The Onyx Review: The Interdisciplinary Research Journal
2017, Vol. 3, No. 1, pp. 53–63

Exploiting Nucleophilic Attack in Chemotherapy

Pooja Venkatesh
Agnes Scott College

Alkylating agents - a large class of clinically used chemotherapy drugs - target tumors via various DNA-drug inter/intrastrand linking mechanisms. These drugs form electrophilic compounds under physiological conditions, allowing for nucleophilic attack by intracellular macromolecules, including DNA. This review will focus on electrophilic addition by the nucleotide guanine, whose nitrogen and oxygen atoms facilitate covalent linkage between the reactive pharmaceutical and the DNA strand. Three examples of alkylating agents, mechlorethamine, mitomycin C, and lomustine, will be discussed. The first chemotherapeutic drug, mechlorethamine, forms reactive aziridinium rings, following loss of a chloride ion in aqueous solution. The resultant cation is then free to alkylate N-6 of guanine. The second compound, mitomycin C, undergoes bioreduction and ring-opening steps, ultimately forming DNA adducts after bond formation to N-2 of guanine. Finally, lomustine spontaneously decomposes in cells forming an alkylating and carbamoylating agent. While the carbamoylating compound can react with lysine residues to inactivate DNA repair enzymes, the alkylating agent is free to attach to O-6 of guanine. While this class of drugs has proven to be highly effective in chemotherapy, alkylating agents are notorious for their cytotoxicity and poor molecular selectivity because of their reactive electrophilic nature. Significant research into drug development must be conducted to improve clinical outcomes and reduce the deleterious side effects of these agents.

Introduction to alkylating agents:

Nonhormonal alkylating agents were the first class of effective anticancer drugs developed. Pharmaceuticals within this class form covalent linkages to DNA, thereby halting DNA synthesis and cancerous cell proliferation.[1] Of note, the term "alkylating agent" inaccurately describes the chemistry of these drugs, as they do not add organic alkyl groups to target nucleotides. Instead, the entirety of the electrophilic, active form of the drug, attaches to DNA in a nucleophilic addition reaction.[1] Often, the portion of the molecule directly involved in the reaction is composed of inorganic elements. The mechanisms of three subclasses of alkylating agents will be detailed here.

Research into alkylating, anticancer drug development began after physicians observed that World War I soldiers exposed to sulfur gases displayed lymphoid aplasia, or disrupted development of lymphoid tissue.[2, 3] The early 1900s marked a time period of significant chemical warfare research and the first large scale uses of toxic mustard gases.[4] Nitrogen mustards were developed in the 1930s and found to inhibit cell division of lymphoid tissue, bone marrow, and gastrointestinal cells in affected soldiers.[4] This led scientists to investigate the potential of nitrogen mustards in inhibiting cancer proliferation. A group of Yale scientists conducted the first chemotherapy clinical trials in 1942 using nitrogen mustards on a sample of terminal lymphosarcoma patients.[4] Success of these first clinical trials began common use of chemotherapy agents in the treatment of tumors.[5] Today, mustards are frequently used as anticancer agents with common drugs including cyclophosphamide, ifosfamide, and chlorambucil - all of which share a bischloroethyl substituent (see Figure 1).[1] An additional example, mechlorethamine (also referred to as

chlormethine) will be reviewed here. Chlormethine is in clinical use for treatment of chronic lymphocytic and myelogenous leukemia, bronchogenic carcinoma, and advanced Hodgkin lymphoma among other specialized forms of cancer.[6] Drugs of this class react in the body to form highly electrophilic aziridinium ions that are then capable of DNA crosslinking, thereby terminating mitosis in dividing cells.

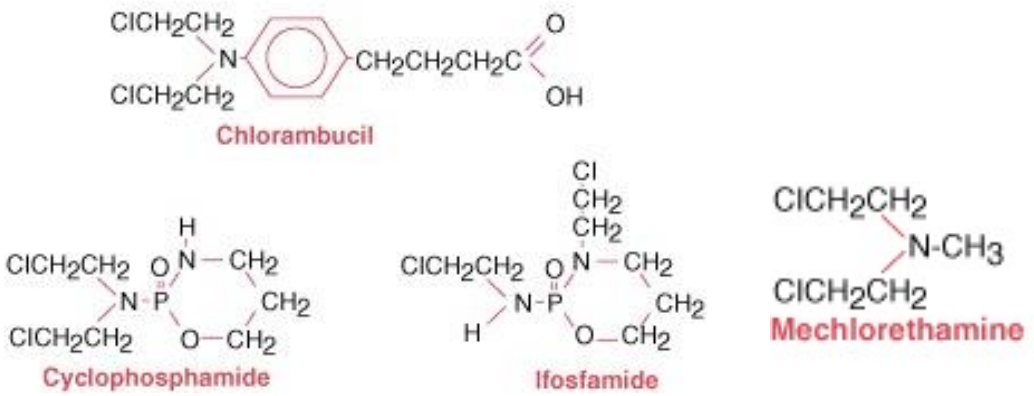

Figure 1: Examples of Currently Used Nitrogen Mustards [7]

Naturally occurring aziridine-containing compounds are a second subclass of widely used chemotherapeutic agents that are closely related to the nitrogen mustards. One of the most toxic antitumor drugs in current clinical use is the aziridine mitomycin C (MMC) (see Figure 2).[8] MMC was discovered and purified from the gram-negative bacterium *Streptomyces caespitosus* in 1958 and has been studied for its significant antibacterial and anticancer properties.[8, 9, 10] The compound is capable of crosslinking DNA at specific adenine and guanine nucleotide motifs during the late G_1 or S phases of cell division, as well as preventing gene expression by binding inducible promoter sequences.[9] MMC is considered a prodrug as it is converted into the active electrophile after intracellular reduction and ring-opening steps.[8] The pharmaceutical is currently being used for treatment of advanced or metastasized gastric cancers and pancreatic adenocarcinoma.[11]

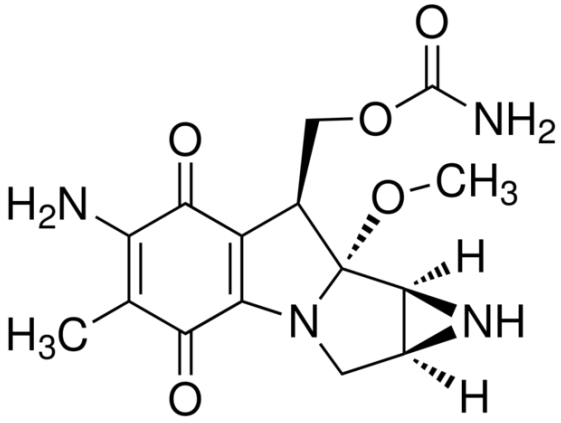

Figure 2: Structure of Mitomycin C [12]

As a third subclass, examples of nitrosoureas will also be analyzed as commonly used chemotherapy drugs. This class of chloroethyl-containing anticancer agents was first developed in the 1960s and consists of prodrugs that decompose after administration to form an electrophilic alkylating agent and a carbamoylating agent. While the alkylating compound forms interstrand DNA linkages, the second

compound carbamoylates lysine residues on intracellular proteins and has been demonstrated to inactivate DNA repair enzymes.[8] Examples of frequently used nitrosoureas include lomustine, carmustine, and streptozotocin. The mechanism of lomustine (structure shown in Figure 3) will be outlined in this paper. This drug is currently used in the treatment of multiple myeloma, primary brain tumors, and lymphomas.[1, 13]

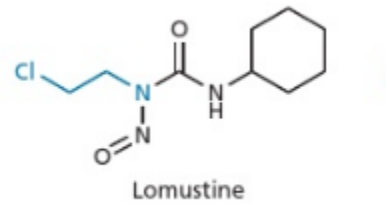

Figure 3: Structures of nitrosourea lomustine [8]

While there are multiple nucleophilic sites on DNA where attachment to alkylating agents can occur, this review will focus on addition to nitrogen-7, nitrogen-2, and oxygen-6 of guanine (see Figure 4 for numbering convention). Covalent bonding to DNA not only results in steric blocking, preventing transcriptional and replication enzymes from accessing the strands, but can also result in incorrect base pairing. This is possible because guanine exists as two tautomers (constitutional isomers), one keto and one enol form. Under normal physiological conditions, the keto isomer is preferred, allowing for correct base pairing with cytosine. However, addition of certain alkylating agents onto guanine shifts the molecule's equilibrium such that the enol tautomer is preferred. While this may seem inconsequential, the enol isomer is likely to base pair with thymine, rather than cytosine, resulting in miscoded messenger RNA molecules and dysfunctional protein products.[8] This paper will discuss the mechanisms by which the chemotherapeutic drugs mechlorethamine, mitomycin C, and lomustine add covalent linkages to guanine residues in actively replicating cells.

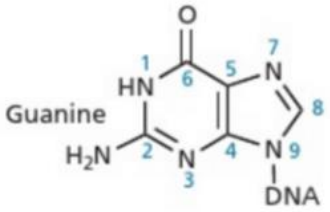

Figure 4: Structure of the Guanine Nucleotide [8]

Discussion:

As mentioned prior, the DNA nucleophilic sites of focus in this paper are N-7, N-2, and O-6 of guanine (see Figure 4). Of these sites, N-7 is of particular interest in cancer research because of its high reactivity relative to other sites of alkylation. This is so because the lone pair on the nitrogen can readily be donated to electrophilic substances, like the nitrogen mustards to be discussed. While guanine adducts at location N-7 readily occur, they are not considered pro-mutagenic as linkages at this site are unstable and often do not disrupt normal base pairing. Half lives of N-7 guanine adducts typically range from 2 hours to 150 hours, a relatively short period compared to other DNA-chemical bonds.[14]

Unlike N-7 of guanine, covalent linkages at O-6, methylation in particular, is highly mutagenic. This is so because addition of electron donating groups at oxygen shifts

equilibrium of the nucleotide such that the enol tautomer is preferred. Consequently, polymerases wrongly insert a thymine, instead of cytosine, opposite the guanine resulting in miscoded DNA, mRNA, and protein.[15] Alkylation by drugs and environmental carcinogens at this site is uncommon, however when adducts are formed to the oxygen they are most potent. Unfortunately, extended clinical use of alkylating agents that target O-6 often results in chemoresistant tumors that upregulate expression of the DNA repair enzyme, methylguanine-DNA-methyltransferase (MGMT), capable of transferring attached methyl groups, on guanine, to cytosine residues.[16]

Lastly, N-2 adducts also promote incorrect guanine base pairing, but not to the extent of O-6 adducts. Studies have demonstrated that the "Y" family of translesion polymerases in mammals is capable of synthesizing error-free, complementary DNA strands despite the presence of covalent linkages at N-2.[17] This means while N-2 adducts often occur due to environmental carcinogen exposure or are specifically targeted in chemotherapy, tumors can upregulate expression of translesion polymerases to bypass the mutagenic effects of alkylation at N-2.[18]

A prime example of anticancer nitrogen mustards is 2-chloro-N-(2-chloroethyl)-N-methylethanamine (mechlorethamine), shown in Figure 5 below. With a molecular weight of 156.05 g/mol, this molecule acts as an alkylating agent following intramolecular rearrangement.[19] Under physiological conditions, chlormethine spontaneously cyclizes to form an aziridinium ion.[20] As depicted in Figure 6, the lone pair on the central nitrogen of the drug forms a bond to a distal carbon, displacing the previously attached chlorine.[8] Of note, this type of intramolecular displacement is an example of anchimeric assistance, a key characteristic of chlormethine that allows the drug to be such an effective alkylating agent.[21] Having formed this highly electrophilic ion, chlormethine can now induce DNA-protein or DNA-DNA crosslinks. Covalent linkages to DNA can form after N-7 of guanine attacks the slightly positive carbon, breaking the C-N bond in the molecule and neutralizing the prior +1 charge. Because chlormethine is a symmetrical molecule, the lone pair on the central nitrogen can repeat the above process by attacking the remaining chlorine-bonded carbon, causing removal of the chloride ion. Once the aziridinium ion is formed, N-7 of a guanine residue on the other DNA strand can now attack the slightly positive carbon forming a second covalent drug-DNA linkage. This interstrand bond formation results in severe steric hindrance of the region of DNA, preventing binding of replication and transcription enzymes to the site of alkylation.[8, 20] This mechanism of action is depicted visually in Figure 5 below.[8] Structurally symmetrical drugs within the nitrogen mustard family follow a similar cross linking mechanism.

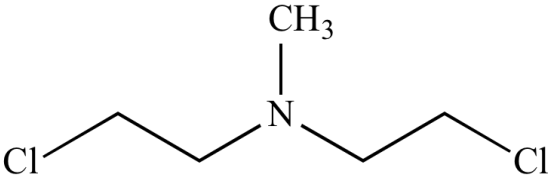

Figure 5: Structure of mechlorethamine [22]

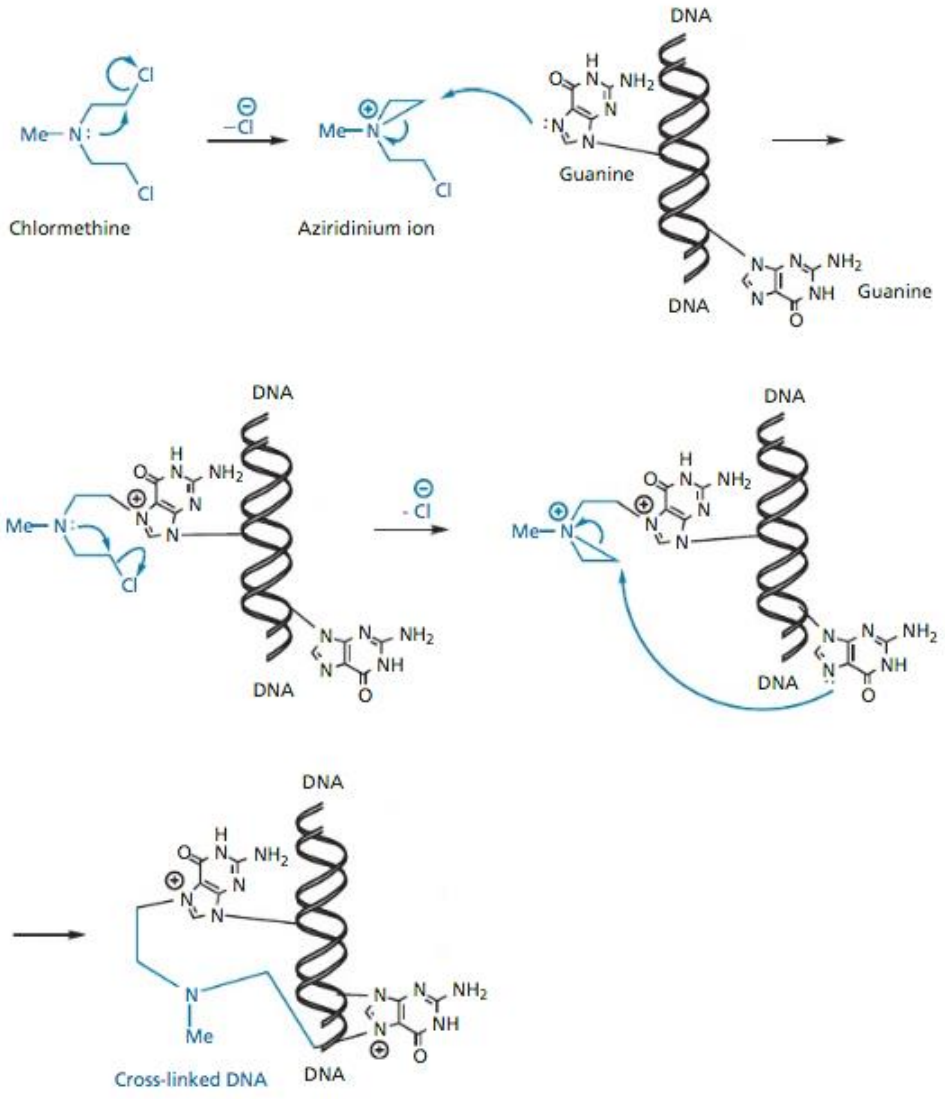

Figure 6: Chlormethine Mechanism of Action [8]

The aziridine-containing drug, mitomycin C, is converted into an active alkylating agent under physiological conditions, making the compound a prodrug.[8] Compared to nitrogen mustards, MMC has a far more complicated, asymmetrical molecular structure composed of a quinone ring system, pyrrolo[1,2-a]indole, carbamate, and aziridine (see Figure 7).[10] Activation of MMC begins with intracellular reduction of the quinone ring to a hydroquinone. Under normoxic conditions, reduction is catalyzed by the flavoenzyme NAD(P)H:quinone oxidoreductase I.

Encoded by the NQO1 gene, this enzyme forms an active homodimer with two FAD molecules bound per subunit. Two reduced NADH or NADPH cofactors are then utilized to catalyze a reductive two electron transfer from NAD(P)H to cytosolic quinones.[23] *In vitro* this reaction was determined to be pH-sensitive, with reaction rates being highest under slightly acidic conditions.[24] Xenograft mouse models found correlations between NQO1 genetic overexpression and MMC sensitivity, as well as correlations with cytochrome P450 reductases, suggesting multiple enzymes can catalyze MMC quinone reduction *in vivo*.[23]

Under acidic conditions, the methanol substituent on MMC is protonated and cleaved from the ring.[10] Following loss of the methanol, the reduced compound undergoes a spontaneous aziridine ring opening to form the active alkylating agent. The nucleophilic NH_2 substituent of guanine N-2 can then attack C-1 of MMC, forming the first covalent linkage between the drug and DNA (see Figure 7 for MMC carbon numbering). Release of NH_3 and CO_2 from the ring carbamate group occurs as N-2 of a guanine residue on the other strand of DNA attacks C-10 of MMC. Completion of this step results in MMC being covalently attached to both strands of DNA at guanine motifs.[8] This mechanism of action is illustrated in Figure 8 below.

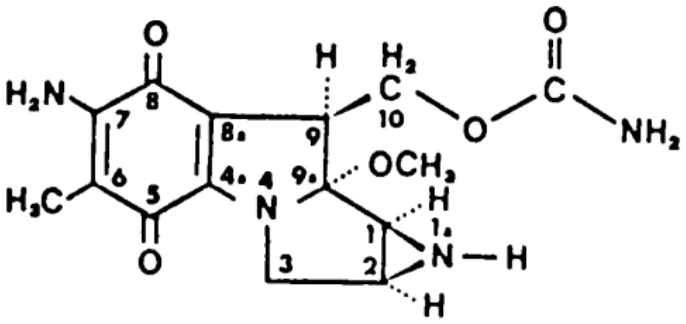

Figure 7: Structure of Mitomycin C [10]

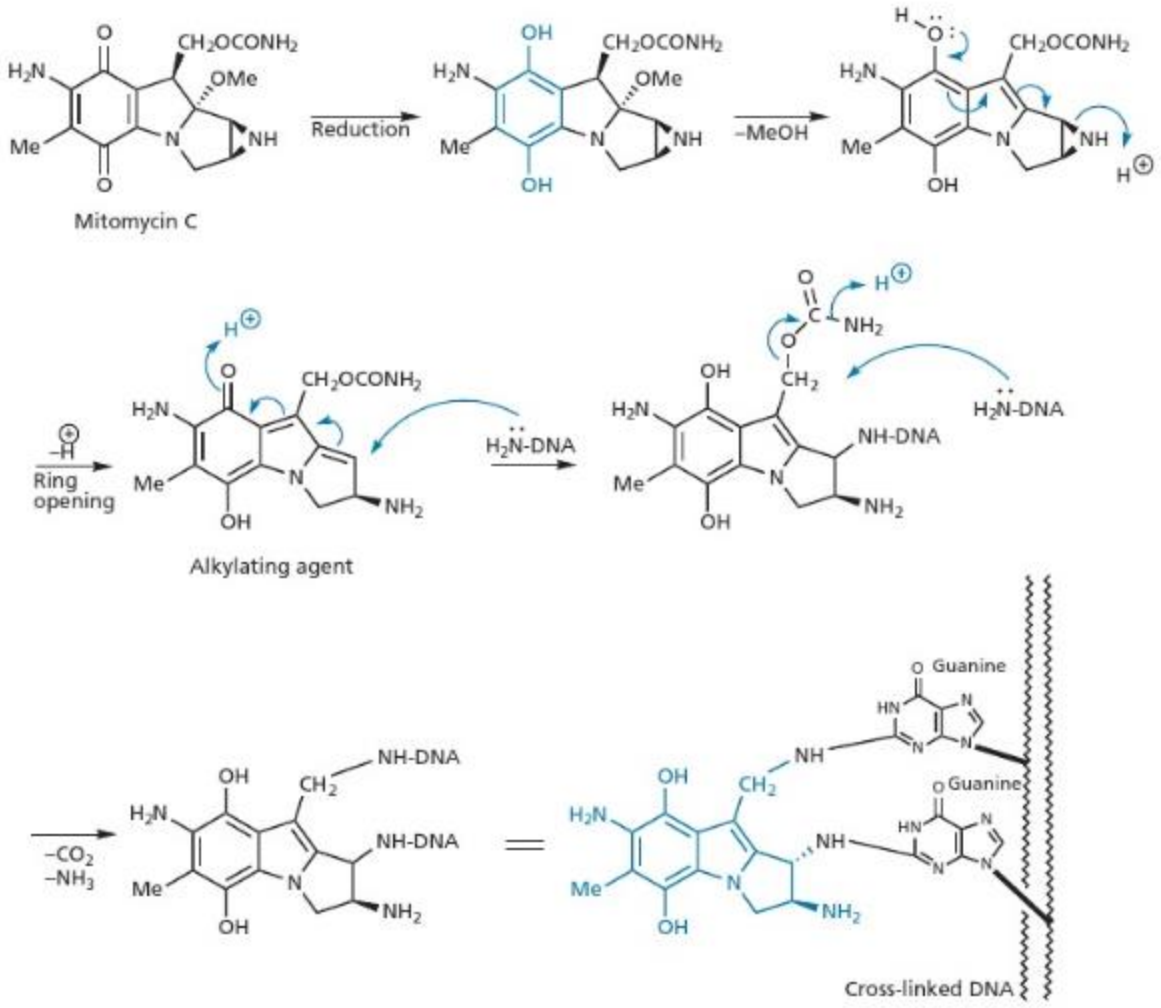

Figure 8: Mitomycin C Mechanism of Action [8]

As a last mechanism for review, the nitrosourea alkylating agent lomustine has a third unique method of DNA covalent linkage. Following spontaneous decomposition in the body, lomustine forms an alkylating and carbamoylating agent. In aqueous media, the process begins when the oxygen of a hydroxide ion attacks the amide hydrogen causing spontaneous release of an isocyanate group and 2-chloroethyl diazene hydroxide.[25] While the isocyanate group can now carbamoylate lysine residues on nearby proteins, inactivating them, the alkylating group undergoes further decomposition. Release of a hydroxide ion and N_2 from 2-chloroethyl diazene hydroxide leaves a highly electrophilic carbonium ion. Unique to the nitrosoureas, O-6 of guanine is the preferred alkylation site. Acting as a Lewis base, oxygen donates a pair of electrons to an empty p orbital on the carbonium ion resulting in a DNA-drug crosslink. Dissociation of a chloride ion from the attached drug allows for a second DNA strand linkage.[8,]

[25]

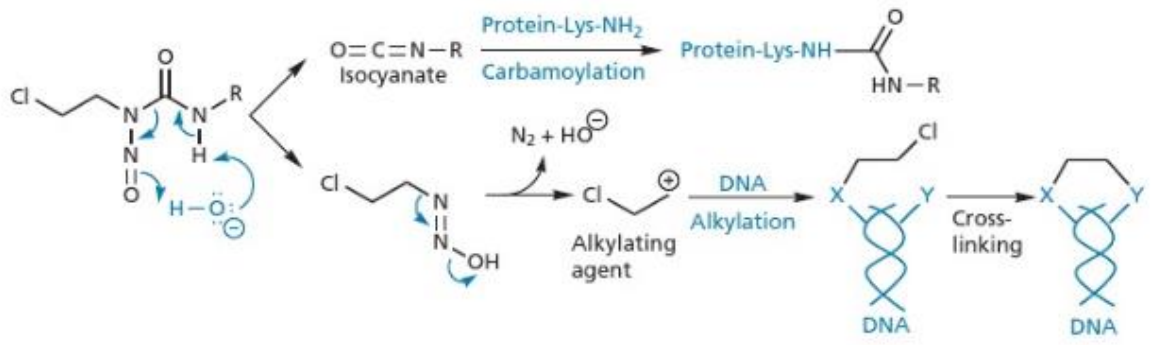

Figure 9: Lomustine Mechanism of Action [8]

Overall, while the chemotherapeutic agents reviewed have significant organic components, the majority of their alkylating activity originates from their inorganic substituents. Covalent addition to DNA residues is further facilitated by inorganic nitrogen and oxygen nucleophiles present on guanine nucleotides. While the three described drugs all alkylate DNA, their mechanisms are quite different from one another. Nitrogen mustards have symmetrical bischloroethyl substituents that initiate aziridinium ion formation, mitomycin C undergoes bioreduction and aziridine ring-opening, and lomustine spontaneously decomposes in solution to form an alkylating and carbamoylating agent. Alkylating agents have proven to be highly effective in treating cancers, however further drug development is necessary to reduce cytotoxicity and increase molecular selectivity of these drugs.

Conclusion:

Ultimately, research into the development and anticancer properties of the nitrogen mustards, aziridine compounds, and nitrosoureas has allowed for great strides in chemotherapy. However, the drugs reviewed, mitomycin C in particular, are known for their serious side effects and high toxicities. Chemotherapy, as a whole, must continue to be heavily researched. While electrophiles are effective alkylating agents, they are also highly reactive molecules that can alkylate proteins, lipids, and other vital components of cells. Furthermore, antitumor drugs are poorly selective and tend to have drastic effects on all proliferating cells in the body.

While the drugs reviewed have well established mechanisms of action, new drugs must regularly be developed to combat the increasing chemoresistance displayed by aggressive cancers. Resistance to mechlorethamine often occurs due to cellular adaptations decreasing active transport of the molecule into the cell. An alternative mechanism for resistance is inactivation of the agent, as performed by aldehyde dehydrogenase enzymes on nitrogen mustards. This enzyme detoxifies the primary metabolites of the mustard compounds rendering them non-reactive. Strong associations between resistance and glutathione (GSH) levels have also been made, suggesting that GSH may protect cancer cells from exposure to highly electrophilic substances.[1] Lu *et al.* demonstrated overexpression of gamma-glutamylcysteine synthetase, a GSH synthesizing enzyme, in melphalan-resistant liver cancer cells.[26] Furthermore, three isozymes of glutathione S-transferase (GST) have been shown to be capable of conjugating GSH to aziridinium ions, preventing alkylation of nitrogen mustards to DNA. Tumor resistance to nitrosourea alkylation is possible through upregulation of the DNA repair enzyme O6-alkylguanine-DNA-alkyltransferase (O6-AT) that

catalyzes the removal of covalently attached groups at O-6 of guanine residues, undoing the interstrand DNA crosslinks of nitrosoureas drugs.[1]

Severe toxicity due to alkylating agents affects multiple physiological systems in receiving patients including hematopoietic, gastrointestinal, gonadal, and pulmonary toxicities.[1] As mentioned, extended research must be conducted to alleviate the severe side effects of chemotherapy drug use and improve drug efficacy.

References

1. Colvin, M. Alkylating Agents https://www.ncbi.nlm.nih.gov/books/NBK12772/ (accessed Apr 27, 2017).
2. Adair, F. E.; Bagg, H. J. Experimental and Clinical Studies on the Treatment of Cancer by Dichlorethylsulphide (mustard gas) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1398743/?page=1 (accessed Apr 27, 2017).
3. Bradbury, S.; Micklem, H. S. Amyloidosis and Lymphoid Aplasia in Mouse Radiation Chimaeras https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1920353/ (accessed May 1, 2017).
4. Pechura, C. M. History and Analysis of Mustard Agent and Lewisite Research Programs in the United States https://www.ncbi.nlm.nih.gov/books/NBK236059/ (accessed Apr 27, 2017).
5. Mechlorethamine https://pubchem.ncbi.nlm.nih.gov/compound/mechlorethamine#section=Pharmacology (accessed Apr 27, 2017).
6. Mechlorethamine Hydrochloride https://www.cancer.gov/about-cancer/treatment/drugs/mechlorethaminehydrochloride (accessed Apr 27, 2017).
7. Colvin, M. Structures of nitrogen mustards currently used in therapy https://www.ncbi.nlm.nih.gov/books/NBK12772/figure/A12437/?report=objectonly (accessed Apr 27, 2017).
8. Patrick, G. L. An Introduction to Medicinal Chemistry 5th Edition Graham L Patrick http://www.academia.edu/22983454/An_Introduction_to_Medicinal_Chemistry_5th_Edition_Graham_L_Patrick_PDF_Deth_/ (accessed Apr 27, 2017).
9. Li, N. Y. K.; Chen, F.; Dikkers, F. G.; Thibeault, S. L. Dose-dependent effect of mitomycin C on human vocal fold fibroblasts https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4113207/ (accessed Apr 27, 2017).
10. Verweij, J.; Pinedo, H. M. Mitomycin C: mechanism of action, usefulness and limitations. https://www.researchgate.net/publication/21025071_Mitomycin_C_Mechanism_of_action_usefulness_and_limitations (accessed May 1, 2017).
11. Mitomycin C https://www.cancer.gov/about-cancer/treatment/drugs/mitomycinc (accessed Apr 27, 2017).
12. Mitomycin C https://www.mpbio.com/product.php?pid=02100498&country=223 (accessed Apr 27, 2017).
13. Carmustine https://www.cancer.gov/about-cancer/treatment/drugs/carmustine (accessed Apr 27, 2017).
14. Boysen, G.; Pachkowski, B. F.; Nakamura, J.; Swenberg, J. A. The Formation and Biological Significance of N7-Guanine Adducts https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2739241/ (accessed May 1, 2017).
15. Warren, J. J.; Forsberg, L. J.; Beese, L. S. The structural basis for the mutagenicity of O6-methyl-guanine lesions http://www.pnas.org/content/103/52/19701.full (accessed May 1, 2017).
16. Fan, C.-H.; Liu, W.-L.; Cao, H.; Wen, C.; Chen, L.; Jiang, G. O6-methylguanine DNA methyltransferase as a promising target for the treatment of

temozolomide-resistant gliomas http://www.nature.com/cddis/journal/v4/n10/full/cddis2013388a.html (accessed May 1, 2017).
17. Washington, M. T.; Minko, I. G.; Johnson, R. E.; Haracska, L.; Harris, T. M.; Prakash, L. Efficient and Error-Free Replication past a Minor-Groove N2-Guanine Adduct by the Sequential Action of Yeast Rev1 and DNA Polymerase ζ http://mcb.asm.org/content/24/16/6900.long (accessed May 1, 2017).
18. Choi, J.-Y.; Angel, K. C.; Guengerich, F. P. Translesion Synthesis across Bulky N2-Alkyl Guanine DNA Adducts by Human DNA Polymerase κ http://www.jbc.org/content/281/30/21062.full (accessed May 1, 2017).
19. Mechlorethamine https://pubchem.ncbi.nlm.nih.gov/compound/mechlorethamine#section=Computed-Properties (accessed Apr 27, 2017).
20. Michaelson-Richie, E. D.; Ming, X.; Codreanu, S. G.; Loeber, R. L.; Liebler, D. C.; Campbell, C.; Tretyakova, N. Y. Mechlorethamine-Induced DNA-Protein Cross-Linking in Human Fibrosarcoma (HT1080) Cells https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3208907/ (accessed Apr 27, 2017).
21. Komarova, B. S.; Tsvetkov, Y. E.; Nifantiev, N. E. Design of α-Selective Glycopyranosyl Donors Relying on Remote Anchimeric Assistance. https://www.ncbi.nlm.nih.gov/pubmed/26785933 (accessed Apr 27, 2017).
22. Mechlorethamine http://web.chem.ucla.edu/~harding/IGOC/M/mechlorethamine.html (accessed May 1, 2017).
23. Siegel, D.; Yan, C.; Ross, D. NAD(P)H:Quinone Oxidoreductase 1 (NQO1) in the Sensitivity and Resistance to Antitumor Quinones https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3482497/ (accessed May 1, 2017).
24. Siegel, D.; Gibson, N. W.; Preusch, P. C.; Ross, D. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. https://www.ncbi.nlm.nih.gov/pubmed/1701346 (accessed May 1, 2017).
25. Pratt, W. B.; Ruddon, R. W. *The Anticancer Drugs*, 2nd ed.; Oxford University Press: New York, NY, 1994.
26. Lu, S. C. Regulation of Glutathion Synthesis https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2704241/ (accessed May 3, 2017).

Submitted September 5, 2017
Accepted with revisions October 16, 2017
Accepted November 1, 2017