of small-molecule inhibitors of BER proteins such as APE1, PARP-1, and Pol β have been used in combination chemotherapy with DNA-damaging agents [39]. AP sites as the crucial intermediates in BER pathway could also be the target of anticancer drugs. A small alkoxyamine, MX, works as a BER inhibitor by chemically modifying AP sites induced by DNA-damaging agents and has been proposed as an anticancer agent in combination with alkylating agents such as temozolomide [43]. Thus, blocking the repair of AP sites could be a promising approach to eliminating drug resistance and improve the chemotherapeutic outcome of DNA-damaging agents.

1.2.1 DNA-damaging agents in chemotherapies

Chemotherapeutics agents have been used in cancer treatment for decades and are still the most prevalent treatment, since the first drug, nitrogen mustard was used for curing lymphoma and leukemia tumors during world War II [44]. In the 1960s, the mechanism of killing cancer cells by nitrogen mustard was elucidated. The ability of nitrogen mustard to kill tumors is based on its property of DNA damaging. When cells have the high level of DNA damage, DNA
damage response and repair pathways are activated to maintain genome integrity and prevent transferring damaged DNA to daughter cells. If damaged DNA was not repaired prior to S phase in cell proliferation, the progress of DNA replication would be blocked by damaged bases. This would subsequently lead to accumulation of deleterious DNA double-strand breaks (DSBs). DSBs are principally repaired by either NHEJ or HR pathway. If left unrepaired, cells will be arrested or undergo apoptosis [35] (Figure 5). In normal cells, cell-cycle checkpoints are responsible for sensing DNA defects then activating DNA damage response and repair pathways to induce either cell cycle arrest, DNA repair, or cell apoptosis. However, human cancer cells often ignore cell cycle checkpoints, continuing DNA replication in the presence of damaged DNA [34]. This increases the likelihood of formation of DSBs and cell death during DNA replication when cancer cells are exposed to DNA-damaging agents. Therefore, this confers cancer cells higher susceptibility to DNA damages. Moreover, human tumors typically show aberrations in DNA damage response or repair. Inactivation of DNA damage response was observed in various cancers, due to mutation and deletion of P53 gene or decreased expression of ataxia telangiectasia mutated (ATM)), BRCA1, and MRN complex [35, 45, 46]. However, in some cases, proteins involved in ATM-mediated DNA damage response are upregulated, and this induces resistance of cancer cells to DNA-damaging agents. Also, many cancers have deficiencies in a certain DNA repair pathway and may be more susceptible to a specific type of DNA damage [35].
DNA-damaging agents introduce DNA damages including base modifications, crosslinks and single-strand breaks (SSBs). Each type of lesions is processed by a specific repair pathway such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and direct repair (DR). Unrepaired damaged DNA could stall DNA replication fork and induce double-strand breaks (DSBs). In response to DSBs, DNA damage responses are activated to promote cell cycle arrest, cell apoptosis or DSB repair by homologous recombination (HR) /non-homologous end joining (NHEJ)/alternative NHEJ (alt-NHEJ). The figure was modified from a figure in the reference [35].

A large number of antitumor drugs targeting DNA have been developed and clinically used in the treatment of cancers. Based on the mechanism of action, DNA-damaging agents can be classified into three categories including DNA-reactive agents, antimetabolites and topoisomerase poisons (Table 1). DNA-reactive agents represent a class of antitumor compounds that directly react with DNA leading to modified bases or cross-linking of DNA. For example, DNA alkylating agents include the nitrosoureas family of compounds (nimustine, carmustine and lomustine) and triazene compounds (dacarbazine, procarbazine and temozolomide) [47]. These drugs directly alkylate DNA causing replication fork collapse and consequently killing cancer cells. Another example of DNA reactive agents is cisplatin, a
platinum-containing drug, which forms an adduct in DNA inducing the formation of intrastrand crosslinks [48]. Cisplatin has been successfully used to treat various types of cancers such as testicular, bladder, cervical, ovarian, lymphomas, etc [49]. Another class of DNA-targeted drugs is antimetabolites that mimic normal cellular metabolites interfering with DNA synthesis and replication and leading to cell apoptosis. A number of nucleobase analogs such as 5-fluorouracil (5-FU) are widely used as antimetabolite anticancer drugs. The cytotoxicity of 5-FU results from its misincorporation in DNA as well as inhibition of thymidylate synthase [50]. In addition, other antimetabolites such as pemetraxed, methotrexate and aminopterin can inhibit enzymes involved in DNA synthesis to prevent cancer cell growth [51, 52]. The third class of DNA-damaging agents is topoisomerase poisons. Topoisomerases including topoisomerase I and II are enzymes responsible for overwinding or unwinding the supercoiled structure of DNA, and they are essential for DNA replication and transcription. Etoposide, an inhibitor of topoisomerase II, works as a cytotoxic antitumor drug by targeting DNA-topoisomerase complex, inhibiting religation of strand breaks and resulting DSBs [53, 54].

Table 1 DNA-damaging agents used in the chemotherapy.

<table>
<thead>
<tr>
<th>Category</th>
<th>DNA-damaging agents</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-reactive agents</td>
<td>Alkylating agents: Nimustine, Carmustine, Lomustine, Dacarbazine, Procarbazine and Temozolomide.</td>
<td>Alkylation of bases Crosslinks</td>
</tr>
<tr>
<td></td>
<td>Platinum-based agents: Cisplatin, Carboplatin, and Picoplatin</td>
<td>Crosslinks</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>5-Fluorouracil, Pemetraxed and Methotrexate</td>
<td>Inhibition DNA synthesis</td>
</tr>
<tr>
<td>Topoisomerase poisons</td>
<td>Etoposide, Doxorubicin, Daunorubicin, Camptothecin and Mitoxantrone.</td>
<td>Topoisomerase poison</td>
</tr>
</tbody>
</table>
One major limitation of the use of DNA-damaging agents in cancer chemotherapy is drug resistance. The development of resistance is the primary cause of failure of chemotherapies. Tumors acquire resistance to drugs in a number of ways. In some cases, drug resistance results from decreasing drug uptake or increasing drug efflux. In many cases, tumors become resistant to DNA damaging agents by overexpressing proteins in DNA repair pathways and increasing tolerance to DNA damage. Therefore, targeting DNA repair pathway is a potential and rational strategy to overcome resistance and enhance antitumor efficacy of DNA-damaging agents.

1.2.2 BER inhibitors in combination chemotherapy

Most cancer cells have defects in either DNA or DNA repair pathway [55]. DNA damage is mostly processed through multiple repair pathways. In cancer cells, deficiency in one repair pathway is compensated by an alternative pathway. Disrupting DNA repair may block backup repair in cancer cells and lead to cell death [56]. But normal cells will be intact because they have proficient compensatory repair system (Figure 6). Therefore, interrupting DNA repair pathway is a promising strategy to kill cancer. The purpose of use of DNA-damaging agents in cancer chemotherapy is to increase the level of DNA lesions and overload DNA repair capacity. The majority of damaged DNA induced by DNA-damaging agents is repaired through base excision repair pathway, which is essential for both normal and cancer cells.
Figure 6 DNA repair in normal and cancer cells. Normal cells process DNA damage by multiple pathways (A and B), while cancer cells defective in pathway A repair DNA by compensatory pathway B. When the pathway B is inhibited, both pathways are defective in cancer cells. This would cause cell death. However, normal cells can survive because the pathway A can compensate the inhibition of pathway B. The figure was modified from figures in the references [35, 57].

Tumor's sensitivity to DNA-damaging agents is affected by BER imbalances as a consequence of alterations in the activity or expression level of BER enzymes. Mice deficient in alkyladenine-DNA glycosylase (AAG) and mice with overexpressed AAG have been used to study the consequence of an imbalance of BER initiation step [58, 59]. AAG excises damaged bases formed by alkylating agents and initiates BER, resulting in the accumulation of toxic intermediates AP sites. Overexpression of AAG has been reported to sensitize mice to MMS treatment. And Aag^{−/−} mice showed remarkable resistance in response to alkylating agents, compared to wild-type mice [60]. However, inhibition of BER initiation does not always exhibit protection against all alkylating agents [61, 62]. In addition, imbalance of downstream BER can
also alter the sensitivity of cells to DNA-damaging agents. Kelley group found the elevated expression of APE1 in human osteosarcoma samples conferred resistance to antitumor treatments including DNA alkylating agent chemotherapy and ionizing radiation, while osteosarcoma cell lines treated with APE1-siRNA exhibited sensitivity to DNA-damaging agents due to decreased level of APE1 proteins [63]. Moreover, it was reported that cells defective in polymerase β showed increased sensitivity to DNA alkylating agents [64]. Altogether, these results indicate that imbalance of BER is essential for the sensitivity and resistance to therapeutic damaging agents. Therefore, blocking BER downstream pathways provides a potential approach to enhance the cytotoxic effect of DNA-damaging agents in cancer treatment and conquer drug resistance. There has been growing interest in the development of small molecules inhibitors targeting BER pathway. Many BER inhibitors are in development or on the market.

APE1 is the major AP endonuclease in human, which incises DNA backbone at an AP site generated by DNA glycosylase and produces a single-strand break that is further processed by downstream enzymes. Many small molecules have been identified to inhibit APE1 activity. Some of them have shown the ability to increase cell killing by DNA alkylating agents in melanoma and glioma cancer cells [65]. However, they are still in the preclinical stage [52, 63, 66, 67]. APE1 is a multiple functional enzyme. In addition to DNA repair function, APE1 also has the redox function to regulate transcription of genes. E3330 is a specific small inhibitor for APE1 redox function, which has been used for the treatment of many cancers at the stage of clinical trials [40, 68]. However, it does not affect DNA repair function of APE1.
Pol β has two functions including lyase and polymerase activities to process the remaining breaks with the 5’dRP terminus created by APE1. As the step of removing 5’dRP residue by Pol β is rate limiting in BER pathway, Pol β is an attractive candidate for inhibition of BER. Moreover, it has been reported that overexpression of Pol β is the cause of resistance to irradiation (IR) treatment and DNA-damaging agents therapies in various cancers [35, 69]. A lot of natural compounds such as oleanolic acid, edgeworin, harbinatic acid and stigmasterol were observed to inhibit the activity of Pol β in biochemical assays [41, 70]. Unfortunately, they showed little effect on cytotoxicity of DNA-damaging drugs. Jaiswal group identified two small-molecule inhibitors of Pol β, NSC666715 and NCS124854, both of which are able to greatly enhance the cytotoxic effect of temozolomide in vitro and in vivo at a low concentration [42, 71]. Additionally, in combination with temozolomide, the two compounds promote killing of MMR-deficient cancer cells that are resistant to alkylating treatment [66]. However, additional in vivo studies are required before the NCS compounds move to clinical trials.

DNA ligase is essential for DNA repair and replication. In human, DNA ligase I and III are responsible for linking of 3’-hydroxyl and 5’-phosphate terminus at DNA nick to accomplish base excision repair. Small molecules were identified to inhibit DNA ligases based on the crystal structures [72]. For example, compounds 67 and 189 were observed to enhance drastically the cytotoxicity of methyl methanesulfonate (MMS) in breast tumor cells which showed a higher level of expression of DNA ligase I than normal tissues [73].

1.2.3 Methoxyamine as a BER inhibitor in combination therapy

The inhibitors discussed in section 1.2.2 are targeting enzymes involved in BER pathway. Another approach to inhibiting BER pathway is to modify AP sites chemically by a small
alkoxyamine, MX, as AP sites are important intermediates in BER pathway. MX has worked as a BER inhibitor for many years since Liuzzi et al. first utilized MX as a novel tool to investigate the enzymatic reactions in BER pathway in 1985 [74]. The mechanism of disruption of BER by MX has been documented. It has been found that MX binds to AP sites faster than APE1 [75]. The MX-bound AP site substrate could be not recognized by APE1 or be refractory to the cleavage activity of APE1; thereby it protects AP sites against the cleavage of APE1 [8]. However, Wilson group reported that MX-bound AP sites could be excised by APE1 with a much lower turnover number than intact AP sites. Instead, MX was shown to block the lyase activity of Pol β and interrupt Pol β-dependent short-patch BER [76]. They found that MX-bound AP sites can be efficiently repaired by long-patch BER rather than NER pathway indicated in previous reports [77]. It is still not clear whether MX-AP adducts can be repaired and how cells process these adducts.

MX has been used as a part of combination treatment to enhance the cytotoxic effect of DNA-damaging agents in a variety of cancers. It reacts with AP sites induced by DNA-damaging agents such as alkylating agents to form MX-AP adducts, which escape repair and stall replication fork resulting in cell death (Figure 7). Gerson group first explored the use of MX in antitumor treatment. They found that MX increased the therapeutic efficacy of DNA alkylating agents, TMZ and BCNU, in colon cancer cell lines as well as human colon tumor mouse xenograft [43, 78]. Colon cancer cells with mismatch repair deficiency are resistant to TMZ treatment, but MX sensitizes them to TMZ treatment. Moreover, this group showed that MX coupling with other DNA-damaging agents is also able to improve their antitumor efficacy. For example, MX greatly potentiates the cytotoxicity of pemetrexed in some solid tumors [79]. And
the combination of MX with fludarabine improves chemotherapeutic efficacy in chronic lymphocytic leukemia [80]. Targeting AP sites by MX as a strategy to enhance antitumor efficacy of chemotherapy are also attractive to other investigators. Several groups demonstrated that combing MX with TMZ treatment can overcome the resistance of glioma cell lines to TMZ treatment [81]. Clinical trial studies of using MX (TRC102) in combination chemotherapy are underway or have been completed. The completed phase I clinical trial is to evaluate the combination treatment of TRC102 with pemetrexed in lung tumor and other solid tumors, and phase 1 studies of TRC102 coupling with TMZ and fludarabine are ongoing.

**Figure 7 MX enhances cell killing by DNA damaging agents.** DNA damaging agents such as temozolomide (TMZ) and pemetrexed introduce DNA lesions, which cause replication fork collapse and cell death if they are unrepaired. However, if cancer cells remove lesions by BER pathway, cells can survive and display resistance to these agents. MX has the ability to block BER pathway by binding to AP sites and inhibiting APE activity. Coupling MX with DNA damaging agents overcomes resistance and increases cell death.
MX targets not only BER pathway but also inhibits topoisomerasers. Topoisomerasers are essential for the processes of DNA replication, transcription and chromosome segregation. They regulate the topology of DNA by cutting one or two strands of a DNA-double helix and then reannealing strand breaks. Based on the number of strand cut in DNA, topoisomerasers are divided into type I (top I) and II (top II). Interfering with topoisomerasers is considered as one effective strategy for cancer therapy. Many topoisomerase inhibitors have been developed into antitumor therapeutic agents such as etoposide, camptothecin, mitoxantrone and doxorubicin, because they increase the level of DNA breaks associated with topoisomerasers and lead to accumulation of DNA damage and chromosome aberrations [82]. Many evidences have shown that AP sites are topoisomerase poisons and are able to induce cell death. When AP sites are located at the cleavage site of topoisomerasers, the DNA lesions prevent religation of transient DNA breaks mediated by topoisomerasers, increase enzyme-DNA cleavage complex and convert transient double-strand breaks into permanent DNA breaks through DNA replication process [83-85]. Under normal conditions, deleterious AP sites can be rapidly repaired through BER and do not poison topoisomerasers. If AP sites are unrepaired, the persistence of AP sites will result in topoisomerase-mediated cytotoxicity (Figure 8). Since MX blocks the repair of AP sites, it would indirectly poison topoisomerase. In vitro studies indicated that MX-bound AP sites considerably elevated topoisomerase II-mediated cleavage. Furthermore, the combination treatment of TMZ and MX increased top II expression as well as DNA double-strand breaks in colon cancer cells. And the result of the collocation of top II protein and γ-H2AX suggested that the accumulation of DNA breaks is associated with induction of top II expression after TMZ and
MX treatment [8, 43]. These findings demonstrate that MX-AP sites are capable to poison topoisomerase stimulating topoisomerase-mediated cytotoxicity.

![Diagram](image)

**Figure 8 Model for topoisomerase poison by AP site intermediates of BER.** Topoisomerase inhibitors such as etoposide work as antitumor drugs and kill cells by promoting accumulation of DNA breaks mediated by topoisomerase. AP sites arising from the repair of damaged DNA by BER can also act as topoisomerase poison, by preventing ligation of topoisomerase-associated transient DNA breaks and leading to the permanent existence of DNA breaks. The figure was modified from a figure in the reference [83].

### 1.3 B-cell lymphomas and abasic sites

B-cell non-Hodgkin lymphomas (B-NHL) are the sixth most common type of cancers, which have various subtypes. The majority of B-NHL originate from germinal centers, in which a DNA mutator enzyme, activation-induced deaminase (AID) initiates the processes of somatic hypermutation (SHM) and class switch recombination (CSR) for antibody maturation, by deaminating cytosines into uracils in immunoglobulin (Ig) loci [86-88]. AID also involves in B cell
lymphomagenesis and constitutively expresses in many B-cell lymphomas [89-91]. Recently, two papers successively demonstrated that the constitutive expression of AID in B-cell lymphomas leads to the accumulation of uracils in their genomes [90, 92] and the high level of AID expression positively correlates with an excess of genomic uracils. In BER pathway, uracil-DNA glycosylases remove uacils forming AP sites. The high level of uracils in B-cell lymphoma cells must lead to high level of formation of AP sites intermediates. An imbalance in the downstream of BER repair enzymes may cause the AP sites to persist. Therefore, it is highly possible that these B-cell lymphoma cells with an excess of uracils would have the high level of AP sites intermediates accumulation in their genomes.

1.3.1 Antibody maturation

Innate immunity and adaptive immunity constitute the human immune system, which is responsible for protecting the body from infection. Innate immunity works as a first defense line and eliminates foreign organisms through physical barriers, chemicals and innate immune cells. But it lacks specificity and long-term protection. Adaptive immunity also called acquired immunity is mediated by antibodies and cell surface receptors. Unlike innate immunity, the adaptive immune system is in response to a specific pathogen and creates memory cells to protect the body from infection by the same pathogen for a lifetime. Also, it recruits antibodies made by B lymphocytes to recognize and combat particular foreign objects. Antibodies encoded by immunoglobulin gene are glycoproteins with a heterodimer consisting of two heavy chains and two light chains. The variable domains are responsible for binding specific antigens, while the remaining part composed of constant domains determines antibody isotypes. A human without antigen stimulation can produce more than $10^{12}$ antibodies allowing binding to
different antigens, although the total number of genes in a human genome is fewer than 50,000 [86]. After stimulation by antigens, the immune system can create antibodies with higher affinity to antigens. The question is how the immune system makes a vast number of antibodies with a limited number of genes.

The amazing diversity of antibodies results from the V (D) J recombination, SHM and CSR processes. During V (D) J recombination, variable (V), diversity (D) and joining (J) segments in Ig genes are rearranged to create a unique combination during early B cell development [93]. When B cells are exposed to antigens, further expansion and maturation of antibodies are generated through the mechanisms of SHM and CSR. SHM is a process of introducing point mutations in V (D) J segment of Ig genes to increase antibody affinity to the particular foreign antigen. The rate of SHM is almost $10^6$ fold higher than the normal mutation rate (about $10^{-9}$ per base pair) in the genome, but this mutational process is restricted to V (D) J region [87]. B cells with somatic mutations that produce antibodies with better affinity to antigens are positively selected for further cell expansion, while mutated B cells secreting antibodies that show decreased affinity are eliminated through cell apoptosis [94]. CSR is a genetic rearrangement of switch regions of constant segments in heavy chains to change antibody isotypes and improve effector function of antibodies. This recombination is achieved by creating double-strand breaks in two different S regions and joining DNA ends together. The first antibody isotype (IgM) can be switched to different isotypes (IgA, IgG, IgD and IgE) through this exchange of constant segments [32] (Figure 9).
24

Figure 9 Molecular mechanism of antibody maturation. Different rearrangements of V, D, J segments located in the upstream of immunoglobulin gene allow B cells generating an enormous primary antibody repertoire. After exposure to antigens, AID introduces mutations in recombined VDJ region to improve the affinity of antibodies, and this process is called somatic hypermutation (SHM). Red sticks within the variable region indicate point mutations. During class switch recombination (CSR), the AID-mediated mutations cause DNA double-strand breaks in two different switch regions (S) of constant gene segments, and the two switch regions join together releasing intervening DNA fragment as a circle. CSR process is essential for switching the antibody from IgM to another isotype. The figure was modified from a figure in the reference [95].

1.3.2 Activation-induced deaminase (AID)

Antibody maturation requires an enzyme called activation-induced deaminase (AID) [96], which is primarily expressed in germinal center B cells [97]. AID is a DNA-cytosine deminase [98], directly targeting single-stranded DNA and converting cytosines into uracils to initiate SHM and CSR processes [99](Figure 10). Post V (D) J recombination, AID-mediated deamination in DNA introduces point mutations at high frequency in the V (D) J segment during SHM. However, the
conversion of C: G pairs into U:G pairs by AID is just the initiative step of SHM. To acquire different types of mutations in Ig locus, SHM process also includes the downstream cellular pathway of processing uracils in DNA.

![Figure 10 Deamination of cytosines into uracils by AID.](image)

AID-mediated deamination is not the only pathway to create uracils in the genome. Uracils can also be introduced through spontaneous water-mediated deamination of cytosine and misincorporation of dUMP during DNA replication [100]. Cells remove uracils in the genome by uracil-DNA glycosylases through BER pathway. Mammalian cells have four different types of uracil-DNA glycosylases including uracil-N glycosylase (UNG), single-strand-specific monofunctional uracil (SMUG1), methyl-binding domain glycosylase 4 (MBD4) and thymine DNA glycosylase(TDG) [101]. UNG plays a major role in removal and repair of genomic uracils, and SUMG1 often works as the backup system [102]. The U: G mismatches mediated by AID are processed in several different pathways (Figure 11). Through BER, uracil-DNA glycosylases excise uracils restoring C: G pairs. If uracils are unrepaired, through simple DNA replication, U: G mispairs lead to C/G to T/A transition mutations [90]. That is because replicative DNA
polymerases treat U as T and inserts A across from U template [98]. Besides transition mutations, transversion mutations are also found in Ig locus. Hence, DNA replication alone cannot contribute to all types of hypermutations on Ig genes of activated B cells. And there must be other mechanisms leading to SHM. In error-free BER pathway, UNG removes uracils generating AP sites, which are further processed by the downstream enzymes APE1, Pol β and ligase to maintain genome integrity. Alternatively, if the resulting AP sites are not repaired, DNA replicative polymerases cannot copy them. But they could be bypassed by TLS polymerases, which could insert any of nucleotides across from AP site template and introduce either transition or transversion mutations at C: G pairs during SHM [103]. It was shown that the frequency of mutations in Ig genes was significantly reduced in activated B cells from a mouse with deficiency of Pol θ [104]. Other TLS polymerases such as Pol η and Rev1 may also be involved in SHM of Ig gene [105, 106].

Sequence analysis of SHM has showed that mutations in Ig genes occur not only at C: G pairs, but also at A: T pairs. Through DNA replication and error-prone TLS processes, the mutations are only restricted to C: G pairs not A: T pairs. A number of evidence showed that mismatch repair pathway is implicated in SHM to introduce mutations at A: T pairs. Although replicative polymerases have the ability of proofreading, some replication errors still escape correction. Cells have developed a conserved repair pathway, MMR to remove incorrect base pairs [107, 108]. In human cells, MMR pathway starts with the recognition and binding of mismatches by a dimer of MSH2 and MSH6, followed by the excision of the incorrect base and its surrounding nucleotides by the exonuclease (Exo1). The resulting strand gap generated by Exo1 is then repaired by TLS polymerase and ligase [108]. Several reports showed that the total
frequency of SHM reduced either in MSH2−/− mice or MSH6−/−mice [69, 109, 110]. More interestingly, in the MMR defective mice, the frequency of hypermutations at C: G pairs increased while the mutations at A: T pairs decreased [111, 112]. These data suggest that MMR pathway is involved in SHM to introduce mutations at A: T pairs.

Figure 11 Mechanism of somatic hypermutation initiated by AID. AID converts C to U in VDJ region. The U: G mispairs can be processed in four different ways. Uracils are excised by uracil DNA glycosylase (UNG), forming AP sites. Through BER, remaining AP sites are further processed by AP endonuclease (APE) and other downstream BER proteins to restore C: G pair. Alternatively, these AP sites can be bypassed by translesion synthesis (TLS) polymerase, leading to transition and transversion mutations at C: G pairs. If uracils are not removed, A is inserted across from U during replication, resulting in C to T transition mutation. Also, the U: G mispairs can be processed through mismatch repair (MMR), introducing either transition or transversion mutations at A: T or C: G pair. The figure was modified from figures the references [95, 113].
During CSR, AID targets the Sµ region and one of the downstream S regions and introduces uracils in two switch regions [114, 115]. UNG is recruited to remove these uracils generating AP sites in the S regions. The resulting AP sites are processed by APE forming single-strand breaks [32]. AID can attack both strands of DNA in a close proximity and create DSBs. The DSBs in two S regions generated by AID are joined through NHEJ eliminating the intervening sequence as a circle [32]. For examples, in a class switch to Ig G, AID deaminates cytosines into uracils in Sµ region and Sγ region and induces the formation of DSBs in the two switch regions. The DSBs are processed by NHEJ to accomplish CSR. A series of reports suggest that MMR also plays a role in the formation of DSBs during CSR.

1.3.3 Germinal center reaction and B-cell lymphoma

Germinal centers (GCs) are structures in secondary lymph nodes which are formed after the exposure of naïve B cells to antigens [116]. GCs are involved in the maturation of B cells that generate antibodies with high affinity to antigens. The histological structure of GCs consists of the dark zone and light zone. In the dark zone, B cells are activated after exposure to antigen and undergo proliferation and SHM, while in the light zone antigen-selected B cells undergo CSR [117, 118]. The GC B cells producing antibodies with low affinity against antigen will be eliminated through apoptosis. But the mature B cells with high-affinity antibodies will differentiate into plasma B cells that secret antibodies against infection, as well as memory B cells for protecting the body against the same antigen in the future. In GCs, AID is upregulated to induce mutations and DSBs in Ig genes and promote SHM and CSR processes. GC reaction is essential for the immune system, but may also be implicated in the B cell lymphomagenesis [119].
Gene expression profiles reveal that Burkitt lymphomas are derived from dark zones of GCs, while follicular lymphomas and diffuse large B-cell lymphomas (DLBCL) originate from light zones [120] (Figure 12). The chromosomal translocations between Ig gene and proto-oncogenes are a hallmark of GC-derived B-cell lymphomas [121]. These translocations may lead to dysregulation of oncogenes promoting lymphomagenesis. Antibody maturation events including V (D) J recombination, SHM and CSR are associated with such translocations [122-125]. For example, errors in V (D) J recombination mediate translocations of IgH locus with BCL2 locus found in follicular lymphoma [88, 126]. During CSR, DSBs breaks are not only generated in Ig switch regions, but also in Myc locus. This aberrant CSR promotes translocations of Ig gene with Myc gene resulting in Burkitt lymphoma [127]. Also, translocations can arise from mistakes in SHM process that lead to off targeting of non-Ig genes such as BCL-6. Many studies confirm that dysregulation of BCL6 is involved in the pathogenesis of DLBCL [128, 129].
**Figure 12 Cellular origin of GC-derived B-cell lymphomas.** After naïve B cells are exposed to an antigen, these cells enter the germinal center, where AID-mediated SHM and CSR take place and mature B cells with high affinity to the antigen are formed and differentiated into memory B cells and plasma B cells. AID-dependent aberrant SHM and CSR may lead to chromosomal translocations and lymphomagenesis. Burkitt lymphoma originates from dark zone of GC whereas follicular lymphoma and diffuse large B-cell lymphoma (DLBCL) originate from the light zone of GC. The figure was adapted from figures in the references [88, 119].

1.3.4 AID and GC-derived B-cell lymphoma

Since AID mediates SHM and CSR processes, AID must be associated with chromosomal instability and malignant transformation of B cells in GCs. Much evidence shows that AID plays an essential role in B-cell lymphomagenesis. Sequencing data revealed that AID could act on genes outside of Ig locus. AID-mediated deamination is found in many genes associated with B-cell lymphomagenesis such as Myc, Pax5, Pim1, Ebf1 and BCL6. As a consequence, AID could induce mutations or DNA double-strand breaks in off-target genes, promoting chromosomal translocations between Ig gene and oncogenes. Ramiro et al. showed that translocations of IgH with c-myc were observed in AID-proficient mice but not detected in AID-deficient mice. This result suggests that AID expression is necessary for translocations of IgH/c-myc. Furthermore, the overexpression of AID promotes the development of B-cell lymphoma and leukemia in a bone marrow transplantation mouse model [130]. However, it is not clear how AID targets non-Ig genes and contributes to B-cell lymphomagenesis.

AID expression is strictly regulated in normal B lymphocytes. However, aberrant expression and off-targeting activity of AID in B cells may induce tumorigenesis. Cytokines secreted from immune cells such as IL-4 and TGF-β, increase AID expression in B cells. HIV-associated Burkitt lymphomas have the high level of AID expression [131]. Also, expression of AID elevated by Epstein-Barr virus (EBV) was shown to be associated with the development of
Burkitt lymphomas [132]. Many studies demonstrated that there is a strong correlation between B-cell cancers and aberrant expression of AID. A lot of B-NHL arise from germinal center showed constitutive expression of AID [89-91]. Recently, Bhagwat group examined AID expression in cancer cell lines derived from germinal centers including Burkitt lymphoma, follicular lymphoma and diffuse large B-cell lymphoma, as well as cell lines not derived from germinal centers including multiple myelomas (MM), T-cell lymphomas and classical Hodgkin’s lymphoma (CHL). AID expression level was determined by RT-PCR and AID enzymatic activity assay. It was observed that GC-derived B-NHL cell lines express AID at a high level. Furthermore, AID was also overexpressed in FL patient tumor samples as well as DLBCL patient tumor samples, whereas AID was expressed at a low level in marginal zone lymphoma (MZL) patient samples whose origin is the post-germinal center.

1.3.5 Genomic uracils in B-cell lymphoma

Recent studies on sequencing of a variety of cancer genomes revealed that the C-T transitions are the most common mutations in human tumors. This suggests that deamination of cytosines into uracils mediated by enzymes could be a potential cause of tumorigenesis. Since AID is one of major enzymes converting cytosines into uracils and its expression is correlated with B-cell cancers, AID could be a potential endogenous source of mutagenesis and contribute to the mutational signature in B-cell cancers. Therefore, studies of genomic uracil load would help understanding how AID promotes the development of cancers. Bhagwat and Krokan groups successively demonstrated that the constitutive expression of AID leads to accumulation of uracils in the genome of B-cell lymphomas, although they quantified the level of genomic uracils with different techniques. Compared to normal human B cells and non-lymphoma
cancer cell lines, B-NHL cell lines with higher levels of AID expression show higher levels of uracils in their genome. Human B-NHL cell line, Ramos-7 overexpressing AID displayed largely increased uracils in their genome, while knockdown of AID decreased genomic uracil load. Mouse B cell lymphoma CH12F3 cells transfected with AID-EYFP fusion gene showed a much higher level of uracils than control cells with EYFP gene following stimulation. These results indicate a positive correlation between AID expression and uracil load.

AID introduces uracils in B-cell genome. WT mouse B cells and human B cells with ex vivo stimulation showed an increase in AID expression correlating with a similar increase in UNG2 expression. Therefore, no significant accumulation of genomic uracils was observed in these cells, due to the perfect balance between the creation of uracils by AID and excision of uracils by UNG2. In contrast, in UNG2−/− mice, the genomic uracil load substantially increased following stimulation. Other uracil-DNA glycosylases including SMUG1, TDG and MBD4 are less responsible for excision of uracils produced by AID. These observations indicate that UNG2 primarily contributes to the removal of uracils and maintenance of the balance of genomic uracils introduced by AID. There is an inverse connection between UNG2 expression and uracil level.

In normal primary B cells, elevated AID expression does not lead to genomic uracil accumulation due to simultaneously increased UNG2 repair capacity. However, mouse B-cell lymphoma cell line CH12F3 with stimulation showed uracil accumulation in their genome, although the expression and enzymatic activity of AID and UNG2 increased in a similar pattern to those in WT splenocytes. Moreover, human B-NHL cell lines expressing the high level of AID showed an increase in either UNG2 and SMUG1 gene expression or uracil excision activity of
the nuclear extract. However, compared to normal B cells, genomic uracil levels in these cells are still much higher, despite of elevated capacity of uracil removal. When UGI, a UNG enzyme inhibitor, was added in the enzymatic reaction of uracil excision, the ability of nuclear extract to excise uracil was almost abolished. This suggested that UNG2 was the enzyme responsible for removing uracils in these cells. A good balance between uracil generation and removal results in low level of uracil accumulation in the genome of normal B cells. However, B-cell lymphoma cells seem to lose this balance and lead to accumulation of uracils. This indicates that the function of UNG2 is not effective enough to excise uracils in B-cell cancer cells, although there is an increase in its expression and uracil elimination activity of the nuclear extract. A number of studies demonstrated that UNG2 has to be recruited by some protein factors when it acts on gene locations where AID introduces uracils. It has been suggested that Rev1 protein could play such role in the recruitment of UNG2. Additionally, UNG2 action could be affected by chromatin structure; thereby chromatin modifiers may also be involved in the recruitment process. Downregulation of these factors could be the reason for the ineffectiveness of UNG2 function in these B-cell cancers. Furthermore, Burkitt lymphoma cell line, Raji cells expressed AID at the high level. But the number of uracils did not significantly change over several passages, suggesting that B cell lymphoma cells may keep a new steady-state level of uracils [92].

1.3.6 AP sites in B cell lymphomas

In BER pathway, damaged bases are recognized and excised by a specific DNA glycosylase, resulting in the generation of AP sites which are processed by AP endonuclease. UNG is the major uracil-DNA glycosylase removing genomic uracils which are misincorporated into DNA or introduced by enzymes or drugs. Pemetrexed, a folate antimetabolite, works as a
chemotherapy drug for the treatment of non small cell lung cancer (NSCLC) by increasing uracil misincorporation in DNA and resulting in genomic instability. Uracils arising from pemetrexed treatment are removed by UNG enzyme. The UNG proficient cells displayed increasing number of AP sites with increased pemetrexed treatment. However, the level of AP sites always stayed a low level in the UNG defective cells treated with different concentration of pemetrexed [79]. Therefore, AP sites levels in cells treated with pemetrexed depend on UNG expression.

UNG plays an essential role in the processes of either SHM or CSR, by processing U:G mispairs induced by AID. In UNG proficient mice, mutations at C: G pairs of Ig loci include 65% transition mutations and other random mutations, while the UNG-deficient mice showed 95% mutations at C: G are transitions. The dramatic shift to transition mutations suggested that the deficiency of UNG inhibits the formation of AP sites which are further copied by TLS polymerase causing random mutation at C: G pairs. Moreover, in the absence of UNG, the spectrum of Ig isotypes from serum was altered, and CSR was greatly inhibited [133]. This could be explained by the fact that CSR is also via the generation of AP sites by UNG. Therefore, AP sites resulting from the excision of uracils by UNG are crucial intermediates for the processes of antibody diversifications. The absence of UNG prevents the development of GC-derived B cell lymphomas in a murine model, suggesting UNG might play an active role in the lymphomagenesis by promoting the formation of deleterious AP sites and DNA strand breaks intermediates [134]. This also indicates that the disruption of downstream BER may drive the accumulation of UNG-mediated DNA lesions and contribute to the pathogenesis of B-cell lymphoma. Therefore, B-cell lymphoma cells with an excess of uracils would be expected to contain the high level of AP sites accumulation in their genomes.
1.3.7 B-cell lymphoma therapy

The treatment for B-cell lymphomas includes chemotherapy and radiation or their combination, depending on the type and the stage of B-cell lymphomas. DLBCL is the most common type of B-NHL, and it is a very aggressive type of cancer. In most cases, DLBCL is treated with R-CHOP, which refers as combination chemotherapy of a monoclonal antibody (rituximab) and four chemotherapeutic drugs (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone) (Figure 13). Follicular lymphoma is a slow-growing type of lymphoma, but it is difficult to cure completely. In the early stage, follicular lymphoma responds very well to radiation therapy. In the advanced stage, the most widely used treatment is chemotherapy including R-CHOP and R-CVP (rituximab, cyclophosphamide, vincristine and prednisone). Burkitt lymphoma usually grows very quickly and can spread to brain and spinal cord. This type of lymphoma has to be treated with intensive chemotherapy that combines at least 4 chemotherapy drugs with rituximab or radiation therapy.
Figure 13 Structures of CHOP chemotherapy (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone).

1.4 Scope and significance

As AP sites are the most prevalent types of lesions in DNA and many DNA-damaging agents can directly or indirectly induce the formation of AP sites, it is of great interest to detect and quantify AP sites. The measurement of AP sites will be very helpful to evaluate the effect of DNA-targeted chemotherapeutic agents and thereby improve therapies. Although a lot of different techniques have been used to label, identify and quantify AP sites, these techniques suffer from drawbacks such as cumbersome protocols, low sensitivity, high background, use of radioisotopes, incompatibility with a clinical setting, use of a large amount of DNA, etc. Thus, there is a critical need to develop a more versatile technique for labeling and detecting AP sites with high sensitivity. One goal of this project is to develop a novel and sensitive tool for
quantification of AP sites in cellular DNA. To achieve this aim, a small synthetic alkoxyamine with a terminal alkyne moiety was developed as a new tool for AP sites quantification in this work. This alkoxyamine allows reacting with AP sites and linking with biochemical tags through click chemistry for detection and quantification. In addition to quantification of AP sites in vitro, this chemical is also used to label AP sites in vivo and allows directly detecting AP sites induced in situ by DNA-damaging agents.

Another use of alkoxyamines is in combination chemotherapy. Alkylating agents such as TMZ are against cancer cells by methylating DNA bases and DNA backbone. However, cells can excise the products of TMZ treatment such as 7-methylguanine and 3-methyladenine using DNA glycosylases, repair resulting AP sites and suppress the effects of TMZ. Drug resistance is one big obstacle for chemotherapy. The alkoxyamine that has been used in combination chemotherapy to overcome the drug resistance is MX. MX potentiates the cytotoxic effects of TMZ by reacting with the AP sites formed through the action of DNA glycosylases and blocking the cleavage activity of AP endonuclease. The second goal of the project is to develop other alkoxyamines as BER inhibitors to enhance anticancer efficacy of chemotherapy. This work may provide new drug candidates to overcome resistance to chemotherapeutic agents and enhance antitumor efficacy.

B-NHL cell lines and patient tumors derived from germinal center exhibit constitutive expression of AID. Moreover, the high level of AID expression has been demonstrated to coordinate with the high level of uracil accumulation in the genome. However, DNA repair is also active, and cells attempt to remove uracils by uracil-DNA glycosylase and create AP sites intermediates. Therefore, the excess of uracils in these B-cell lymphomas may result in AP sites
accumulation if there is an imbalance in the downstream of BER repair enzymes. In combination chemotherapy of MX with an alkylating agent, the use of alkylating agents is aiming to promote genome instability and to create AP sites that are targeted by MX. The high levels of endogenous AP sites in B-cell lymphoma could also be the target of MX or other alkoxyamines. Therefore, alkoxyamines alone could be used to block the repair of an excess of AP sites in B cell cancers and cause cell death. The last goal of this project is to develop alkoxyamines as antitumor drugs for selectively killing B-cell lymphomas. Alkoxyamines could be potential drugs for the specific treatment of B-cell cancers.

1.4.1 Rationale

The rationale of this project is that its successful completion will provide a new versatile tool to label and quantify AP sites in cellular DNA in multi-tier plate format without the use of proteins. The novel technique of AP site labeling will be useful to study genomic instability caused by endogenous or exogenous agents. In addition, the inhibitory effect of alkoxyamines on base excision repair could be the basis of their use in combination with DNA-damaging agents for anti-cancer treatment. This work will strengthen the chemotherapeutic efficacy of killing cancers. Finally, this project will provide novel antitumor candidates for selectively treating B-cell cancers that have the high level of deleterious AP sites in the genome.

1.5 Specific aims of research

Aim 1) A novel chemical is developed as a versatile tool for labeling and quantifying AP sites in cellular DNA with high sensitivity.

Both MX and ARP are well-known alkoxyamines that covalently link with AP sites. While MX does not allow tagging of AP sites, ARP is bulky, contains biotin as the only tag and requires
proteins and enzymes for its use. To create a more versatile tool for labeling AP sites, I designed small alkoxyamines with a terminal alkyne (Figure 14). This class of chemicals can tag AP sites through the reaction between the aldehyde residue of AP sites and alkoxyamines. Also, the terminal alkyne functional group allows linking a variety of biochemical tags through click chemistry for visualization and detection. Classic click chemistry is a chemistry of copper-catalyzed azide-alkyne cycloaddition, which is a well-established bioorthogonal reaction that creates stable triazoles and has been used to label sugars, proteins, DNA and other biomolecules both in vitro and in situ [135-137]. The reactivity of these chemicals with AP sites is determined using synthetic DNA oligomers containing a single AP site. Moreover, they can be developed into a new method for detection and quantification of AP sites in cellular DNA. Additionally, they can be used to label AP sites in vivo and visualize AP sites locus in cells. The smallest alkoxyamine with a terminal alkyne, AA3, is first synthesized and used.

Figure 14 Labeling AP sites with small alkoxyamines. (A) The general structure of alkoxyamines(AA) with a terminal alkyne. (B) Use of click chemistry to label AA-adducted DNA

\[
\text{H}_2\text{N}^-\text{O}^-\text{R}^-\equiv
\]

\[
\text{HCl} \cdot \text{H}_2\text{N}^+\text{O}^-\equiv
\]

\[
\text{Click reaction (CuBr/TBTA)}
\]

\[
\text{Biotin / Fluorescent-AP site}
\]
( ★ can be biotin, a fluorophore or any other molecule). (C) The structure of O-2-Propynylhydroxylamine hydrochloride, AA3.

Aim 2) Inhibitory effect of AA3 on AP endonuclease is examined, and AA3 is applied in combination chemotherapy to increase antitumor efficacy of DNA-damaging agents.

MX is known as a BER inhibitor. The reaction of MX with AP sites has been demonstrated to inhibit the first enzyme in the repair of AP sites, AP endonuclease [12], and this is the basis of its proposed use as part of anti-cancer combination chemotherapy. It has been shown that coupling alkylating agents such as TMZ with BER inhibitor MX increases the efficacy of killing tumor cells. AA3 is expected to block the action of AP endonuclease and inhibit the repair of AP sites. To test that, synthetic oligomers containing an AP site are used to assess the inhibition of AA3 on APE-1. Based on its effect on the activity of APE-1, it is used to treat cancer cell lines in combination with DNA-damaging agents to improve the effectiveness of killing tumors.

Aim 3) Cytotoxicity of AA3 in B-cell lymphoma cells is examined, and the molecular mechanism of AA3 action is elucidated.

If AP sites are chemically modified by alkoxyamines, they will escape repair by BER pathway and the alkoxyamine-AP site adducts would block DNA replication in subsequent cell cycle eventually causing cell death. If B-cell lymphoma cells contained an excess of AP sites as I anticipated, alkoxyamines could also target these AP sites and kill B-cell lymphoma cells without the assistance of other DNA damaging agents. The cytotoxicity of AA3, MX and ARP is evaluated in B-cell lymphoma cell lines, as well as normal human B cells and non-B cell lines. Also, the
molecular mechanism of cytotoxicity of alkoxyamines in B cell lymphoma cells is elucidated. Analogues of AA3 are synthesized to improve drug potency.
CHAPTER 2: MARTIAL AND METHODS

Portions of the text in this chapter were reprinted or adapted from DNA repair journal, Vol 27, Wei, S., Shalhout, S., Ahn, Y., Bhagwat, A.S., A versatile new tool to quantify abasic sites in DNA and inhibit base excision repair, Pages 9-18, Copyright (2015), with permission from Elsevier.

2.1 Cell lines and tissue samples

2.1.1 Human cell lines

Human cervical cancer cell line (HeLa), breast cancer cell lines (MCF-7 and MDA-MB-453) and embryonic kidney cell line (HEK293T) were obtained from ATCC (American Type Culture Collection). Human lung cancer cell line (A549) was kindly provided by Dr. Young-Hoon Ahn (Wayne State University). Human Burkitt lymphoma cell lines (Raji and Daudi) and diffuse large B-cell lymphoma cell line (Toledo) were obtained from ATCC. Human Burkitt lymphoma cell line (Ramos 1) was kindly provided by Dr. Alberto Martin (University of Toronto). Primary human epidermal keratinocytes (HEKn) was obtained from Thermo Fisher Scientific. All B-cell lymphoma cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (Fisher Scientific). HeLa, A549 and HEK293T were cultured in DMEM medium (HyClone) with 10% FBS and 1% penicillin-streptomycin. MCF-7 was grown in MEM medium (HyClone) supplemented with 10% FBS and 1% penicillin-streptomycin, while MDA-MB-453 (ATCC) were grown in Leibovitz's L-15 medium (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin-streptomycin. All human cells were incubated at 37°C in a humidified incubator with 5% CO₂ in T-25 cm² flasks, T-75 cm² flasks or 48-well plates (CytoOne, USA scientific). All cell lines are summarized in Table 2.

2.1.2 Primary human B cells
Normal apheresis blood samples collected from healthy Red Cross donors were kindly provided by Dr. Martin Bluth (Wayne State University). The purified B cells were isolated with Easy Sep™ direct human B cell isolation kit (Stem Cell Technologies). Human B cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin and activated with 1 µg/mL anti-CD 40 antibody (Peprotech) and 50 ng/mL IL-4 (Peprotech).

**Table 2 Summary of human cell lines used in this research project.**

<table>
<thead>
<tr>
<th>Number</th>
<th>Cell lines</th>
<th>Type of Cell Lines</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HeLa</td>
<td>Human cervix carcinoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>2</td>
<td>MCF-7</td>
<td>Human breast carcinoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>3</td>
<td>MDA-MB-453</td>
<td>Human breast carcinoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>4</td>
<td>A549</td>
<td>Human Lung cancer</td>
<td>Dr. Young-Hoon Ahn, Wayne State University</td>
</tr>
<tr>
<td>5</td>
<td>HEK293T</td>
<td>Human embryonic kidney</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>6</td>
<td>Raji</td>
<td>Human Burkitt lymphoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>7</td>
<td>Daudi</td>
<td>Human Burkitt lymphoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>8</td>
<td>Toledo</td>
<td>Human diffuse large B-cell lymphoma</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>9</td>
<td>Ramos 1</td>
<td>Human Burkitt lymphoma</td>
<td>University of Toronto</td>
</tr>
<tr>
<td>10</td>
<td>HEKn</td>
<td>Human epidermal keratinocytes</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
</table>
2.2 AP site labeling reaction

A 6-carboxyfluorescein-(6-FAM-) labeled oligonucleotide U-17-mer (5’-6-FAM-ATTATTAUCCATTATT-3’, Integrated Device Technology) was used for the AP site labeling reaction. The oligomer (4 pmol) was incubated with E. coli uracil DNA glycosylase (UDG, 1 unit, New England Labs) at 37°C for 30 minutes in reaction buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT and 1 mM EDTA to create AP site containing DNA. The alkoxyamine [methoxyamine, MX (Sigma-Aldrich); aldehyde reactive probe, ARP (Dojindo Laboratories) or AA3] was added into the oligomer solution at indicated concentrations and incubated at 37°C for another 30 minutes. In some experiments, the pH of the solution was adjusted with HCl or NaOH solution following removal of uracil and the pH was confirmed using pH paper. Following the reaction of alkoxyamine, unlabeled AP sites were reduced by the addition of NaBH₄ (Sigma-Aldrich) to 100 mM and further incubation for 5 minutes. If this step was omitted, I observed significant degradation of the DNA (data not shown). The reactions were stopped by adding formamide, loading dye and heating to 95°C for 5 minutes. The DNAs were electrophoresed in 20% polyacrylamide gels containing 7M urea and scanned using a Typhoon 9210 phosphorimager (GE Healthcare). Product bands in images were quantified using ImageJ software.

In experiments involving competition between alkoxyamines, AP site containing DNA was reacted with the first alkoxyamine at 37°C for 30 minutes. This was followed by the addition of the second alkoxyamine and incubation for an additional 30 minutes. The reactions were stopped, and the products were analyzed as described above.

2.3 Click reaction
Biotin azide (prepared in Ahn lab) or Cyanine5 (Cy5) azide (Lumiprobe) was added to a solution of AA3-linked 17-mer to 0.5 mM followed by the addition of a freshly prepared solution of CuBr/TBTA (1:10 in DMSO/t-BuOH 3:1, 0.5 mM, Sigma-Aldrich). The mixture was shaken at 45°C for 1 hour. The DNAs were electrophoresed and analyzed as described above.

![Figure 15 Structures of biochemical tags. (A) The structure of biotin azide. (B) The structure of Cy5 azide.](image)

2.4 Genomic DNA isolation

Cells were harvested by centrifugation and lysed by incubation for 1 hr at 37°C in Tris-EDTA buffer (TE) containing 2 μg/ml of RNase A and 0.5% SDS, followed by incubation with
Proteinase K (100 μg/ml, Qiagen) at 56°C for 3 hours. The DNA was isolated by phenol/chloroform extraction and ethanol precipitation and dissolved in TE.

2.5 Creation of AP sites in genomic DNA by heat and acid treatment

Genomic DNA was digested with HaeIII (New England Biolabs) and endogenous AP sites in DNA were reduced by the addition of NaBH₄ to 100 mM. The reducing agent was removed by gel filtration, and this DNA was incubated in sodium citrate buffer (10 mM sodium citrate, 10 mM NaH₂PO₄, 10 mM NaCl, pH 5.0) at 70°C for various lengths of time (0, 15, 30, 45, or 60 minutes). The DNA was rapidly chilled on ice and filtered using MicroSpin G-25 columns (GE Healthcare) and precipitated with ethanol.

2.6 AP site quantification assay

2.6.1 AP site quantification assay using ARP

ARP or AA3 was added to a solution of genomic DNA to 2 mM and the DNA was incubated at 37°C for 30 minutes. In parallel, a 75 base pair duplex DNA with one uracil (5'-T₃7UT₃7-3'/5'-A₃7GA₃7-3') was treated with UDG to create AP sites and was also treated with ARP or AA3. This served as an AP site standard. All DNAs were purified by phenol/chloroform extraction and ethanol precipitation, followed by MicroSpin G-25 column (GE Healthcare).

The ARP-tagged DNA was heated at 95°C for 5 minutes, prior to transfer to a positively charged nylon membrane. The DNA was UV cross-linked to the membrane using a Beckman UV Stratalinker 1800. The membrane was then incubated with Starting Block Blocking Buffer (Fisher) at room temperature for 1 hour, followed by the incubation of streptavidin-conjugated horseradish peroxidase (HRP, Thermo Scientific) in blocking buffer at room temperature for 30 minutes. After washing with Tris-buffered Saline containing Tween-20 (TBS-T) for 15 minutes,
the membrane was incubated in SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) for five minutes. The emitted light was captured by FluorChem Imaging System (Alpha Innotech). The resulting images were analyzed using ImageJ software. When Cy5-Streptavidin was used, the fluorescence was quantified using Typhoon 9210 phosphorimager (GE Healthcare) (Figure 16).

**Figure 16 Schematic outline of AP sites quantification assay using ARP.** Digested DNA is treated with ARP, and ARP-labeled DNA is transferred to a positively charged nylon membrane. The membrane is incubated with streptavidin-conjugated horseradish peroxidase (STV-HRP), followed by incubation with Chemiluminescent Substrate. The emitted light is detected by FluorChem Imaging System. Alternatively, the membrane with DNA is incubated with streptavidin-Cy5 and scanned using Typhoon phosphorimager.

**2.6.2 AP site quantification assay using AA3**

AA3-tagged DNA was linked with Cy5 azide using the click reaction and purified by ethanol precipitation and filtration through a MicroSpin G-25 column (GE Healthcare). The Cy5-labeled DNA was heated at 95°C for 5 minutes before being transferred to a positively charged nylon membrane. The membrane was scanned using a Typhoon 9210 phosphorimager (GE Healthcare). The images were analyzed using ImageJ software. Alternately, the fluorescence of
AA3-Cy5 tagged samples was directly measured using Synergy H1 Hybrid Reader (BioTEK) and the fluorescence intensities were obtained directly from the instrument.

![Diagram of AP sites quantification using AA3](image)

**Figure 17 Schematic outline of AP sites quantification using AA3.** Digested DNA is treated with AA3, followed by incubation with Cy5 azide. Cy5-labeled DNA is spotted onto a positively charged nylon membrane, which is scanned using Typhoon phosphorimager. Alternatively, Cy5-tagged DNA is transferred to a microplate, and the fluorescence of DNA samples is measured using Synergy H1 Hybrid Reader.

2.6.3 Quantification of AP sites in vivo labeled by AA3

Cells were cultured in the growth medium supplemented with AA3 at indicated times. Genomic DNA was isolated using the protocol described in section 2.4, followed by restriction digestion with HaeIII (New England Biolabs). Digested DNA was labeled with Cy5 azide through click reaction, prior to being transferred to a positively charged nylon membrane. The membrane was scanned and analyzed as described above.

To quantify AA3 in vivo binding in dead and viable cells, Daudi cells were treated with 5 mM AA3 for 5 hours. The dead cells and viable cells were separated using apoptotic cell isolation kit (PromoKine) and collected separately. Genomic DNA was extracted from the cells, fragmented with HaeIII restriction digestion and labeled with Cy5 azide for quantification as described above.

2.7 AP endonuclease activity assay

The cleavage activity of AP endonuclease APE-1 (1 unit, New England Biolabs) was assayed using a 6-FAM labeled oligomer (4 pmol) containing a single uracil. The uracil was
excised using UDG to create an AP site and the AP site was labeled with an alkoxyamine as described above. The APE-1 reaction was performed in the reaction buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9) at 37°C for 1 h. DNA products were stabilized by incubation with NaBH₄ and analyzed by gel electrophoresis as described above.

2.8 Killing of cancer cells by AA3

2.8.1 Trypan blue exclusion assay

Cells were stained with Trypan blue (HyClone) and counted by TC20™ automated cell counter (Bio-Rad Laboratories) which provides the total cell count and cell viability. Cell viability was determined using this assay.

2.8.2 Killing of HeLa cells by combination chemotherapy

DNA alkylating agents including methyl methanesulfonate (MMS, Sigma-Aldrich) and temozolomide (TMZ, Sigma-Aldrich) were used in combination chemotherapy. MMS was dissolved in phosphate buffered saline (PBS). TMZ was dissolved in sterile Dimethyl sulfoxide (DMSO). MX or AA3 was dissolved in sterile water, and the pH was adjusted to 7 using NaOH solution. The solutions of MMS, MX and AA3 were freshly prepared for each cytotoxicity experiment, while TMZ stock was stored at -20°C. HeLa cells were seeded in 48-well tissue culture plates at 3x10⁴ to 6x10⁴ cell/mL cell density and grown overnight in DMEM medium with 10% FBS and 1% penicillin-streptomycin. Cells were treated with a DNA alkylating agent (MMS or TMZ) or as a combination of a DNA alkylating agent with MX or AA3 at indicated concentrations. Cell viability was measured after 24-hour treatment.

2.8.3 Killing of B-cell lymphoma cell lines by alkoxyamines
B-cell lymphoma cell lines (Raji, Ramos 1, Daudi and Toledo) were seeded in 48-well tissue culture plates at 2x10^5 to 5x10^5 cells/mL cell density and cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin. Cells were treated with alkoxyamines (MX/AA3/ARP) at indicated concentrations and harvested after 24 hours. Cell viability was determined.

In MX pretreatment experiment, Daudi cells were treated with MX at 10mM for 6 hours, followed by the incubation with AA3 at 1mM for overnight. Cells were harvested, and their viability was determined.

2.8.4 Killing of normal human B cells by AA3

Normal human B cells were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin and stimulated with 1 µg/mL anti-CD 40 antibody and 50 ng/mL IL-4. After three days, cells were seeded in 48-well plates at 2x10^5 to 5x10^5 cell/mL cell density, treated with AA3 at indicated concentrations and harvested after 24 hours. The viability of cells was determined.

2.8.5 Killing of non-B cells by AA3

HeLa, MCF-7, MDA-MB-453, A549, HEK293T and HEKn cells were seeded in 48-well tissue culture plates at 3x10^4 to 6x10^4 cell/mL cell density and grown overnight in DMEM medium with 10% FBS and 1% penicillin-streptomycin. Cells were treated with AA3 at indicated concentrations and harvested after 24 hours. The viability of cells was determined.

2.9 Immunofluorescence microscopy and quantification of fluorescence intensity per nuclei
Daudi cells were treated with 5mM AA3 for 5h or 50μg/ml Phleomycin (Sigma-Aldrich) for 24 hours and harvested by centrifugation. The cell pellet was resuspended in a hypotonic solution 75mM KCl, incubated at 37°C for 15 minutes and spun onto microscope slides using a StatSpinCytofuge (Beckman Coulter). After fixation with cold methanol, cells were blocked with 3% FBS in Tris-buffered saline (TBS) and incubated with 2μg/mL anti-phospho-histone H2A.X (Ser139) antibody solution (Millipore). The slides were washed with TBS, followed by the incubation with 1μg/mL goat anti-mouse antibody conjugated to Fluorescein isothiocyanate (FITC) (Millipore). Then, the slides were mounted with ProLong® Gold AntifadeMountant with DAPI (Thermo Fisher Scientific) after washing with TBS and visualized using a fluorescence microscope. The fluorescence intensity per nuclei was analyzed and quantified using ImageJ software. Untreated cells served as a negative control, while cells treated with phleomycin were a positive control.

2.10 Cell synchronization

Cells were treated with or without 100μM L-Mimosine from Koa hoale seeds (Sigma-Aldrich) for 24 hours to arrest cells at G1 phase. Half of Mimosine-treated cells were harvested by centrifugation, resuspended in the fresh growth media and cultured for another 4 hours to allow cells to reenter S phase. The remaining Mimosine-treated cells and untreated cells were also cultured for another 4 hours. Cells were seeded in a 48-well plate and treated with AA3 at 5mM for 5 hours. The viability of cells was determined by Trypan blue exclusion assay.
To confirm whether cells were arrested at G1 phase, cell cycle was analyzed by flow cytometry with Propidium iodide (PI, Sigma-Aldrich). Briefly, suspension cells were transferred into a FACS tube and pelleted by centrifugation. Cell pellet was washed with cold Dulbecco's Phosphate-Buffered Saline (DPBS, HyClone) and resuspended into 0.5mL cold DPBS. Cold 70% ethanol (4mL) was added dropwise into cell suspension solution to fix cells. After incubation at 4 °C for 1 hour, cells were pelleted by centrifugation, washed with cold DPBS and incubated with 0.5mL PI staining solution at 37 °C for 30min. Cell cycle analyses were performed by BD LSRFortessa™ cell analyzer (BD Biosciences). The data of flow cytometry was analyzed by FlowJo software.

2.11 RNA isolation and real-time PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized with ProtoScript First Strand cDNA synthesis kit (New England Biolabs) and amplified by HotStartTaq polymerase (SA Biosciences). The primers used for RT-PCR were described in the previous paper [92]. RT-PCR was carried out using an Applied Biosystems 7500 fast real-time PCR system.

2.12 Synthesis of alkoxyamines

2.12.1 Synthesis of O-2-Propynylhydroxylamine hydrochloride (AA3)

AA3 was synthesized according to figure 18. Propargyl bromide (11.3 mmol, Sigma–Aldrich) was added drop wise into a mixture of tert-butyl-N-hydroxycarbonate (3.7 mmol, Sigma–Aldrich) and sodium carbonate (7.4 mmol) in N, N-dimethylformamide (DMF). The reaction mixture was stirred overnight at 70°C, washed with water and extracted with ethyl acetate three times. The combined organic solution was washed with saturated aqueous
sodium chloride (50 mL), dried over sodium sulfate and concentrated. The crude residue was purified by silica gel column chromatography (hexane: ethyl acetate = 10:1) to give the intermediate product tert-butyl N-(2-propynyl)-carbamate. HCl solution (4 M in 1, 4-dioxane, 2 mL, Sigma–Aldrich) was added to a concentrated solution of tert-butyl N-(2-propynyl)-carbamate (2.1 mmol) in dichloromethane in an ice bath and stirred for approximately 20 min. The white precipitate was filtered and recrystallized with diethyl ether and ethanol to give O-2-propynylhydroxylamine hydrochloride (AA3, 0.08 g). Its structure was confirmed by $^1$H NMR and $^{13}$C NMR.$^1$H NMR (400 MHz, CD$_3$OD) $\delta$: 4.748 (s, 2H, CH$_2$), 3.376 (t, 1H, CCH, J = 2.4 Hz); $^{13}$C NMR (500 MHz, CD$_3$OD), $\delta$: 79.8 (CH$_2$), 74.6 (C), 62.3 (CH).

![Figure 18 Scheme of synthesis of AA3.](image)

2.12.2 Synthesis of O-propylhydroxylamine hydrochloride (AA4)

1-Bromopropane (11.3 mmol, Sigma-Aldrich) was added dropwise into a mixture of tert-butyl-N-hydroxycarbamate (3.7 mmol, Sigma-Aldrich) and sodium carbonate (7.5 mmol) in DMF. The reaction mixture was stirred overnight at 90 °C, washed with water and extracted with ethyl acetate three times. The combined organic layer was washed with saturated aqueous sodium chloride (50 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The crude residue was purified by silica gel column chromatography (hexane: ethyl acetate = 13:1) to give the intermediate product tert-butyl N-(propyloxy)-carbamate. HCl (4M in 1, 4-dioxane, 2 mL, Sigma-Aldrich) was added to a concentrated solution of tert-butyl N-(propyloxy)-carbamate
(2.1 mmol) in dichloromethane in an ice bath and stirred for approximately 30 min. The mixture was concentrated and the resulting oil product was recrystallized using diethyl ether/ethanol as the solvent to give $O$-propylhydroxylamine hydrochloride (AA4, 0.07g). Its structure was confirmed by $^1$H NMR.

![Figure 19 Scheme of synthesis of AA4.](image)

2.12.3 Synthesis of $O$-(3-butynyl) hydroxylamine hydrochloride (AA5)

Diisopropyl azodicarboxylate (DIDA, 40% solution in toluene, 0.0011mol, Sigma-Aldrich) was added dropwise to a stirred solution of the 3-butyn-1-ol (0.001 mol, Sigma-Aldrich), $N$-hydroxyphthalimide (0.001 mol, Sigma Aldrich) and triphenylphosphine (TPP, 0.001 mol, Sigma-Aldrich) dissolved in anhydrous tetrahydrofuran (THF, 2.5 mL). After stirring 24 hours at 45°C, the solvent was removed. Ether was added to the residue to precipitate triphenylphosphine oxide and diethyl hydrazinedicarboxylate, which were filtered off. The filtrate was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (hexane: ethyl acetate = 8:1) to give the intermediate product $N$-(but-3-ynyloxy) phthalimide. A mixture of the $N$-(but-3-ynyloxy) phthalimide (0.5 mmol) and hydrazine monohydrate (0.53 mmol, Sigma-Aldrich) was vigorously shaken at room temperature for 15 min. Diethyl ether (2 mL) was added, and the mixture was shaken for another 15 min before it was filtered. The residue was washed again with 3 x 3 mL portions of ether. HCl (4M in 1, 4-dioxane, 1 mL, Sigma-Aldrich) was added dropwise to the combined ethereal filtrate to precipitate out the $O$-(3-
butynyl) hydroxylamine hydrochloride (AA5, 21.5 mg) which was filtered, washed with ether and recrystallized using 2-propanol/ether as the solvent [138]. Its structure was confirmed by $^1$H NMR.

![Diagram of synthesis of AA5](image)

**Figure 20 Scheme of synthesis of AA5.**

2.12.4 Synthesis of O-(2-azidoethyl) hydroxylamine hydrochloride (AA6)

To a stirred solution of N-hydroxyphthalimide (3.06 mmol, Sigma Aldrich) in DMF (3.75 mL), triethylamine (6.74 mmol, Sigma Aldrich) and 1-bromo-2-chloroethane (14.42 mmol, Sigma-Aldrich) were added, and the mixture was stirred overnight at 55°C. The solid was filtered and the precipitate was washed with DMF until it became white. The filtrate was diluted with ethyl acetate and the organic layer was extracted with 1M HCl (x2) and brine. The organic phase was dried with anhydrous Na$_2$SO$_4$, filtered and evaporated under reduced pressure. To remove the excess alkyl bromide the product was further purified using silica gel column chromatography (hexane: ethyl acetate = 8:1) and recrystallized with ethyl acetate/diethyl ether to afford 2-(2-chloroethoxy)isoindoline-1, 3-dione (0.55 g) as a white solid. Its structure was confirmed by $^1$H NMR.

To a stirred solution of 2-(2-chloroethoxy) isoindoline-1, 3-dione (0.55 g, 2.43 mmol) in DMF (4 ml), NaN$_3$ (1.41 g, 21.69 mmol) was added and the reaction mixture was stirred for 2 days at 55 °C. The reaction was diluted with CHCl$_3$ and H$_2$O, and the organic layer was extracted
with CHCl₃ (x2) and the organic layers were dried over anhydrous Na₂SO₄, filtered and evaporated under reduced pressure. Then it was further purified using silica gel column chromatography (hexane: ethyl acetate = 8:1) and recrystallized using ethyl acetate/ hexane to afford 2-(2-azidoethoxy) isoindoline-1, 3-dione (0.30 g) as a white solid. Its structure was confirmed by ¹H NMR.

A mixture of the 2-(2-azidoethoxy)isoindoline-1,3-dione (1.3 mmol), hydrazine monohydrate (7.78 mmol, Sigma Aldrich) and dichloromethane (3 mL) was vigorously shaken at room temperature for 15 min. Diethyl ether (6 mL) was added and the mixture was shaken for another 15 min before it was filtered. The residue was washed again with 3 x 3 mL portions of ether. Ethereal HCl (4M in 1, 4-dioxane, 2 mL, Sigma-Aldrich) was added drop wisely to the combined ethereal filtrate to precipitate out the O-(2-azidoethyl) hydroxylamine hydrochloride (AA6, 0.28 g) which was filtered, washed with ether and recrystallized using 2-propanol/ether as the solvent. Its structure was confirmed by ¹H NMR.

![Scheme of synthesis of AA6](image)

Figure 21 Scheme of synthesis of AA6.
CHAPTER 3: RESULTS

Portions of the text and figures in this chapter were reprinted or adapted from DNA repair journal, Vol 27, Wei, S., Shalhout, S., Ahn, Y., Bhagwat, A.S., A versatile new tool to quantify abasic sites in DNA and inhibit base excision repair, Pages 9-18, Copyright (2015), with permission from Elsevier.

3.1 Reactivity of a versatile chemical AA3 with AP sites in DNA

To create a more versatile tool for labeling AP sites, I designed and synthesized a small alkoxyamine with an alkyne group, AA3. I combined the abilities of both MX and ARP into AA3, which should react with AP sites in, but also allow linking AP sites to biochemical tags like biotin or fluorescent tags through click chemistry for quantification (Figure 22). The reactivity of AA3 toward AP sites in DNA and was examined using a synthetic DNA oligomer containing a single AP site. All reaction products were separated by denaturing polyacrylamide gel.

Figure 22 Use of click chemistry to label AA3-AP site DNA (★ can be biotin, a fluorophore or any other molecule).

3.1.1 Labeling of AP sites using AA3

To demonstrate that AA3 reacts with AP sites, we showed that it inhibits the ability of ARP to label AP sites. AP sites were created by the excision of uracils by uracil-DNA glycosylase
in a synthetic oligomer. They were reacted with ARP and the products separated on a
denaturing gel. The linking of ARP to DNA caused a shift in mobility of oligomer[33](Figure 23A;
lane 3). This shift was eliminated completely if the AP sites were pretreated with AA3 (Figure
23A; lane 6). This shows that AA3 forms a stable adduct at AP sites and blocks the reaction of
ARP. Treatment of the oligomer with AA3 alone did not create an observable shift in oligomer
mobility (Figure 23A; lane 5) probably because of the small size of AA3 compared to ARP (MW
71.0 vs. 331.4). However, treating the oligomer with the two chemicals in the reverse order still
created the mobility shift (Figure 23A; lane 4) suggesting that ARP-DNA adducts are stable and
cannot be replaced with AA3.

AA3 is both versatile and more efficient than ARP in labeling AP sites. AP sites reacted
with AA3 can be labeled with an appropriate azide using click chemistry. Following a reaction
with AA3, biotin azide was used to routinely convert greater than 70% of the AP sites to
biotinylated form (Figure 23A, lane 7). Prior treatment of the AP sites with a reducing agent,
sodium borohydride, eliminated labeling showing that AA3 reacts with only the unreduced form
of the AP site (Figure 23B). AA3 reacted equally well with AP sites in ssDNA and dsDNA
converting an overwhelming majority of substrate to a product (Figure 23C). It may be possible
to increase the reaction yield through further optimization of the click reaction [139]. In
contrast, ARP converted less than 20% of the substrate to the biotinylated product (Figure 23A,
lanes 3, Figure 23C, lanes 2 and 6).

To show that AA3 can be also used to label AP sites with different tags, we replaced
biotin azide with Cy5 azide in the click chemistry. Using this strategy, 67% of AP sites in the
oligomer were labeled with the fluorescent label (Figure 23D, lane 4). As a large number of
fluorescent dyes and other molecules are commercially available in azide form, it should be possible to choose appropriate labels for AP sites based on the intended application.
Figure 23 Reactivity of AA3 toward AP sites in DNA. A scheme for each experiment is shown at the top of each part of the figure. (A) Inhibition of ARP reaction by AA3. Briefly, a 5'-6-FAM labeled DNA was incubated with UDG to create AP sites and reacted with AA3 and ARP in the indicated order. For the reaction in lane 7, DNA was incubated with UDG and AA3, followed by reaction with biotin azide using click chemistry. (B) The 17-mer DNA containing a single AP site was reacted with either ARP (lane 2) or AA3 followed by click reaction with biotin azide (BA; lane 3). This created characteristic gel retardation of the DNA (indicated by arrows). When the AP site was treated with sodium borohydride prior to ARP or AA3 reaction these bands disappeared (Lanes 5 and 6, respectively). (C) Comparison of reactions of AA3 with AP sites in
double-stranded DNA (lane 3 and 4) and single-stranded DNA (lane 7 and 8). Reactions of ARP with double- and single-stranded DNA are shown in lanes 2 and 6, respectively. (D) Labeling of AP sites with Cy5 using AA3. AP sites were reacted with AA3 followed by click reaction with biotin azide (lane 3) or Cy5 azide (lane 4).

3.1.2 AA3 has better reactivity profile than ARP

We were surprised that ARP labeled <20% AP sites under the standard conditions (Tris buffer, pH 8.0). To investigate this, the pH of the reaction buffer was changed after uracil excision, but prior to addition of ARP. When the products were quantified by gel electrophoresis the gel showed that ARP reacts well with AP sites only at acidic conditions (Figure 24A). We consistently found that with 0.2 μM oligomer and 5 mM ARP <20% of the substrate was converted to product at pH 7 or 8 (Figure 23 and 24A). In contrast, AA3 was much more reactive with AP sites over the pH range of 4 to 8, and converted 57% or greater AP sites to AP-AA3 adduct (Figure 23 and 24B). Therefore, AA3 is more suitable for in situ labeling of AP sites in cells than ARP.
Figure 24 pH dependence of reaction of ARP and AA3 with AP-sites. A scheme for each experiment is shown at the top of each part of the figure. (A) Reactions of ARP (5 mM) with AP sites at pH 4, 5, 6 and 7 (lanes 3 through 6). (B) Reactions of AA3 (5 mM) with AP sites at pH 4, 5, 6 and 7 (lanes 3 through 6) followed by the addition of biotin azide.

Under physiological conditions, AA3 was more reactive toward AP sites than ARP even at much lower concentrations. Increasing the ARP concentration from 5 to 10 mM resulted in an increase in product formation from 13% to 20% (Figure 25A), while AA3 converted greater than that amount of substrate to product even at 1 mM (Figure 25B). The results from three
independent experiments are presented in Figure 25C. They show that AA3 was much more reactive at pH 7 toward AP sites than ARP over a range of concentrations and AA3 was about as reactive toward AP sites at 1 mM as ARP was at 10 mM (Figure 25C).
Figure 25 Concentration dependence of reactivity of ARP and AA3 with AP sites at pH 7. (A) DNA was incubated with UDG to create AP sites and reacted with 2, 1, 5, or 10 mM ARP (lanes 2 through 5). (B) DNA was incubated with UDG to create AP sites and reacted with 1, 2, 5, or 10 mM AA3 (lanes 2 through 5) followed by click reaction with biotin azide. (C) Quantification of the reaction products of different concentrations of ARP and AA3 with AP sites at pH 7. Mean and standard deviation of triplicate samples is shown.

3.2 A technique for quantification of AP sites by AA3

As ARP has been used to quantify AP sites in genomic DNA of normal cells [27, 28, 140] and cancer cell lines [27, 141], we wished to compare the use of AA3 in a similar setting. When ARP was used, the DNA adducts were quantified using streptavidin-conjugated horseradish peroxidase [27], and when AA3 was used the adducts were tagged with Cy5 for quantification. In both cases, the samples were spotted on a nylon membrane using a dot-blot apparatus and the light or fluorescence from each dot was quantified. The comparison was done in three different ways.

3.2.1 AA3 can be used to quantify genomic aldehydic lesions and AP sites

First, genomic DNA was extracted from HeLa cells and the AP sites were quantified using ARP or AA3. It should be noted that ARP and AA3 would react with all aldehydic lesions in DNA including intact unoxidized AP sites, AP sites cleaved by AP endonuclease, oxidized AP sites and formamidopyrimidines which result from alkylation or oxidation of purines in DNA [27, 142]. Such lesions occur routinely in cellular DNA, and hence the sites labeled by ARP or AA3 in HeLa DNA are referred simply as aldehydic lesions in DNA.

The results of these experiments are shown in Figure 26. The ARP-based method gave about twice as many aldehydic lesions in HeLa as AA3 (Figure 26A). A previous study of HeLa DNA using ARP reported [141] somewhat higher number of aldehydic sites (~20 per 10^6 bp). The
differences in those numbers and the numbers obtained in our study may be due to methods of DNA preparation, age of DNA used and use of different AP site standards. The Mendez et al study used depurinated pBR322 as the standard[141], while we used a synthetic oligomer containing uracil that was treated with UDG as the AP site standard (Figure 26C).

In a second set of experiments, HeLa DNA was first treated with sodium borohydride to reduce preexisting aldehydic lesions and make them resistant to ARP or AA3. The DNA was then heated under acidic conditions to create new AP sites through depurination. The depurination reaction was terminated at various times and the AP sites were quantified using the two chemicals as described above. The data showed there was a linear time-dependent increase in the number of AP sites and the two methods gave comparable numbers for AP sites at all time points (Figure 26B, left panel).
Figure 26 Quantification of aldehydic lesions and AP sites using ARP and AA3. (A) HeLa DNA was labeled with ARP and vacuum-spotted onto a nylon membrane. Aldehydic lesions in this DNA were quantified using streptavidin-conjugated horseradish peroxidase (HRP) and detected using a chemiluminescent substrate (open bar). In parallel, aldehydic lesions in HeLa DNA were also labeled with AA3 and quantified by reaction with Cy5 azide followed by applying DNA to a nylon membrane (black bar). AA3-labeled aldehydic lesions reacted with Cy5 azide were also quantified in solution using a microplate reader (gray bars). Mean and standard deviation of triplicate samples is shown. (B) HeLa DNA was pre-treated with NaBH₄ to reduce endogenous aldehydic lesions and AP sites were generated by heat and acid treatment for different lengths of time. The AP sites were labeled using ARP or AA3 for labeling, and the DNA was spotted onto a nylon membrane and the membranes scanned to quantify AP sites (Left). AP sites in the same DNAs were also quantified by reacting them successively with AA3 and Cy5 azide, and measuring fluorescence intensity directly using a microplate reader (right). Mean and standard deviation of triplicate samples is shown for each time point. (C) A 75-mer DNA containing a single uracil was treated with UDG to create AP site. Different dilutions of this DNA were treated with ARP or AA3 as indicated. This was followed by treatment with HRP-streptavidin
followed by incubation with chemiluminescent substrate and the light intensity was quantified (left panel), or reacted with Cy5 azide and fluorescence intensity quantified (center and right panel). When AA3 was used, the reaction samples were either spotted on a nylon membrane (center panel) or measured directly using a microplate reader (right panel).

3.2.2 Sensitivity and ease of use of AA3

To determine whether AA3-based quantification of AP sites was as sensitive as ARP-based quantification, we treated different amounts of a DNA oligomer containing AP sites with ARP or AA3 and quantified the products. The membrane images from the two parallel experiments are shown in Figure 27A. The sample containing $10^8$ AP sites was visible using ARP-chemiluminescence assay, but the sample with $10^7$ sites could not be detected above background. In contrast, $10^7$ AP site sample could be detected in the image from AA3-fluorescence assay. Adjusting the image brightness did not change the relative sensitivities of AA3 and ARP. The membrane containing ARP-labeled samples have much higher background than the membrane with AA3-labeled samples (Figure 27B). Thus under these conditions, AA3-based detection of AP sites has a lower background and greater sensitivity than ARP.

However, the readout for the comparison of two techniques used to quantify AP sites in Figure 27A was different, and I wanted to eliminate this variable in the comparison. To accomplish this, I labeled different amounts of the AP site-containing DNA duplex with ARP and then bound it to Cy5-streptavidin. In parallel reactions the DNA was reacted with AA3 followed by reaction with Cy5 azide. Both sets of samples were spotted on nylon membranes and Cy5 fluorescence was quantified. The results show that both methods result in a linear relationship between the number of AP sites and Cy5 fluorescence (Figure 27C), but the use of AA3 results in lower background and hence greater signal-to-noise ratio (Figure 27C, inset).
One problem of using ARP to quantify AP sites is the difficulty of separating unbound protein (HRP or streptavidin) from that bound to DNA. In contrast, the AA3-based method does not use a protein and hence it is possible to eliminate unreacted AA3 from the much larger AA3-DNA by a G-25 mini-column. The Cy5 fluorescence can then be directly measured using a microplate fluorometer (Figure 27D). I performed this simplified procedure on endogenous aldehydic lesions in HeLa DNA and on AP sites created by heat and acid treatment, and the results were comparable to those obtained by the other two methods (Figure 26A, and 26B, right panel). Thus the use of AA3 simplifies AP site quantification (Figure 27D).
Figure 27 Sensitivity of detection of AP sites using ARP and AA3. (A) Image of the nylon membranes where different amounts of DNA containing a synthetic oligomer with one AP site were labeled using either ARP-HRP (top) or AA3-Cy5 (bottom). (B) The brightness levels in images shown in Figure 27A were adjusted using Adobe Photoshop to show more signal. With this adjustment, $10^8$ AP sites can be detected using the ARP/HRP protocol, while $10^7$ sites can be detected using the AA3/Cy5 azide protocol. (C) Synthetic duplex containing one AP site was labeled with either ARP or AA3. Different dilutions of ARP-labeled DNA was spotted on a
membrane and bound with Cy5-streptavidin. AA3-labeled DNA was reacted with Cy5 azide, and different dilutions were spotted on a membrane. Cy5 fluorescence was quantified in each case and is plotted against the number of AP sites in each spot.

3.3 AA3 inhibits APE-1

The reaction of MX with AP sites is known to inhibit its repair by AP endonuclease [74] and this is the basis of its proposed use as part of anti-cancer combination chemotherapy [65]. To find out whether ARP and AA3 similarly inhibit AP endonuclease APE-1, I treated an oligomer containing an AP site with MX, ARP or AA3 and then challenged the DNA with APE-1. The results showed that while MX and AA3 were very effective in blocking the action of APE-1, ARP was a poor inhibitor of the enzyme.

When AP sites were reacted with ARP at pH 7 and the DNA was then treated with APE-1, about 90% of the DNA was cleaved by the enzyme showing poor protection of AP sites by ARP (Figure 28A, lane 8). This is probably because of the poor reactivity of ARP at pH 7 (Figure 24A). In contrast, both MX and AA3 protected an overwhelming majority of AP sites at pH 7 (Figure 28A, respectively lanes 4 and 6). When the pH of ARP reaction was lowered to 5, protection of AP sites against cleavage by APE-1 increased to 26% (Figure 28B, lane 8). Under the same conditions, the protection by MX and AA3 was ~100% (Figure 28B, lanes 4 and 6). Thus, MX and AA3 protect AP sites against APE-1 cleavage equally well at both pH conditions.
Figure 28 Inhibition of APE-1 activity by MX, ARP or AA3. A scheme for each experiment is shown at the top of each part of the figure. (A) DNA containing an AP site was reacted with MX, AA3 or ARP at pH 7. This was followed by incubation of DNA with APE-1. (B) DNA containing an AP site was reacted with MX, AA3 or ARP at pH 5. This was followed by incubation of DNA with APE-1.
3.4 AA3 kills cells containing DNA base damage

Inhibition of base-excision repair has been proposed as a strategy for anti-cancer chemotherapy [143, 144]. In particular, it has been shown that coupling treatment of cancers with alkylating agents such as MMS or TMZ with BER inhibitor MX increases killing of tumor cells [43, 65]. To determine whether AA3 is also able to enhance killing cells treated with an alkylating agent, I combined AA3 treatment of HeLa cells with MMS or TMZ treatment.

3.4.1 AA3 enhances cell killing by MMS

When HeLa cells were treated with a low concentration of MMS (0.5 mM), very little loss of viability was observed after one day (Figure 29A). Also, treatment of HeLa cells with AA3 alone did not kill HeLa cells (data not shown). However, when HeLa cells were treated with 0.5 mM MMS and different concentrations of AA3, cell viability decreased with increasing concentration of AA3 dropping to about 50% survival at 10 mM AA3 (Figure 29A). We then directly compared the ability of MX and AA3 to enhance killing by MMS at this concentration using six independent cultures for each chemical. The results showed that while MX did enhance killing by MMS, AA3 had a stronger lethal effect (Figure 29B).
Figure 29 Killing of HeLa cells by combining MMS with MX or AA3. (A) HeLa cells were treated with 50 µM MMS and different concentrations of AA3 and cell killing was determined by Trypan blue exclusion assay. Each circle represents the result from one independent culture and the broken line connects median values at each AA3 concentration. (B) HeLa cells were treated with only MMS (50 µM) or with MMS and MX (10 mM) or AA3 (10 mM), and cell killing was quantified. The results are from six independent cultures, and the mean and standard deviation are shown in each case.

3.4.2 AA3 enhances cell killing by temozolomide

HeLa cells were treated with 700µM TMZ for 24 hours, and cell viability did not significantly decrease. Also, 10 mM MX or AA3 alone did not kill HeLa cells after one-day treatment. When cells were treated with 700 µM TMZ and 10 mM AA3, cell viability dropped to 20% (Figure 30). The result shows that AA3 potentiated the cytotoxic effect of TMZ. Surprisingly, the combination of TMZ and MX treatment did not significantly kill HeLa cells. Higher concentration of MX may be required to kill cells treated with 700µM TMZ. The results suggest that AA3 could more effectively enhance cell killing by TMZ.
In both cases, the difference between the killing enhancement caused by MX and AA3 is statistically significant and suggests that AA3 is better than MX as a component in anti-cancer combination chemotherapy regimen. It is unclear why AA3 kills MMS-treated cells better than MX, despite the fact that both chemicals appear to be equally effective at inhibiting APE-1.

![Figure 30 Killing of HeLa cells by combining TMZ with MX or AA3.](image)

Figure 30 Killing of HeLa cells by combining TMZ with MX or AA3. HeLa cells were treated with only TMZ (700 µM) or with TMZ and MX (10 mM) or AA3 (10 mM), and cell killing was quantified Trypan blue exclusion assay. The mean and standard deviation are shown in each case.

3.5 AA3 kills B-cell lymphoma cells

3.5.1 High levels of AP Sites in B-cell cancers

AP sites in genomic DNA from a number of different human cancer cell lines and two types of normal primary human cells were quantified using a previously described procedure [145], and the results were normalized to the number of AP sites in the HeLa genome. The three GC-derived B-NHL cell lines (Toledo, Raji and Daudi) express AID at high levels [92] and
have about 10- to 35-fold higher levels of AP sites in their genomes compared to HeLa (Figure 31A). In contrast, all the non-B cell lines (A549, HEK293, MDA-MB-453 and MCF-7) had low AID expression (Figure 31B) and low levels of AP sites compared to the B-NHL lines (Figure 31A).

It was previously shown that, in contrast to B-NHL cancers, normal B cells from murine spleen or human tonsils do not accumulate uracils in their genomes [92]. This suggested that normal B cells may contain fewer AP sites than the B-NHLs. Quantification of AP sites in the genomes of B cells from normal human volunteers showed that they had slightly higher levels of AP sites than HeLa cells, but lower than the three B-NHL cell lines (Figure 31A). Additionally, AP sites in primary neonatal human keratinocytes (HEKn) cells were determined, and these were comparable to non-B cell lines. A comparison of AP site levels in the B-NHL lines with non-B cells or normal human B cells showed that differences between these groups of cells were statistically quite significant (Figure 31A). These results confirm the prediction that cell lines derived from cancers that express AID at high levels should accumulate more AP sites than normal B cells and non-B cell lines.
Figure 31 Quantification of levels of AP sites in B-NHL cell lines and non B-NHL cells. (A) Comparison of levels of AP sites of three B-NHL cell lines (Toledo, Raji and Daudi) with non B-cell lines (HeLa, A549, HEK293T, MDA-MB-453, MCF-7 and HEKn) and human B cell. The level of AP sites of each cell line is shown relative to that of HeLa cell (set at 1). The average level of AP sites of three B-NHL cells is significantly higher than that of five non B-cell lines (****p<0.0001), and it is also significantly higher than two normal human cells (B cell and HEKn) (***P<0.0005). (B) AID expression levels in B-NHL and non-B cell lines. The levels of AID expression are shown relative to gene expression levels of TBP. The asterisk (*) indicates undetectable AID expression.

3.5.2 B-cell lymphoma cell lines are sensitive to AA3

As alkoxyamines are expected to react with AP sites [28], I wondered whether AA3 would react with excess AP sites found in B cell cancer lines causing cell death. To test this, four GC-derived B-NHL cell lines were treated with AA3 at different concentrations for 24 hours and the cell viability was determined using the trypan blue exclusion assay. The results are shown in Figure 32. All four cell lines were sensitive to sub-millimolar concentrations of AA3 and at 1 mM, AA3 reduced cell viability by about 50% to 80% (Figure 32A). Greater killing was observed in the
millimolar range of concentrations and at 10 mM the viability of all the cell lines was reduced to below 30% (Figure 32B). Daudi, a Burkitt lymphoma-derived cell line, was consistently most sensitive to AA3. Its viability was typically reduced to about 10% at 2 mM AA3 (Figure 32B) and hence it was used as a positive control in subsequent experiments.

![Graphs showing killing of B-NHL cell lines by AA3.](image)

**Figure 32 Killing of B-NHL cell lines by AA3.** (A) Killing B-NHL cells by AA3 at sub-millimolar concentration. Daudi, Ramos1, Raji and Toledo (2x10^5 cells/mL seeding density) were treated with 0, 250, 500, 1000 µM AA3 for 24 hours and cell viability was determined by Trypan blue exclusion assay. (B) Killing B-NHL cells by AA3 at millimolar concentration. Daudi, Ramos1, Raji and Toledo (5x10^5 cells/mL seeding density) were treated with 0, 2, 5, 7.5, 10mM AA3 for 24 hours. Viability data was normalized to cell viability of untreated cells (set to 100). The mean and standard deviation are shown in each case.

3.5.3 Lack of sensitivity of normal cells and non-B cancer lines to AA3

To determine whether normal B cells were sensitive to AA3, human B lymphocytes were isolated from the blood of normal volunteers and stimulated to divide for three days using IL-4 and anti-CD40 antibody [146]. These cells and the HEKn cells were treated with 5 or 10 mM AA3 for 24 hours, and cell viability was determined. While neither type of normal cells was significantly killed by AA3, Daudi cells treated in parallel cultures were highly sensitive to the
chemical (Figure 33A). This shows that AA3 is much more toxic to Daudi cells than normal human B lymphocytes or primary keratinocytes and this correlates well with the presence of a much higher number of AP sites in Daudi cells (Figure 31A).

Next, I tested the five non B-NHL cell lines for sensitivity to AA3 and found that none was very sensitive to the chemical (Figure 33B). At the highest concentration of AA3 tested, 20 mM, there was slight sensitivity to the drug, but the reduction in viability was only 10-15% (Figure 33B). These results suggest that the low levels of AP sites found in these cell lines make them insensitive to AA3.

![Figure 33 Cytotoxicity of AA3 in non B-NHL cells.](image)

(A) Cytotoxicity of AA3 in normal human B cells and primary keratinocytes (HEKn). Normal human B cells isolated from blood of healthy donors, HEKn and Daudi were treated with AA3 at 5 or 10 mM and cell killing was determined after one day. (B) Cytotoxicity of AA3 in non B-NHL cell lines. Non-B-cell cancer cell lines (HeLa, MCF-7, MDA-MB 453 and A549) and human embryonic kidney cell line (HEK293T) were treated with AA3 at 1, 2, 5, 10, 20mM AA3 for 24 hours and cell viability was determined. Viability data was normalized to cell viability of untreated cells (set to 100). The mean and standard deviation are shown in each case.

3.5.4 AA3 reacts with AP sites in genomic DNA in vivo

AA3 has alkyne functionality that can be used to link it with a fluorescent label through click chemistry [145]. I used this property to determine whether genomic DNA in Daudi cells
treated with AA3 has the chemical linked to the DNA. HeLa or Daudi cells were treated with AA3 for 5 or 24 hours, and DNA was extracted from the cells. This DNA was reacted with Cy5 azide and Cy5 fluorescence was quantified (Figure 34A). There was a small, but significant, increase in fluorescence linked to HeLa DNA following 5-hour treatment, but little additional increase was seen when the AA3 treatment was extended to 24 hours (Figure 34A). Compared to HeLa DNA, the increase in fluorescence intensity linked to Daudi DNA was much greater following both 5 and 24 hours of AA3 treatment (Figure 34A). In a different experiment, I used the chemical ARP, which also labels AP sites [147, 148]. Daudi cells were treated with AA3 and cellular DNA was extracted and labeled using ARP. When the amount of AP sites labeling by ARP was quantified using Cy5-Streptavidin, DNA from AA3-treated cells showed much lower levels of fluorescence than untreated cells (Figure 34B). These results are consistent with the higher levels of AP sites in extracted Daudi DNA compared to HeLa DNA (Figure 31A) and suggest that AA3 reacts with AP sites in DNA in vivo.

As AA3 kills Daudi cells, I wished to determine whether there was a difference in the amount of AA3 bound to DNA of cells killed by AA3 compared to the living cells. Following a 5-hour treatment of Daudi cells with 5mM AA3 (50% killing), the dead cells were separated from the viable cells using an apoptotic cell isolation kit. DNA was isolated from both the cell populations and reacted with Cy5 azide, and the fluorescence was quantified. As expected, Cy5 azide readily reacted with DNA from dead cells showing the presence of covalently linked AA3 in its DNA (Figure 34C). Surprisingly, DNA from non-apoptotic Daudi cells showed the same fluorescence intensity as the untreated cells (Figure 34C). This result suggests that the cells that
are not killed by AA3 either prevent the chemical from reaching their genome or are able to repair AA3-DNA adducts.

Figure 34 AA3 binds to AP sites in genomic DNA in vivo. (A) Quantification of AP sites of DNA in vivo bound to AA3. HeLa and Daudi cells were treated with AA3 for 5 and 24 hours. DNA was extracted and labeled with Cy5 azide. The fluorescence intensity of Cy5 represents the level of AA3 in vivo binding to DNA. (B) Quantification of AP sites in genomic DNA labeled by ARP in vitro. Daudi cells were treated with AA3 for 24 hours, and DNA was extracted from cells. Genomic DNA was then labeled by ARP, followed by incubation with Streptavidin-conjugated Cy5. The intensity of Cy5 fluorescence represents the level of AP sites labeled by ARP after cells were treated with AA3. (C) Comparison of the amount of AA3 in vivo binding to DNA in cells killed by
AA3 and living cells. Daudi cells were treated with AA3 for 5 hours and dead cells were isolated from living cells. DNA was extracted from dead and living cells separately and labeled with Cy5 azide. The intensity of Cy5 fluorescence indicates the amount of AA3 binding to DNA (**P<0.005, n.s. = no significance).

3.5.5 AA3 causes strand breaks in DNA

It is likely that AA3-AP site adducts in B-NHL cell DNA would block DNA replication causing the collapse of the replication fork and resulting in cell death. Thus AA3 treatment should cause strand breaks in B-NHL cellular DNA. To test this, Daudi cells were treated with AA3 and then stained with anti-γ-H2AX antibodies. Phleomycin-treated Daudi cells served as a positive control in these experiments (Figure 35A, bottom panel). While some untreated Daudi cells showed a few nuclear γ-H2AX foci, other nuclei showed no foci (Figure 35A, top panel). AA3 treatment caused a large increase in γ-H2AX foci in the nuclei of most cells (Figure 35A, center panel). Fluorescence intensity of γ-H2AX per nuclei was quantified using ImagJ software (Figure 35B). More than 85% untreated cells did not show fluorescence of γ-H2AX. In contrast, a moderate and high level of fluorescence intensity of γ-H2AX was detected in about 70% AA3-treated cells.
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Figure 35 γ-H2AX in Daudi nuclei after AA3 treatment. (A) Representative γ-H2AX immunofluorescence images of Daudi cells after 5-hour AA3 treatment. Cells were stained with anti γ-H2AX and nuclei were stained with DAPI. DAPI (blue) images are shown on the left. γ-H2AX (FITC, green) images are shown in the middle. Merged DAPI and γ-H2AX images are shown on the right. Untreated cells served as negative controls (top panel) and phleomycin-treated cells were positive controls (bottom panel). (B) Quantification of γ-H2AX nuclear fluorescence intensity. The intensity of γ-H2AX fluorescence per nuclei was quantified using ImageJ software. Cells were divided into three groups based on the intensity of γ-H2AX fluorescence per nuclei, including the group with higher than 10,000 fluorescence intensity per nuclei (black bar), the group with 1-10,000 intensity (gray bar) and the group with undetectable fluorescence (0) (white bar). Percentage of cells of each group in untreated cells (N=171), AA3-treated cells (N=214) and phleomycin-treated cells (N=152) are indicated.

3.5.6 AA3 cytotoxicity is cell cycle-dependent

It is likely that AA3-AP site adducts in B-NHL cell DNA block DNA replication causing the collapse of the replication fork and cell death. Thus B-NHL cells that enter the S phase should be more susceptible to killing by AA3 than those that do not replicate. To test this idea, we arrested Daudi cells in the G1 phase by treating them with Mimosine. A 24 hour-treatment of cells with Mimosine increased the number of cells in the G1 phase from 21% to 72% and release of the Mimosine block for four hours increased the number of cells in both S and G2
phases (Figure 36A). All three types of cells were treated with AA3 for five hours and cell viability was determined.

Mimosine-treated Daudi cells were not significantly killed by AA3 (Figure 36B) showing that cells in G1 phase are insensitive to AA3. In contrast, the cells that were released from the Mimosine block regained their sensitivity to AA3 (Figure 36B). These results show that cellular DNA replication is required for the ability of AA3 to kill B-NHL cells. In other words, AA3 should only kill cells that undergo cell division. And I analyzed the total number of control cells before and after AA3 treatment to confirm that AA3 kills cells that are proliferating. The total number of cells was determined using Bio-rad TC20 cell counter. It was shown that greater than 50% of an increase in the total cell number of all of four B-cell lymphoma cell lines and normal human B-cells after 24 hours (Figure 36C). This observation confirmed that these cells are proliferating well.
Figure 36 AA3 cytotoxicity is cell cycle dependent. (A) Cell cycle analysis by flow cytometry after Mimosine treatment. Daudi cells were incubated with 100µM Mimosine for 24 hours. After that, treated cells were divided into two parts and grown in the medium with and without 100µM Mimosine separately for 4 hours. Cells were collected, and DNA was stained with propidium iodide for flow cytometric cell cycle analysis. (B) Killing of G1-arrested Daudi cells by AA3. Daudi cells treated with or without Mimosine (Mimosine treated or control) and cells released from Mimosine block (Mimosine removed) were incubated with AA3 for 5 hours and cell viability was examined. Viability data was normalized to cell viability of cells without AA3 treatment (set to 100). The mean and standard deviation are shown in each case (**P<0.005, n.s. = no significance). (C) Analysis of the total cell number of B-NHL cells and normal B cells before and after 24-hour growth. The total cell number was counted using TC cell counter.

3.5.7 Other alkoxyamines do not kill B-NHL cells

Methoxyamine reacts with AP sites and has been used to increase the effectiveness of known chemotherapeutic agents [143, 149]. However, unlike AA3, MX has not been shown to kill any cancer cell on its own. ARP is also known to react with AP sites as AA3 but has also not been shown to kill cancer cells. We tested the possibility that MX and ARP may kill B-NHL cells. Neither MX nor ARP killed Daudi cells (Figure 37A). At the maximum concentrations tested, 10 mM, AA3 reduced the viability of Daudi cells to ~10% while MX or ARP showed no significant effect on cell viability (Figure 37A). These two chemicals also did not significantly kill other B-NHL cells, Raji and Toledo (Figure 37A).

It seemed possible that the lack of sensitivity of Daudi cells and other B-NHLs to MX or ARP may be because these chemicals are not efficiently taken up by the cells or are actively exported by them. To test this possibility, I pre-treated Daudi cells with MX followed by AA3 treatment and compared the effects of this sequential treatment on cell viability to treatment with AA3 or MX alone. The results showed that MX pre-treatment suppressed the toxicity of AA3 (Figure 37B) and strongly suggest that both MX and AA3 react with the same cellular targets, presumably AP sites in DNA.
Figure 37 Killing of B-NHL cell lines by other alkoxyamines. (A) Cytotoxicity of MX or ARP in B-NHL cell lines. Daudi, Raji and Toledo were treated with 10mM MX or ARP or AA3 for 24 hours, and cell killing was analyzed. (B) Inhibition of AA3 cytotoxicity in Daudi cells by pre-treating MX. Daudi cells were treated with 10mM MX for 6 hours, followed by incubation with 2mM AA3. Cell viability was determined after 24 hours and normalized to that of untreated cells (set to 100). The mean and standard deviation are shown in each case (** P<0.005, n.s. = no significance). NT stands for untreated cell.

3.5.8 Alkyne functionality of AA3 is important for its toxicity

The lack of toxicity of MX and ARP for B-NHL cells suggested that the alkoxyamine functionality within AA3 was not sufficient for its biological action. As the only other functional group within AA3 is the weakly reactive alkyne groups connected to the alkoxyamine by a methylene bridge (Figure 38A), variants of this functional group were synthesized and their toxicity toward Daudi cells was tested. In particular, I synthesized and tested molecules in which the alkyne functionality was replaced with a methyl group (AA4) or an azide group (AA6) and a molecule in which the alkyne was separated from the oxygen by an additional carbon-carbon bond (AA5; Figure 38A).
Remarkably, neither compounds lacking the alkyne functionality, AA4 and AA6, was toxic to Daudi cells (Figure 38B). In contrast, AA5, which contains the alkyne functionality killed these cells, but less efficiently than AA3 (Figure 38B). These results suggest that the bioorthogonal alkyne functional group is required for the toxicity of these alkoxyamines toward B-NHL cells, and their effectiveness is modulated by the distance between alkoxyamine and alkyne groups.

![Cytotoxicity of AA3 analogs including AA4, AA5 and AA6.](image)

**Figure 38 Cytotoxicity of AA3 analogs including AA4, AA5 and AA6.** (A) Cytotoxicity of AA3 and analogs in Daudi cells. Daudi cells were treated with different alkoxyamines at different concentration (0, 1, 2, 5, 10mM) for 24 hours. Cell viability was normalized to that of untreated cells (set to 100). The mean and standard deviation are shown in each case. (B) Structures of AA3, AA4, AA5 and AA6.
CHAPTER 4: DISCUSSIONS AND FUTURE DIRECTIONS

4.1 AA3 efficiently labels and detects AP sites in DNA

In this study, I created a small alkoxyamine with alkyne functionality called AA3. The reactivity of AA3 toward AP sites was examined using a synthetic oligomer. However, the linking of AA3 to AP sites did not create a shift in mobility of DNA band due to the small size of AA3. Therefore, I carried out the competition experiment with ARP and the experiment for the direct detection of AA3 binding through click reaction. Both of them confirmed that AA3 labels AP sites of DNA more efficiently than ARP at physiology condition (Figure 23A, 23B). Compared to AA3, ARP is much larger. Hence, the steric hindrance of ARP could inhibit its reactivity toward AP sites. In the competition experiment, the ARP-tagged product was hardly visualized after DNA substrate was first incubated with AA3 (Figure 23A, lane 6), suggesting all AP sites in DNA were bound by AA3. But following the reaction with AA3, biotin azide did not entirely convert substrates to biotinylated form. The reason could be that click reaction is not complete. Further optimization of click reaction needs to be carried out to improve the efficiency of this reaction.

AA3 has a better pH profile than ARP. AA3 converted 57% or more DNA to the product over the pH range of 4 to 7. In contrast, less than 20% of the substrate was labeled by ARP at pH 7 while greater than 70% DNA was tagged by ARP at pH 4 to 5 (Figure 24). This result suggests that ARP can react with AP sites more efficiently at the acidic condition. In the condensation reaction between aldehyde and alkoxyamine, the nucleophilic nitrogen of an alkoxyamine attacks the electrophilic carbonyl carbon of an aldehyde to form as a stable oxime. Under the physiological condition, the reactivity of ARP is relatively low because the nucleophilic addition of ARP is sterically hindered. However, at the acidic condition, oxygen of
the carbonyl group of an aldehyde is protonated. This protonation increases the electrophilicity of carbonyl carbon and confers carbonyl group more susceptible to nucleophilic attack by ARP. Therefore, acids catalyze this condensation reaction and increase the reactivity of ARP toward AP sites in DNA.

A novel technique for quantification of AP sites in cellular DNA using AA3 was developed in the study, and it shows greater sensitivity and lower background than ARP-based ELSA like assay. The membrane from the ARP-based assay displayed much higher background than that from the AA3-based assay (Figure 27A). In the AA3-based detection method, prior to being transferred onto a membrane, processed samples are purified by ethanol precipitation or some spin column, which removes unlabeled fluorescent azides eliminating background. In the ARP-based method, the membrane has to be incubated with streptavidin-conjugated HRP or fluorescence molecule to visualize and detect ARP binding [92, 147, 148]. However, it is hard to completely eliminate non-specific binding of streptavidin protein or fluorescent molecule onto the membrane through the washing step. This problem may result in a considerable background. Furthermore, ARP is less reactive with AP sites in a short oligo (17-mer) (Figure 25). It is much possible that the reactivity of ARP could be much lower when it reacts with genomic DNA. Hence, in addition to high background, the low reactivity could also contribute to the lower sensitivity of ARP-based detection assay.

As AA3 contains alkyne functionality, in vivo labeling of AP sites by AA3 can be detected through click chemistry. To determine whether AA3 binds to AP sites of DNA in vivo, cells were treated with AA3 and AA3 binding was then detected by incubation of genomic DNA with Cy5 azide. In the study, this method was used to examine whether AA3 reacts with AP sites in Daudi
cells. However, neither MX nor ARP has this ability. Although ARP contains a biotin tag for
detection, ARP is bulky and may pass hardly to the cell membrane. Biotin also exists in cells, and
the use of ARP for labeling AP site in live cells would cause non-specific binding. Additionally,
the effectiveness of ARP in labeling AP sites in live cells would be largely affected by its low
reactivity. MX is a small molecule and can easily enter cells through diffusion, but it does not
have any tag. Radioisotope technique has to be used for the detection of MX binding in vivo
[24]. Hence, AA3 is an ideal tool to label AP sites in live or fixed cells and to monitor the
formation of AP sites in situ by DNA damaging agents.

4.2 AA3 inhibits base excision repair and is more effective to enhance the cytotoxicity of DNA
alkylating agents than MX.

MX is well known as a BER inhibitor, which blocks the cleavage of AP sites by AP
endonuclease[8]. I have shown that AA3 inhibits APE1 activity as well as MX but ARP is a poor
inhibitor of APE1 at the physical condition. The failure of inhibition of ARP on APE1 enzyme
could be attributed by the relatively low reactivity of ARP toward AP sites. Since AA3 and MX
inhibit APE1 enzyme equally well, it was expected that AA3 should be as good as MX when it is
used in combination chemotherapy to improve antitumor efficacy of DNA alkylating agents.
Surprisingly, AA3 is more efficient than MX to kill cancer cells treated with MMS or TMZ. One of
the reasons could be that cells process AA3-bound DNA and MX-bound DNA differently. It has
been reported that MX-AP sites in DNA could be removed by long-path BER pathway [77].
Other studies show that MX-AP sites could be excised through NER pathway [77]. These
evidences indicate that it is possible that a part of MX-AP sites could be repaired, although the
mechanism is not clear yet. The differences between MX and AA3 in chemical structure and size
could result in that AA3-AP sites are less susceptible to these repair pathways. Further work needs to be done to clarify why AA3 is better than MX in combination therapy.

When HeLa cells were treated with TMZ and MX, MX failed to significantly increase cell killing by TMZ. Many studies have shown that MX is capable to sensitize brain tumor cells to TMZ treatment [43, 81, 149]. HeLa cells may be not sensitive to TMZ treatment. As a consequence, this concentration of TMZ may not induce enough AP sites. In contrast, AA3 is more effective so that binding a moderate level of AP sites by AA3 may cause cell death. Therefore, increasing the concentration of either TMZ or MX could improve cell killing. In addition to MMS and TMZ, other DNA-damaging agents could also be combined with AA3 to increase antitumor efficacy of chemotherapy. For example, AA3 will be used in combination therapy with pemetrexed, which has been successfully coupled with MX to kill lung and other solid tumors [79, 150].

4.3 AA3 specifically kills B-cell lymphoma cells

Blockage of BER pathway by targeting AP sites could be considered as a potential strategy for cancer therapy. Two recent papers [90, 92] have shown that B-cell lymphomas derived from germinal centers have high levels of genomic uracils induced by AID. When cells endeavor to maintain genomic stability by removing these uracils through the action of uracil-DNA glycosylase, this will lead to the formation of AP sites. Therefore, these B-cell lymphoma cells may have high levels of AP sites accumulation in their genomes, if there is an imbalance of the downstream enzymes. To confirm this, I quantified AP sites in B-cell lymphoma cells. Cell lines derived from Burkitt lymphoma (Raji and Daudi) and DLBCL (Toledo) showed relatively high levels of endogenous AP sites compared to normal human B cells (Figure 31A). In this study,
AP sites were quantified by the AA3-based assay which is based on the reaction between AA3 and the aldehyde residue of an AP site. Therefore, AP sites detected by AA3 in this assay could be aldehydic lesions in DNA including intact AP sites, cleaved AP sites and other sites with the aldehyde moiety [151]. Moreover, AP sites are often created in several different ways. Thus, AP sites in B-cell lymphoma cells reported here are not entirely generated through the excision of uracils by uracil-DNA glycosylases. But up-regulated UNG2 expression upon the induction of uracils observed in these cells [92] could be one major cause for the increase in AP sites. Additionally, I also analyzed AP sites in non-B cells that express AID at very low levels (Figure 31B). All of them show much lower levels of AP sites than B cell cancers (Figure 31A). These results indicate that AID expression may be associated with the formation of AP sites and incomplete downstream repair results in accumulation of AP sites.

As B-cell lymphoma cells have high levels of AP sites in their genome, I hypothesized that AA3 could be used to target these AP sites and kill cells. In this study, I demonstrated AA3 alone can effectively kill B-cell lymphoma cells at a sub-millimolar concentration (Figure 32A), but it is not toxic to normal human cells including B lymphocytes and keratocytes even at high concentration (10mM) (Figure 33A). Although chemotherapy has been the most prevalent strategy for anticancer treatment for decades, one major limitation in the use of current chemotherapeutic agents arises from toxicity to normal cells. Reducing side effects to patients is still one of the main issues that need to be addressed during drug discovery and development. As AA3 is not toxic to normal cells, AA3 could be a potential candidate for antitumor treatment. An additional finding is that AA3 at high concentration (10mM) did not significantly kill other types of cancer cells such as HeLa, MCF-7, A549 and MDA-MB 453 (Figure 33B), suggesting that
AA3 selectively kills B-cell cancers. If AA3 killed cells by targeting AP sites as I proposed, the level of AP sites may be the key factor that determines the sensitivity of cells to AA3 treatment. AP sites detected in these non-B cells are 12 fold lower relative to B-cell cancer cells in average (Figure 31A). This could explain why they are resistant to AA3 treatment. Furthermore, compared to the sensitivity of four B-cell lymphoma cell lines tested in the study to AA3, Daudi cells appear to be more sensitive than other three cell lines (Figure 32A). This result is consistent with the fact that Daudi cells show a higher number of AP sites than others (Figure 31A).

The success of AA3 in killing B-cell cancers encouraged me to treat these cells with other alkoxyamines. MX and ARP were used to treat Daudi, Raji and Toledo cells. However, no significant cell killing by either MX or ARP was observed, except that less than 10% Raji cells were killed by MX (Figure 37A). Conversely, AA3 at the same concentration killed most of these cells. The reasons for the failure of killing cells by ARP could be that the reactivity of ARP with AP sites is much lower than AA3 [151] and ARP may be harder to pass through the cell membrane because of its bulky size. As MX is well known to kill cancers treated with DNA-damaging agents by targeting AP sites and blocking DNA repair [8, 81, 152], I highly anticipated that MX could also kill B-cell cancers showing high levels of AP sites. However, the result of cell killing by MX is extremely surprising. In this project, I have shown that MX enhances cell killing by MMS, but it is not as good as AA3. As I described above, a portion of MX-AP sites may be repaired or removed through a certain pathway. This could also result in that MX kills B-cell cancers less efficiently than AA3. MX even at a high concentration (10mM) did not kill these cells significantly. The resistance of these cells to MX could arise from the complete removal of
MX-AP sites by upregulated repair proteins in these B-cell cancers. In contrast, the high level of AA3 \textit{in vivo} binding to Daudi DNA shown in figure 34A suggests that AA3-adducted DNA could not be removed and thereby it causes cell death. Secondly, drug uptake and efflux could be associated with the failure of killing B-cell cancers by MX. However, MX was shown to inhibit cell killing by AA3 when cells were pretreated with MX (Figure 37B), implying that MX targets cellular DNA and blocks AA3 binding. The result demonstrated that MX could be efficiently taken up by these cells. Therefore, the issue of drug uptake can be ruled out. Further studies are needed to understand the drastic difference between MX and AA3 in the toxicity for B-cell lymphoma cells, despite the fact that they show similarity in reactivity with AP sites.

Understanding the molecular mechanism of AA3 cytotoxicity in B-cell cancers will be useful for development AA3 as a drug for anti-tumor treatment. I first proved that AA3 covalently links to AP sites in B-cell lymphoma cells. Daudi cells treated with AA3 showed a great reduction in AP sites in genomic DNA detected by ARP-based assay (Figure 34B), indirectly suggesting that AA3 binds to AP sites in cells preventing ARP from bindings to genomic DNA \textit{in vitro}. Similarly, when MX is used in combination therapy with DNA-damaging agents like TMZ and pemetrexed, the level of AP sites detected by APR decreased after MX treatment [7, 43, 150].

Also, I directly demonstrated that AA3 binds to DNA \textit{in vivo}, by detecting and quantifying AA3-bound sites in DNA through click chemistry. In this experiment, I observed AA3 \textit{in vivo} binding in both Daudi and HeLa cells after AA3 treatment, but the level of AA3-bound sites in Daudi cells was much higher than that in HeLa (Figure 34A). That is because Daudi cells have more AP sites than HeLa (Figure 31A). Also, it was found the level of AA3 binding in Daudi
cells greatly elevated with the increasing length of AA3 treatment (Figure 34A). One reason could be that it takes more time for AA3 to completely bind to all AP sites of cells. The longer cells are incubated with AA3, the more AA3 binds to DNA. Moreover, lot of DNA from dead cells is often lost during DNA extraction procedure, and cells with high level of AA3 binding are more likely to die or be dying. Therefore, the amount of DNA extracted from cells with high levels of AA3-bound sites is less than that from cells with low levels of AA3-bound sites. Since the percentage of dead cells after 5-hour treatment was lower than that after 24-hour treatment, DNA from dead cells with high level of AA3 binding after 5-hour treatment is diluted more. This could be the second reason that results that the overall level of AA3-bound sites in cells with 5-hour treatment are lower. Thirdly, dead cells are likely to take more AA3, since the membrane of dead cells is more permeable. This could also result in that the level of AA3 binding is higher after 24-hour treatment. Theoretically, AA3 binding should not be observed in cells without treatment. However, the control cells showed a small amount of AA3 binding, which could result from the background of this assay.

In another experiment, MX inhibited cytotoxicity of AA3 in Daudi cells that were pretreated with MX (Figure 37B), further indicating that MX and AA3 share the same target, AP sites. We compared the level of AA3-bound sites in viable cells and dead cells after AA3 treatment. Dead cells showed an extremely high level of AA3 binding, whereas the level of AA3 binding in viable cells is as low as that in control cells (Figure 34C). This result strongly indicates that AA3 binding to DNA is associated with cell death.

There is some evidence showing that MX enhances the cytotoxicity of DNA-damaging agents via induction of DNA breaks and cell apoptosis [43, 80, 144]. The unprocessed MX-AP
adducts block the progression of DNA replication and cause replication fork collapse resulting in the accumulation of DNA breaks. I found AA3 inhibited cell growth. This inhibition could result from the stalled DNA replication fork caused by AA3-DNA adducts. Moreover, when DNA replication was blocked by arresting cells at G1 phase, cells exhibited less sensitivity to AA3 treatment (Figure 36B). It clearly implies that DNA replication process is necessary for AA3 cytotoxicity. I also detected a significant increase in DNA double-strand breaks in AA3-treated cells by immunostaining γ-H2AX (Figure 35). Altogether, these results indicate the molecular mechanism of AA3 action could be that AA3 reacts with AP sites in B-cell cancers and AA3-DNA adducts lead to cell death by blocking DNA replication and inducing DNA breaks (Figure 39).

**Figure 39 The proposed mechanism of AA3 cytotoxicity in B-cell cancers.** AA3 covalently binds to B-cell cancer genome, but the AA3-bound AP sites are resistant to DNA repair. As a result, AA3-DNA adducts cause cell death by blocking DNA replication fork and elevating DNA breaks.

Although AA3 alone can kill B-cell cancers, the IC50 of AA3 in Daudi cells (500μM) is high. Thus, there is a need to improve its potency through combination with other antitumor drugs or chemical modification. AA3 will be coupled with DNA-damaging agents or BER inhibitors to
increase its antitumor efficacy and reduce IC50. To improve drug efficacy via structure modifications, I investigated what functional group is necessary for AA3 toxicity. Since MX and AA3 display different toxicity in B-cell lymphoma cells, what structural difference contributes to their functional difference needs to be examined. Three AA3 analogs were synthesized (Figure 38B). AA4 and AA6 that do not contain the alkyne functionality fail to kill Daudi cells. But AA5 with a terminal alkyne is toxic to Daudi cells although it is slightly less effective than AA3 (Figure 38). This data demonstrated the alkyne functional group rather than the bigger size is responsible for AA3 cytotoxicity in B-cell lymphoma cells. This finding is very surprising since the alkyne group is often considered as an unreactive functional group at physiological conditions. Terminal alkynes are widely used in bioorthogonal chemistry for labeling biomolecules due to their inert property. One previous paper reported a reaction between terminal alkynes and Cysteine in proteases, in which a terminal alkyne reacts with a thiol of Cysteine forming a vinyl thioether linkage [153]. The terminal alkyne group of AA3 may bind to certain proteins such as DNA repair machinery proteins through this reaction and prevent AA3-bound AP sites from being repaired. Further experiments need to be carried out to elucidate how the alkyne functionality contributes to AA3 cytotoxicity in B-cell lymphoma cells.

In summary, the results of this project demonstrated that a small alkoxyamine, AA3, that efficiently label AP sites in cellular DNA can be used in combination therapy coupling with DNA-damaging agents and specifically kill these B-cell lymphoma cells with high level of AP sites. B-cell lymphomas are typically treated with a combination of a monoclonal antibody (rituximab) and at least four chemotherapeutic drugs such as cyclophosphamide, doxorubicin, vincristine and prednisone. In this study, AA3 is able to kill these cancer cells without the assistance of
other antitumor drugs. This work suggests the family of alkoxyamines could be proposed as a unique class of anti-cancer drugs.
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*Portions of the text and figures in this dissertation were reprinted or adapted from DNA repair journal, Vol 27, Wei, S., Shalhout, S., Ahn, Y., Bhagwat, A.S., A versatile new tool to quantify abasic sites in DNA and inhibit base excision repair, Pages 9-18, Copyright (2015), with permission from Elsevier.*

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ABSTRACT

DEVELOPMENT OF A NOVEL CLASS OF CHEMICALS FOR LABELING ABASIC SITES IN CELLULAR DNA AND KILLING CANCER CELLS

by

SHANQIAO WEI

December 2016

Advisor: Dr. Ashok S. Bhagwat
Major: Chemistry (Biochemistry)
Degree: Doctor of Philosophy

Abasic (AP) sites are the most common type of lesions in DNA. Numerous endogenous and exogenous agents and cellular processes can induce the formation of AP sites in DNA. If left unrepaired, the deleterious AP sites cause mutagenesis and cytotoxicity. Methoxyamine is known to react with AP sites and block base excision repair. Another alkoxyamine, aldehyde-reactive probe (ARP) tags AP sites with a biotin and has been widely used to quantify these sites. In this study, I have combined both these abilities into one alkoxyamine, AA3, which reacts toward AP sites with better reactivity than ARP at physiological pH. Additionally, AA3 containing an alkyne functionality is able to tag AP sites with a biotin and a fluorescent molecule through click chemistry. AA3 is used for AP site quantification with greater sensitivity and lower background than ARP. AA3 also inhibits the first enzyme in the repair of AP sites, APE-1, to about the same extent as methoxyamine. Furthermore, AA3 enhances the ability of alkylating agents to kill cancer cells and is more efficient in such combination chemotherapy than methoxyamine.
The majority of B-cell malignancies originate from germinal centers, where the processes of somatic hypermutation (SHM) and class switch recombination (CSR) for antibody maturation are initiated by activation-induced deaminase (AID). AID deaminates cytosines in DNA creating uracils. It has been previously shown that B-cell lymphoma cell lines and patient tumors express AID at high levels and contain high levels of uracils in their genome. These cells also contain uracil-DNA glycosylases that remove uracils forming AP sites. I have demonstrated that B-cell lymphoma cell lines contain much higher levels of AP site accumulation than normal B cells and non-B cell lines. AP sites are able to react with alkoxyamines forming a stable oxime. This principle was used to block the repair of an excess of AP sites in B-cell cancers by alkoxyamines. AA3 effectively kills B-cell lymphoma cell lines that show high levels of endogenous AP sites. In contrast, AA3 is not toxic to normal human B cells, as well as non-B cell lines. However, neither MX nor ARP displays cytotoxicity in these B-cell cancers, although both of them can target to AP sites. Additionally, AA3 links covalently to B-cell cancer genome, and AA3-DNA adducts cause cell death by blocking DNA replication and elevating DNA strand breaks.

To determine which functionality of AA3 is responsible for its cytotoxicity, I designed and synthesized a series of AA3 analogs. Using this approach, I have demonstrated the alkyne functional group is required for AA3 toxicity in B-cell cancers. Overall, this new family of chemicals could be further developed as novel anti-cancer drugs.
AUTOBIOGRAPHICAL STATEMENT

SHANQIAO WEI

Education

Ph.D. Chemistry (Biochemistry) 2011-present
Wayne State University, Detroit, Michigan
Research Advisor: Prof. Ashok S. Bhagwat

M.S. Medicinal Chemistry 2006-2009
Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu China

B.S. Biological Science 2002-2006
East China Normal University, Shanghai, China

Publications


H. Shao, S. Wei, J. Zhao. Method for preparing glycogen ester. CN 200810045480 [P].

Awards and Honors

Biological chemistry graduate students award 2016
Summer dissertation fellowship 2016
Graduate Student Professional Travel Award 2015
Thomas C. Rumble University Graduate Fellowship 2015