Chapter 1 The nitrogen mustard story DNA crosslinkers and the advent of cancer chemotherapy 221022ej3

Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 1

Anti-cancer drugs that crosslink DNA.

The Story of Nitrogen Mustard: From poison gas to anti-cancer drug.

"*. . . and they shall beat their swords into plowshares, and their spears into pruning hooks: nation shall not lift up sword against nation, neither shall they learn war anymore*."
-- Isaia 2:3-4

Isaia's words may not have been the inspiration for how it happened that a poison war gas was "beaten" into an anti-cancer drug, but that nevertheless *is* what happened. It came about by way of a wartime disaster that was part design and part accident or coincidence. It came about, as it were, "from out of the blue." Nor did the admonishment in Isaia's words come to pass, as humans went on to use science to devise ever mightier swords, and one terrible war led to another. But few would have imagined that a poison war gas would lead to some of the most useful drugs for cancer therapy.

On December 2, 1943, in the evening, in the Adriatic harbor of Bari on the Eastern coast of Southern Italy, a still little-known military disaster took place. Some in the United States Navy and Merchant Marine called this World War II event "The Little Pearl Harbor" (Figures 1.1-1.2) (Infield, 1971; Reminick, 2001).

The harbor at Bari was filled that morning with ships waiting to unload their military cargo to supply the Allied push up the Italian boot. No one in the base was

aware that Nazi Luftwaffe bombers were at that moment approaching from the East. The Nazi high command under the direction of General Albert Kesselring had decided that their best chance to slow the Allied advance was to put the Bari harbor out of commission by sinking as many ships as possible while the harbor was crowded with them. The Luftwaffe was by that time pretty much decimated, but General Kesselring was able to assemble enough bombers for a surprise attack.

Figure 1.1. The Italian port of Bari, where the Nazi German attack, known as "the little Pearl Harbor," took place on December 3, 1943.

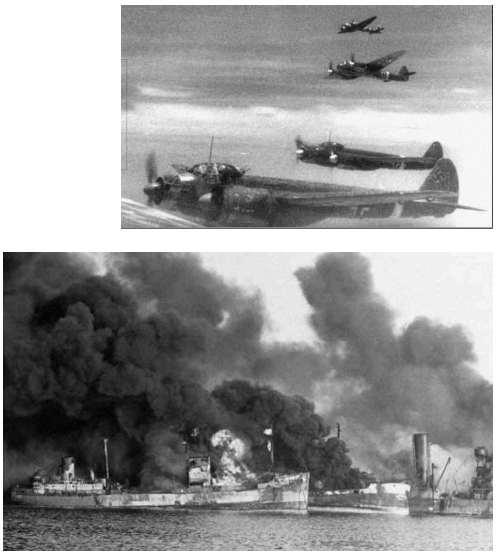


Figure 1.2. German Junkers JU88 bombers (top). Bombed ships exploding in the Bari harbor (bottom). (Source: HistoricWings.com)

And surprise indeed it was. The Allies had discounted the remaining capacity of the Luftwaffe to the extent that they kept the harbor lights on all night to speed the unloading of the ships, didn't make sure their radar was working, and ignored occasional German reconnaissance planes. First to note that something was happening that fateful evening were sailors of the Merchant Marine who saw strange strips of metal foil landing on the decks of their ships (Reminick, 2001). High flying German planes, in advance of the low flying bombers, were dropping metal foil strips to evade and confuse the unbeknownst to them non-functioning Allied radar.

What followed was terrible. Some of the ships blown up were carrying munitions and oil, and the harbor became covered with burning oil in which many sailors were immersed and desperately looking for a way to survive. About 1000 military and merchant marine personnel and about 1000 civilians are estimated to have perished, and 28 of the 30 ships that were in the harbor at the time were sunk or destroyed. The sailors who made it to shore were covered with oil and didn't think it urgent to change clothes or bathe.

During that part of the war, Washington was worried that Nazi Germany might in desperation resort to poison gas. To meet that threat, they made it known that they would retaliate in kind. To back it up, they secretly dispatched ships to deliver mustard gas bombs to key places - one of which, as you will have guessed, was Bari. One of those ships, the John Harvey, a Liberty Ship, was anchored in the port waiting its turn to unload its terrible cargo.

Some who experienced it called the disaster “the little Pearl Harbor”. General Dwight Eisenhower said it was the worst setback on his watch, and cancer researchers felt that it launched the first treatment of cancer with a chemical agent. What happened was recently clarified and filled out in a well-documented book by Jennet Conant (Conant, 2020).

I first heard mention of this World War II disaster in a lecture given during the 1960’s by Dr. Joseph Burchenal, who participated in the secret mustard gas research during the war and later became a leader in the new field of cancer chemotherapy. I learned nothing more about it during the years that I was studying nitrogen mustard at Harvard and at the National Cancer Institute -- until Glen Infield’s little-known 1971 book *Disaster at Bari* was brought to my attention by an Israeli Physician during a course of lectures I was giving in the NIH evening program (Infield, 1971). Conant’s book clarifies and corrects inaccuracies in Infield’s book that were due to difficulties he had acquiring reliable information about the event, which remained hidden in a cloud of silence even years after the war.

Sailors with burns of various degrees were arriving at overcrowded military hospitals, but many of the burns failed to heal as they should. That, together with rumors of smell of garlic, fed suspicions about poison gas, and led to a call for a chemical warfare specialist to come and investigate. The specialist, Lieutenant

Colonel Dr. Stewart F. Alexander (Figure 1.3), soon arrived from North Africa, and was credited with making the connection between mustard gas and a potential anti-cancer drug – although that accolade was ironically to be snatched from him.

Infield's book says that the *John Harvey*'s captain asked the British port commander for priority for unloading his ship, but that secrecy prevented him from revealing why, and his urgent request was denied. Conant's investigations, however, reveal the opposite: port commanders knew about the mustard gas bombs in the *John Harvey*, and it was they who couldn't reveal the secret. Had the medical staffs known about the mustard gas dissolved in the surface layer of oil in the harbor, many lives could have been saved by removing the sailors' contaminated clothing.

Alexander's investigation was hampered by closely held secrecy by those who knew about the delivery of mustard gas bombs. He was nevertheless able to collect undeniable evidence and even pinpointed the *John Harvey* as the source of the mustard gas. He did that by making a crude map of where the ships were located in the harbor and plotting on the map how seriously the casualties from each ship were affected (Figure 1.5). Faced with all that evidence, the British authorities had to admit that poison gas was released. After the disaster, an investigating committee advised that saving lives should be more important than secrecy.

Examining the patients and their medical records, Alexander was surprised that, after the spike in number of deaths that occurred during the first 4 days due to acute injuries, there was a second wave of deaths a few days later (Figure 1.4). But what really "made the hair at the back of [his] neck stand on end" was that many of the patients who survived the first 3 days then had rapidly falling white blood cell counts and died. He had seen that pattern before: in rabbits, in research he had done at Edgewood Arsenal in 1942. After exposure to nitrogen mustard, the rabbits' white blood cell counts plummeted and their lymph nodes "melted away." All of the Bari casualties, like the rabbits, whose white blood cell counts fell to extremely low levels died.

Conant's investigations reveal that German scientists devised nitrogen mustard in their search for a better war gas. It was an improvement over mustard gas in being odorless and devoid of the tell-tale garlic odor, and it was more quickly absorbed through the skin to produce internal injury. Ironically, nitrogen mustard also had an essential advantage as an anticancer drug. In a weak hydrochloric acid solution, it becomes inactive and can safely be injected intravenously. After reaching the blood, it rapidly converts back to its reactive form able to form crosslinks between DNA strands (Kohn et al., 1966). Thus, nitrogen mustard ironically had advantages over mustard gas, both as a war gas and as a therapeutic drug.

Conant's book tells how a sample of the new nitrogen mustard was smuggled out of Nazi Germany early in 1942 and immediately analyzed at Edgewood Arsenal, Maryland and studied for its effects on animals. Through great risk, samples of two compounds had been smuggled out of Germany. They were immediately studied by

Howard Skipper (Figure 2.10), who found them to be potent blistering agents. Chemical analysis revealed one of them to be a chemical relative of mustard gas, the sulfur atom being replaced by a nitrogen (Figure 1.6). The new compound was named nitrogen mustard. The second compound was like nitrogen mustard, except that it had three chloroethyl groups attached to the nitrogen, instead of just two.

Alexander was assigned to study the effects of nitrogen mustard on rabbits. In the course of that work, he made an astounding discovery: nitrogen mustard (but not mustard gas) depleted the rabbits' white blood cells and shrank their lymph nodes. He immediately imagined a possible nitrogen mustard therapy for lymphomas, which are malignant tumors made up of cells that are like white blood cells gone wild.

Alexander began the study on April 13, 1942 and reported his findings on June 30, 1942. Several copies of his report were distributed to leading military doctors and academic scientists who were carrying out classified wartime research on the effects of the mustards. Alexander wanted to pursue his concept of nitrogen mustard as possible treatment for lymphomas, but his research proposal was turned down as "not beneficial" to the war effort.

Researchers at Yale, however, received a copy of Alexander's 1942 report and began investigating the effect of nitrogen mustard on the white blood cell count and lymph nodes in rabbits and other animals. Within a few months, they felt ready to try the drug on a nearly moribund lymphoma patient. They were astonished by a patient's response. His tumors disappeared and he seemed entirely well. But two months later, the tumors reappeared and resisted further treatment. Trial of the drug on other patients, however, were disappointing and further studies languished.

When the Yale scientists later received Alexander's report early in 1944 of the effects of mustard gas on the casualties at Bari, as well as samples of their affected tissues, it spurred new attempts to develop nitrogen mustard as an anticancer drug.

An apparent discrepancy remained: why did mustard gas suppress the white blood counts of the Bari casualties but not of the experimental animals? The difference was likely due to its very slow penetration through the skin. The mustard gas exposures in World War I and in the experimental animals were short-lived exposures of the skin, causing severe burns and blisters, but the mustard gas exposure was not long enough for much of it to penetrate through the skin. The Bari victims, however, were exposed for many hours in their contaminated clothing – which gave time for the toxic stuff to get into the blood stream.

After the war, when Alexander was at last permitted to publish his research, his paper was rejected, because similar results had already been reported by the academic scientists. Eventually however, Alexander was offered the position of assistant director of the new Sloan-Kettering Institute for Cancer Research, but he

declined that enticing opportunity because he had promised to join his father's practice of medicine and cardiology in New Jersey.

Conant lays out the moral complexities of General Motors president Alfred Sloan who sought to allay public criticism of his industrial ties with Nazi Germany by founding what became known as the Sloan-Kettering Institute for Cancer Research. From 1937 to 1941, GM's Opal subsidiary in Germany was manufacturing war machinery for the Nazis, including the engines for the JU88 bombers of the type that were to decimate Bari harbor. GM's continuing profits from its German subsidiary were severely criticized by the American public and press. Sloan sought to restore his reputation by founding the Cancer Research Institute that bears his name.

American scientists invented the name "nitrogen mustard" and dubbed the compound HN2, because it had 2 chloroethyl groups on the nitrogen. The compound with 3 chloroethyl groups on the nitrogen was called HN3. HN2 and HN3 had similar biological activities; wisely, HN2 became the drug preferred for chemotherapy. HN1 was a similar compound, but with just one chloroethyl group on the nitrogen; it was therapeutically worthless for a simple reason: with only one chloroethyl group, it could not form crosslinks. In fact, this turned out to be the first evidence that HN2 and HN3 worked by forming crosslinks: two reactive chloroethyl groups on the nitrogen were need for biological and therapeutic activity. Two reactive groups could bind firmly to two biological molecules, thereby forming a crosslink between them.

Who deserves the credit for triggering the chemotherapy on cancer?

On April 13, 1942, Alexander began his 2-month-long study in which he led a research group to study the effects of HN2 and HN3 on animals, mainly rabbits. He was amazed by the white blood cell depletion the lymph node shrinkage, leaving "shrunken little shells." This had never before been reported in the scientific literature. Moreover, mustard gas did not have these effects (Conant, 2020) – presumably, because in those experiments the mustard gas exposure was not long enough for much of it to penetrate into the internal tissues.

Alexander reported those findings on June 30, 1942 in a secret memorandum: Medical Division Edgewood Arsenal MD Memorandum Report 59, *Preliminary Report on Hematological Changes in the Rabbit Following Exposure to Lethal Doses of 1130* [code name for HN2] (cited by (Conant, 2020), and the classified report was distributed to leading scientists of the National Research Council. Presumably, the academic clinicians who conducted the first pre-clinical studies of nitrogen mustard on lymphomas would have received that report, which was prepared within 4 months after the compounds were smuggled from Germany.

Alexander had wanted to go on to study the effects of nitrogen mustard on lymphomas already in 1942, to see whether the compound would cause those

tumors to shrink as he had seen lymph nodes to shrink. His proposed study however was not approved, because it did not help the war effort (Conant, 2020). It seems therefore that Alexander was the first to propose the nitrogen mustard treatment of lymphomas.

Figure 1.3. Lt. Col. Dr. Stewart Alexander at age 29 was the chemical warfare expert dispatched to Bari, Italy to investigate the suspected poison gas incident consequent to the German bombing of the port on 2 December 1943. His investigation led him to propose nitrogen mustard as treatment for lymphosarcoma. (Source: Jennet Conant, Smithsonian Magazine, September 2020.)

1st day	- 4 deaths	15th day	- 1 deaths
2nd day	- 9 deaths	16th day	- 1 deaths
3rd day	- 11 deaths	17th day	- 0 deaths
4th day	- 8 deaths	18th day	- 0 deaths
5th day	- 4 deaths	19th day	- 2 deaths
6th day	- 4 deaths	20th day	- 1 deaths
7th day	- 5 deaths	21st day	- 0 deaths
8th day	- 9 deaths	22nd day	- 0 deaths
9th day	- 9 deaths	23rd day	- 0 deaths
10th day	- 2 deaths	24th day	- 1 deaths
11th day	- 2 deaths	25th day	- 0 deaths
12th day	- 4 deaths	26th day	- 1 deaths
13th day	- 1 deaths	27th day	- 1 deaths
14th day	- 1 deaths	28th day	- 0 deaths
	After the 28th - 2 deaths		

Figure 1.4. Number of deaths on each day after the bombing, listed by Lt Col. Dr Stewart Alexander. A second wave of deaths occurred on days 8 and 9. (From: Stewart F. Alexander, 1943. “Final Report of Bari Mustard Casualties.” Records of the Office of the Surgeon General. National Archives and Records Administration.)

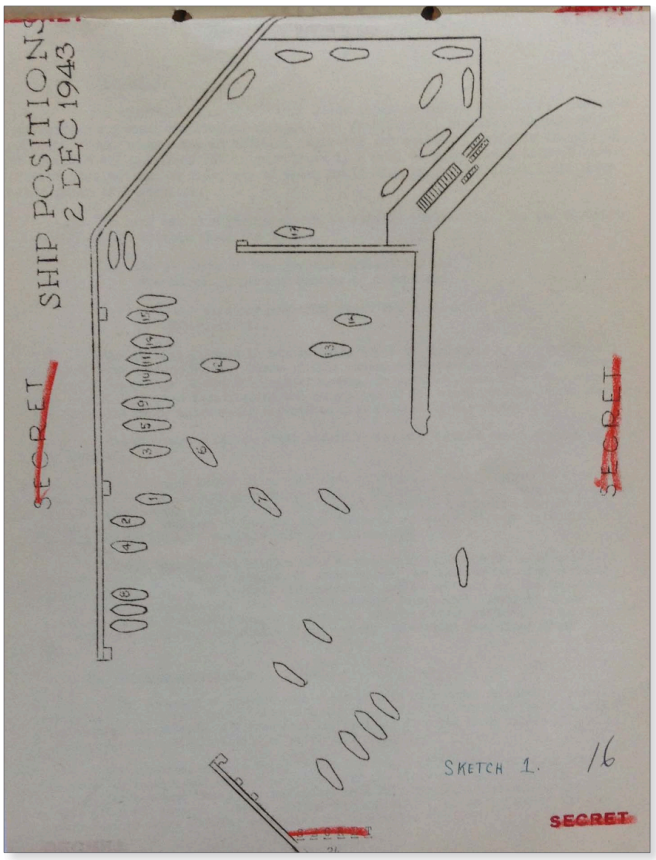
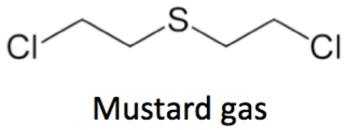
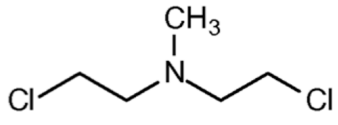


Figure 1.5. Lt. Col. Stewart Alexander's drawing of the positions of ships in Bari Harbor on 2 December 1943. (From: Stewart F. Alexander, "Final Report of Bari Mustard Casualties." Records of the Office of the Surgeon General. National Archives and Records Administration.)



Mustard gas



Nitrogen mustard

Figure 1.6. Nitrogen mustard was found to be like mustard gas in its chemical mechanism of action, with the sulfur atom replaced by a nitrogen.

Mustard gas and the controversy about Fritz Haber

Mustard gas was deployed for the first time in 1917 before the third battle at Ypres. It was developed during World War I by German chemist Fritz Haber (1868-1934) (Figure 1.8) with the idea that it would shorten the war and thus reduce overall casualties. However, it did not shorten the war and only created more misery. Moreover, he was wrong in imagining that poison gas would help Germany win, because the Allies soon countered with their own poison gases and gas masks.

Chlorine and phosgene had been used previously by both sides as poison gas, but these could be protected against with gas masks. The idea behind mustard gas was that it might break the stalemate by sinking into the trenches and be absorbed through the skin even if a gas mask covered the face; mustard gas differed from the previously used poison gases in being able to dissolve in the oily substance of skin and produce disabling burns. Most mustard gas casualties survived, but their burns were often terrible, and many became blind (Figure 1.7). There is no record of what may have happened to them later in life; mustard gas, like radiation, causes mutations and cancer (Panahi et al., 2015).

Figure 1.7. Left, British soldiers blinded by mustard gas, April 1918 (Wikipedia; trcs.wikispaces.com). Right, blisters caused by mustard gas (reference: medscape.com)

Fritz Haber was awarded the Nobel Prize in Chemistry in 1918 for developing the chemical process whereby nitrogen in the atmosphere is used to make ammonia for agricultural fertilizer, which made up for the limited supply from natural sources and saved millions from starvation. However, it also made up for the limited supply of nitrates for the manufacture of explosives and thereby may have prolonged the duration of World War I. The Haber-Bosch process for converting atmospheric

nitrogen to ammonia was produced on an industrial scale from 1910 to the present day.

Haber was demonized as “the father of gas warfare.” He personally supervised the first use of poison gas, chlorine in April 1915. In addition to his belief that it would shorten the war, Haber’s enthusiasm for poison gas may have come from wanting to show that a scientist of Jewish descent was loyal to the German war effort (Dunikowska and Turko, 2011).

Haber’s Nobel Prize, awarded for developing the Haber-Bosch process, was credited for saving millions from starvation. In the 1920 ceremony presenting the Prize to Haber, however, there was no mention of poison gas, either in the presentation speech or in Haber’s acceptance speech. Perhaps the Nobel committee at that time felt it inconclusive whether poison gas was prohibited in warfare. Haber’s view was that "in times of peace, a scientist belongs to the world; in times of war, he belongs to his country" and that "death is death, no matter how it is inflicted." A German military point of view at the time was “War is self-defense that knows no rules” (Deimling, 1930 cited by (Dunikowska and Turko, 2011)) (Friedrich and Hoffmann, 2016).

Those arguments however may not have persuaded Haber’s first wife, Clara Immerwahr (Figure 1.8). On May 1, 1915, there was a party to celebrate Haber’s promotion to captain in recognition of the success of first deployment of chlorine gas, which took place in the battle of Ypres on April 22, 1915. During the party, Clara reportedly had an argument with her husband; some say it was because of her conviction that her husband was misusing science for war. She then left the party and went out into the garden and shot herself in the heart with his revolver. Haber’s views also did not satisfy a public whose outcry about the use of poison gas during the war led to the Geneva Protocol of 1925, which banned the use of chemical or biological weapons.

Clara Immerwahr was an outspoken critic of her husband’s poison gas work, even to the point of being threatened about disloyalty. However, she had been unhappy in her marriage for several years, possibly depressed, and frustrated at being unable to pursue her scientific career (Friedrich and Hoffmann, 2016). Opinions have become polarized about the immediate reason that she shot herself; the truth may lie in a combination of factors.

A German view of the first and second world wars, as well as the period between them, 1914-1945, equated its impact on the country to a “second 30-years’ war” (Stern, 2012). The Haber-Bosch process has been likened to Janus, the 2-faced figure of Roman legend that presided over war and peace (Figure 1.9): credited with saving millions from starvation, but prolonging the war by providing critically needed nitrate for explosives (Stern, 2012). As inventor of the Haber-Bosch process, Fritz Haber also acquired a dual reputation as both benefactor and detractor of human welfare. This incongruity caused Haber’s hometown city of

Breslau (Wrocław) to display his portrait upside down among notable figures who stemmed from that city (Figure 1.9) (Dunikowska and Turko, 2011).

The German military also were two-sided on the use of poison gas: many high-ranking German officers at first detested the use of poison gas (Dunikowska and Turko, 2011).

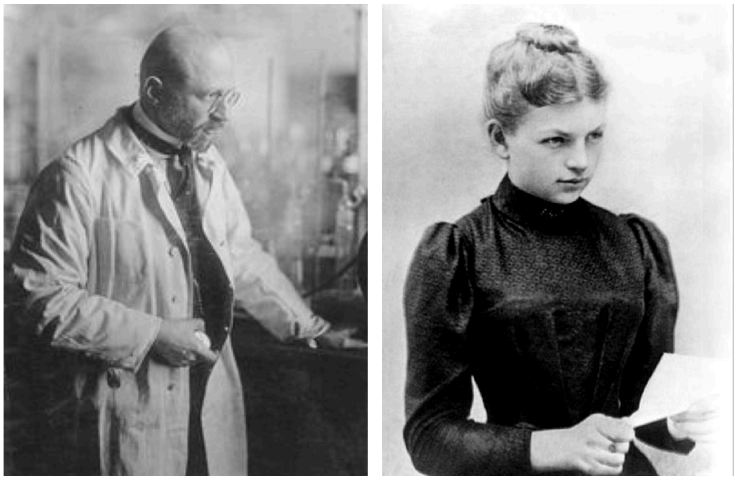
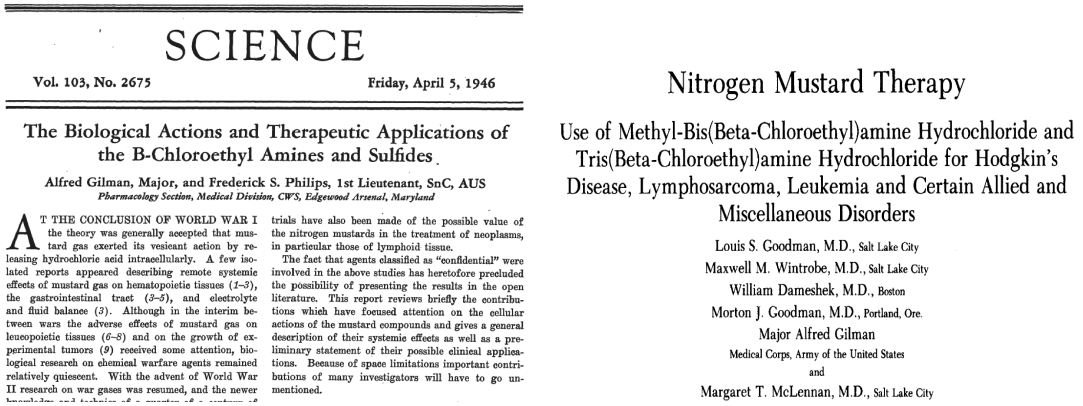


Figure 1.8. Left, Fritz Haber (1868-1934) in his laboratory in 1905 (Bundesarchiv; Wikimedia Commons.) Right, his first wife, Clara Immerwahr (1870-1915), the first woman to be awarded a doctorate in chemistry in Germany. She committed suicide in May 1915 at the age of 44, some say in dismay of her husband's work on poison gas. She was perhaps true to the meaning of her name: Immerwahr = always true.

Figure 1.9.
Left: Roman deity Janus presided over the beginning and ending of conflict, and hence war and peace. Head of Janus, Vatican museum, Rome, from Wikimedia Commons.
Right: Haber′s portrait, upside down in the Salon Slaski in Breslau (Wrocław) (Dunikowska and Turko, 2011) expressed the ambivalent opinions about him .

Nitrogen mustard becomes the first anti-cancer drug.

For many years after World War I, it was thought that mustard gas caused burns by reacting with water in the cells to produce hydrochloric acid. It was only during World War II that organic chemists revealed its more sinister chemistry. The work was carried out by scientists in the United States and was top secret. The remarkable results were not made known until 1946, after the war (Gilman and Philips, 1946; Goodman et al., 1946) (Figure 1.10). Several of the scientists who worked on mustard gas and nitrogen mustard during World War II, including several of the authors of those 2 landmark papers, became leaders in the war against cancer, in accord with *swords into plowshares*.



SCIENCE

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The Biological Actions and Therapeutic Applications of the B-Chloroethyl Amines and Sulfides

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AT THE CONCLUSION OF WORLD WAR I the theory was generally accepted that mustard gas exerted its vesicant action by releasing hydrochloric acid intracellularly. A few isolated reports appeared describing remote systemic effects of mustard gas on hematopoietic tissues (*1–3*), the gastrointestinal tract (*3–5*), and electrolyte and fluid balance (*3*). Although in the interim between wars the adverse effects of mustard gas on leucopoietic tissues (*6–8*) and on the growth of experimental tumors (*9*) received some attention, biological research on chemical warfare agents remained relatively quiescent. With the advent of World War II research on war gases was resumed, and the newer

trials have also been made of the possible value of the nitrogen mustards in the treatment of neoplasms, in particular those of lymphoid tissue.

The fact that agents classified as "confidential" were involved in the above studies has heretofore precluded the possibility of presenting the results in the open literature. This report reviews briefly the contributions which have focused attention on the cellular actions of the mustard compounds and gives a general description of their systemic effects as well as a preliminary statement of their possible clinical applications. Because of space limitations important contributions of many investigators will have to go unmentioned.

Nitrogen Mustard Therapy

Use of Methyl-Bis(Beta-Chloroethyl)amine Hydrochloride and Tris(Beta-Chloroethyl)amine Hydrochloride for Hodgkin's Disease, Lymphosarcoma, Leukemia and Certain Allied and Miscellaneous Disorders

Louis S. Goodman, M.D., Salt Lake City
Maxwell M. Wintrobe, M.D., Salt Lake City
William Dameshek, M.D., Boston
Morton J. Goodman, M.D., Portland, Ore.
Major Alfred Gilman
Medical Corps, Army of the United States
and
Margaret T. McLennan, M.D., Salt Lake City

Figure 1.10. Two landmark papers that ushered in the era of cancer chemotherapy. Both published in1946, they reported the essential findings of the secret studies of mustard gas and nitrogen mustard that were conducted during the war years. The chemical and pharmacologic findings were reported in the paper in *Science* by Alfred Gilman and Fred Philips (left); the remarkable clinical results were reported in the *Journal of the American Medical Association* by Louis Goodman and his colleagues (right). Several authors of these papers became leaders in cancer chemotherapy, hematology and pharmacology.

The application of the new chemistry of alkylation to the new cancer chemotherapy began in 1942, during the war, under a cloak of secrecy; even the identity of the medications was encoded. But it was not until 1946 that the results of the clinical investigations of mustard gas and its chemical relative, nitrogen mustard, were published (Gilman and Philips, 1946; Goodman et al., 1946) (Figure 1.10); the story was further clarified by Alfred Gilman in 1963 (Gilman, 1963). Nitrogen mustard, rather than mustard gas, was used in the biological and clinical studies, because as a crystalline hydrochloride salt it could be freshly dissolved and safely injected. Mustard gas would be very difficult or impossible to use as a drug, but the two substances engage in similar chemical reactions.

Goodman, Gilman and their colleagues during the war found that injections of nitrogen mustard in tumor-bearing mice dramatically reduced the size of the tumors and prolonged the survival of the mice. They did extensive tests in animals to determine the nature of the drug's toxicity and to estimate the dose that would be safe in patients. Only then did they try the drug in cancer patients. Most of their patients had large lymphoma tumors that had become resistant to x-ray treatments and who were not expected to survive much longer (Gilman, 1963; Gilman and Philips, 1946; Goodman et al., 1946). It is here that Stewart Alexander's information from Bari may have had an impact in putting the focus on lymphomas. And that was a fortunate choice, because those cancers were particularly sensitive to drugs like nitrogen mustard, and the clinical responses and promise of the drug were plain to see.

The response of those large tumors to nitrogen mustard must have astounded both physician and patient and given them hope (Figure 1.11) (Goodman et al., 1946, 1984). It was the first time that a chemical agent obliterated a large internal tumor in humans. It was in fact the beginning of the era of cancer chemotherapy. Remarkably, nitrogen mustard was sometimes effective after radiation had failed. The tumor however soon grew back and lost its responsiveness to further treatment. The tumor had become resistant to the nitrogen mustard, as well as to radiation. Nonetheless, the treated patients sometimes lived several months longer than would otherwise have been expected. But the bugaboo of drug resistance was to plague cancer chemotherapy from then on; the reason for the acquired resistance to nitrogen mustard remained enigmatic.

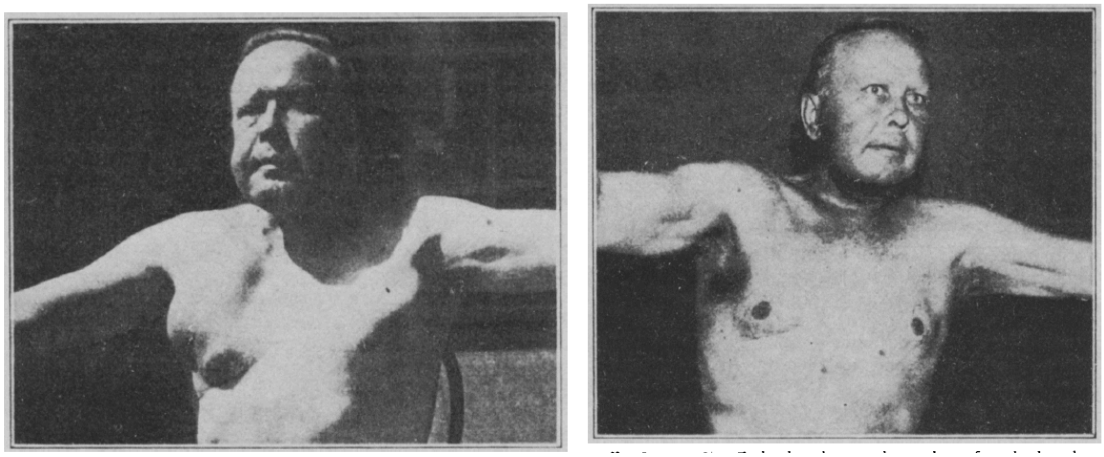

Figure 1.11. This 48-year-old lymphoma patient was one of the first whose tumors shrank after treatment with a drug, nitrogen mustard. This famous case was reported in1946 by Louis Goodman and his colleagues (Goodman et al., 1946). Left, large tumors in armpits, neck and chest as they looked before treatment. Right, after treatment the tumors have disappeared. The full story of this patient and his treatment is told by Vincent DeVita in his book "The Death of Cancer" (DeVita Jr., 2015)

In the initial clinical trial, 67 patients in the last stages of their disease, most of whom had received radiation treatment that was no longer effective, were treated at New Haven Hospital (L. S. Goodman and A. Gilman), Salt Lake County General Hospital (M. M. Wintrobe and M. T. McLennan), and Tufts College Medical School, Boston (W. Dameshek). All of these authors were to become leaders in the new oncology and hematology.

Search for better nitrogen mustards

When the response of lymphoma patients became widely known after the war, it was hoped that better results could be achieved, and resistance perhaps avoided with chemically modified nitrogen mustards. The alkylation chemistry of nitrogen mustards had been worked out during the war and was well understood, and the chemical structure of the nitrogen mustard molecule made it relatively easy to synthesize many active modifications. A huge number of modified nitrogen mustards were synthesized and tested in tumor-bearing mice. Despite massive effort, however, none of the modified nitrogen mustards were distinctly superior in animal tests (Shapiro et al., 1949).

Clinical experience, especially the problem of acquired drug resistance, soon revealed the limitations of what could be achieved with nitrogen mustards and related drugs, and clinicians stopped thinking of "cure" in the context of cancer. Unless a malignant tumor could be eliminated before it spread, there was at the time little hope for more than a brief reprieve.

Of the large number of nitrogen mustards tested, a few did become part of the chemotherapy armamentarium; these will be considered individually after a review of some to the basic science.

Nitrogen mustard may from crosslinks -- but between what?

During the war, chemists learned how nitrogen mustards react, which made it possible to understand the chemical behavior of a variety of related compounds, either natural or synthetic products. The essential reaction, called "alkylation," causes the drug molecule to bind firmly (covalently) to biomolecules such as DNA and proteins. Drugs that work by this mechanism are called "alkylating agents."

What nearly all effective nitrogen mustard-like alkylating agents have in common is a feature that was noted already during the early nitrogen mustard studies. To be effective, the nitrogen mustard had to have 2 reactive sites; when similar molecules with only 1 reactive site were made and tested, the great majority were found to be inactive. It was as if the anticancer and toxic actions required the formation of a crosslink between 2 other molecules; in other words, the effects required the linking together of 2 target molecules (Figure 1.12) (Goldacre et al., 1949; Loveless and Revell, 1949). It was not known what those critical target molecules were, and it took more than a decade to find out.

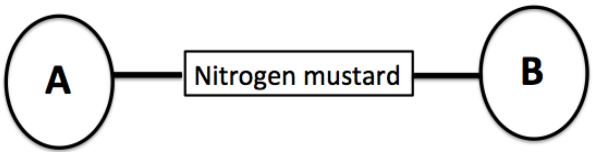

Figure 1.12. An early concept of how nitrogen mustard works was that it forms stable (covalent) crosslinks between 2 important biomolecules, A and B. In 1946 it was not known what A and B might be. The only evidence was that a nitrogen mustard needed 2 reactive sites to be effective.

I first learned of nitrogen mustard alkylation chemistry and the idea of crosslink production in Alfred Gilman's pharmacology lectures at Columbia's College of Physicians and Surgeons in 1952 (Figure 1.13). That idea lingered in my mind until 1960, when I joined Paul Doty's lab at Harvard. Concepts and methods had by that time been developed to permit me to show that the bifunctional reactions of nitrogen mustard formed crosslinks between the paired strands of DNA and was the main factor that killed cells (Kohn and Green, 1966; Kohn et al., 1966). Before proceeding to that story, however, it may be helpful to explains some fundamentals about the chemistry of alkylation and crosslink formation.

Figure 1.13. Alfred Gilman (1908-1984), Professor of Pharmacology, Columbia College of Physicians and Surgeons, was a key figure in the elucidation of alkylation chemistry as it applies to nitrogen mustards. He taught the pharmacology course while I was a medical student there, which is how I first learned about nitrogen mustard and its reaction mechanism.

Alkylation and DNA crosslinking – the chemistry.

The essential property of alkylating agents is the ability to form stable bonds with molecules such as DNA and proteins. Since it has such a fundamental role in drug actions, I will explain how alkylation reactions work. I'll try to explain the essentials in a way that those without much chemistry background could understand. It takes quite a few words to do that, but taken one step at a time, it's pretty simple. We'll take nitrogen mustard as a classic example. So, here goes (in the following, refer to Figure 1.14). If you're familiar with organic chemistry, please just look at Figures 1.14 and 1.15 and skip the rest of this section.

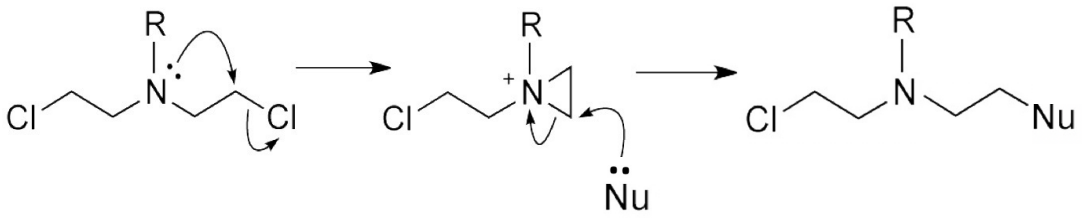

Figure 1.14. Nitrogen mustard (left), showing the unshared electron pair on the nitrogen. The curved arrow shows the unshared electron pair from the N attacking the carbon atom to which the chloride (Cl) is attached. At the same time, the Cl leaves, one electron richer, to become a happy chloride ion. The resulting triangle, consisting of a nitrogen and 2 carbons, is shown on the right. The bond angles however like to be about 109°, whereas confined to 60° in the triangle puts them under much stress. The curved arrows show what happens next: an atom with a loose pair of electrons (here designated Nu, which stands for "nucleophile"), such as the nitrogen at the 7-position of guanine in DNA, can come in, and form a bond with a carbon in the triangle. The triangle opens and the stress is relieved. (By organic chemistry convention, a CH2 is assumed to exist at any angle between two lines.)

A carbon atom has 4 bonds coming out of it. (If you know about such things, you may object: how about double-bonds, pi-bonds and such? Well, for the present purposes, we don't have to trouble with those cases.) The 4 bonds of a carbon atom like to be directed towards the corners of a regular tetrahedron, the carbon atom being at the center of the tetrahedron. That means that the bonds are most stable when the angles between them is about 109°. If three atoms were connected in an equilateral triangle, the bonds would be forced to be at an angle of 60°, which would put a lot of stress on them.

So, what has that to do with forming bonds to DNA? Before we get to that, there is something else to know . . . about nitrogen atoms. Like carbon atoms, they too like to have a tetrahedral configuration of bonds, but they often have only 3 bonds in place; the 4th direction, where a bond could be but isn't, is occupied by "a pair of unshared electrons" (because nitrogen has one more electron out there than carbon does). That unshared pair of electrons has the potential of forming a bond with another atom when there is an opportunity to do so. An opportunity arises when that other atom can create a space for that electron pair to go into.

With those ideas in place, let's look at the business end of the nitrogen mustard molecule. The only atoms that we have to be concerned with are –N–C–C–Cl. (To make up the 4 bonds, each carbon atom also has 2 hydrogen atoms bound to it, which are not shown.)

Now here is another important idea about alkylation reactions, it's called "the leaving group." In this case, the leaving group is just the chlorine atom (Cl) with an extra electron to make it a happy chloride ion. The main thing about a leaving group, you may not be surprised to learn, is that it tries to leave the molecule. But, and this is the key, when it leaves, it carries with it *both* electrons in the bond. A leaving group will only work as such if it attracts electrons much more strongly than the atom from which it is leaving. In this case, the Cl has a much stronger affinity for the electrons in the C-Cl bond than the C does.

However, the Cl can't leave right away, because it would make the C very unhappy: a C cannot tolerate an empty place where there could be a bond. Here is where the N, with its unshared electron pair, comes in; it bends around and takes the place of the Cl; thus, the Cl can make off with the extra electron and becomes a very happy chloride ion. What is left is an N bound to where the Cl was bound before it left . . . which creates a triangle of bonds. As already said, a triangle of bonds puts the carbon atoms under stress. That arrangement of 2 C's and 1N in a triangle, would very much like to open up; in technical terms, it has a lot of energy in it (Figure 1.14).

Now at last we come to how the bond to DNA forms. There is one atom in the base-pairs of DNA that has the greatest possibility of providing an unshared electron pair to relieve the stress in the now activated nitrogen mustard (the 3 atoms in the stressed triangle). That DNA atom is a nitrogen, the so-called N7 atom of the guanines in DNA. What happens is that this "GN7" atom binds to one of the C's in the triangle, releasing the N from the stressed triangle. The stress is relieved, and we are left with a stable bond between the nitrogen mustard atoms and DNA:

$Cl–CH_2–CH_2–N(CH_3)–CH_2–CH_2–GN7–DNA.$

Finally, the second $Cl–CH_2–CH_2–N(CH_3)–$ part of the nitrogen mustard can engage a GN7 on the opposite DNA strand and carry out the same sequence of reactions described above. The result is a stable crosslink between the 2 DNA stands, linked together through atoms from the nitrogen mustard (Figure 1.15).[1]

There are many variations of this theme in the mechanisms of DNA damage and repair. The preceding concepts and explanation can help to understand those different cases.

[1] I have omitted some details about the charges on the atoms, which however are not needed to understand the essentials. When the chloride ion leaves with the extra electron, it has a negative charge; that leaves behind a positive charge, which resides in the now 4-bonded nitrogen atom in the triangle. When the bond to DNA forms, that positive charge is transferred to the attached guanine.

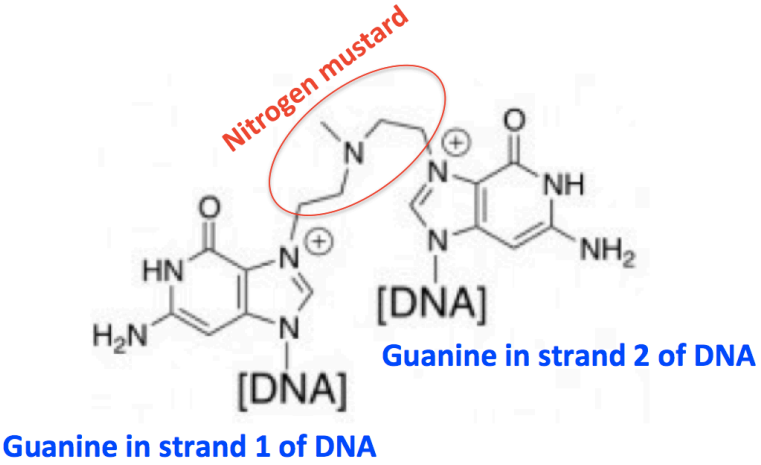

Figure 1.15. A DNA inter-strand crosslink, showing how nitrogen mustard links the 2 DNA strands. The crosslink is between a guanine in one DNA strand and a guanine in the opposite DNA strand. The nitrogen mustard moiety is attached to the nitrogen at position 7 of each guanine. The crosslink prevents the separation of the two strands that must happen when DNA is replicated.

The DNA crosslinking story

To recapitulate, the ability of nitrogen mustard to bind important molecules in the cell had been established in the 1940's, and the alkylation mechanism that brings it about had been elucidated. The observation that 2 reactive groups were needed for potent effects on cells and animals suggested that the drug worked by crosslinking something in the cell (Figure 1.12) (Goldacre et al., 1949; Loveless and Revell, 1949). The question remained: what was the important target that was being crosslinked?

In the late 1950's Paul Doty's laboratory at Harvard had elucidated how the 2 strands of DNA come apart when heated and how they come back together when cooled to let the complementary DNA bases (A-T; G-C) find each other again (Figure 1.16).

Figure 1.16. Paul Doty (1920-2011), Mallinckrodt Professor of Biochemistry at Harvard, developed the principles of the DNA helix-coil transition, the process whereby the paired strands of the DNA helix come apart and re-associate.

When I joined Doty's lab in 1960 and looked over their most recent data, it was apparent that the DNA strands would remain separated only if heated past a critical temperature where strand separation was complete. If the temperature was a little below that point, the strands would separate partially, but almost instantaneously snap back when cooled; the complementary base pairs would be confined to a small region and could quickly find each other again. Only if the temperature was above that critical point would the strands remain separated. As long as even a small region of the strands remained together, the separated regions of complementary sequences could quickly find each other again.

That brought to mind what I had learned from Alfred Gilman in his medical school pharmacology lectures in 1953 about the nitrogen mustard reaction mechanism and that to be effective the molecule had to be able to firmly link 2 sites. It seemed possible that nitrogen mustard linked the DNA strands so that they could not separate completely, in same way as heating to a sub-critical temperature.

The new concepts developed in Doty's lab of how complementary DNA strands dissociate and re-associate suggested how we could test the idea that nitrogen mustard forms crosslinks between the 2 strands in the DNA helix, thereby preventing the strands from separating completely. I found that even one crosslink could keep the strands connected and near each other when all the base pairs had dissociated, and the normal base-paired double helix could quickly reassemble when the base-pair-separating conditions were reversed, because all the complementary bases would remain in a small region of space (Kohn et al., 1966).

The same year that I joined the Doty lab, in 1960, Brookes and Lawley reported that mustard gas can bind to the nitrogen atom at position 7 of guanine (Brookes and Lawley, 1960). We thought that nitrogen mustard could do the same. The two

alkylating groups on nitrogen mustard could bind to guanines in DNA, and, moreover, if nitrogen mustard's 2 alkylating groups each bound to one of the strands in a DNA double helix, the 2 strands would be crosslinked and unable to separate completely.

Lawley and Brookes however reported that the bond between the mustard and the DNA guanine was not very stable. Therefore, instead of using heat to separate the strands, as had been the general practice, I separated them by briefly making the solution alkaline (pH12). After neutralizing the solution, the DNA strands remained separated -- unless they were crosslinked. Thus, normal DNA would end up single-stranded, whereas crosslinked DNA would end up as normal double-stranded helix.

We needed a way to measure how much of the DNA remained intact helix after the procedure and how much was separated single strands. The clearest way to make that measurement was by means of the analytical ultracentrifuge (Figure 1.17). Using that remarkable instrument and other physical-chemical methods, I was able to prove that nitrogen mustard indeed crosslinked the DNA and that even a single crosslink would allow the double helix to quickly re-associate (Figure 1.18) (Kohn et al., 1966).

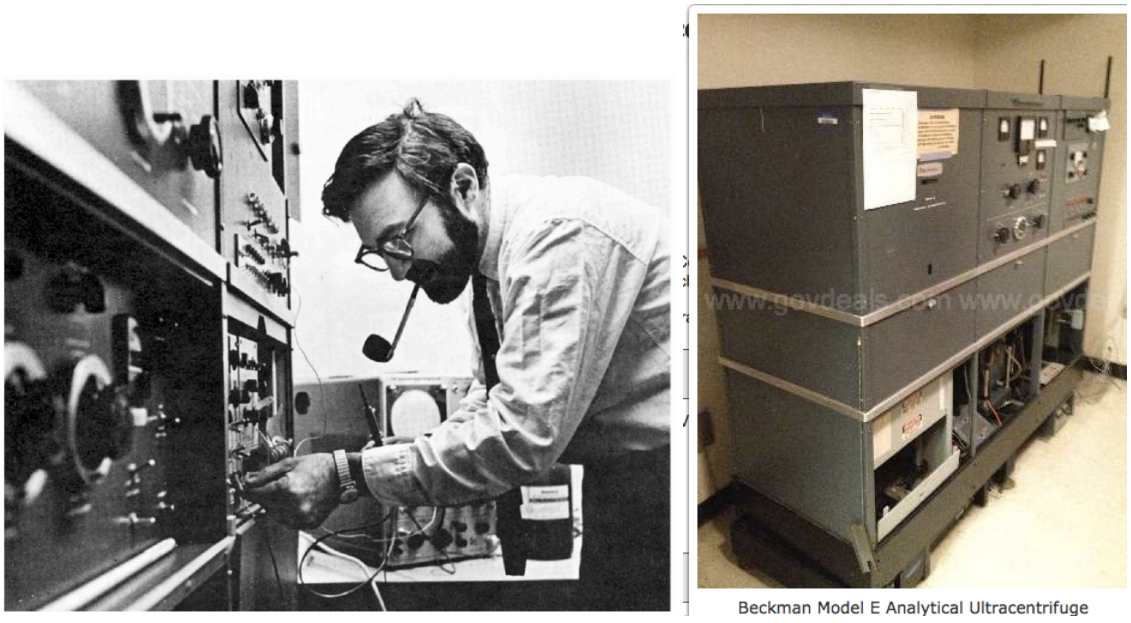

Figure 1.17. The analytical ultracentrifuge was a mainstay in DNA research from the about 1953 until about 1980. Left, an ultracentrifuge at the University of Connecticut being tended in 1968 by my former college roommate, David Yphantis, who became a leader in the development of the technology (Correia et al., 2004). Right, this ultacentrifuge may have been the very one I used in the Doty lab in 1960; it has the same sign taped to it. It was sold at auction in 2014 for $105, presumably for its parts (at NIH, in 1968 or so, we bought a new one for about $7000).

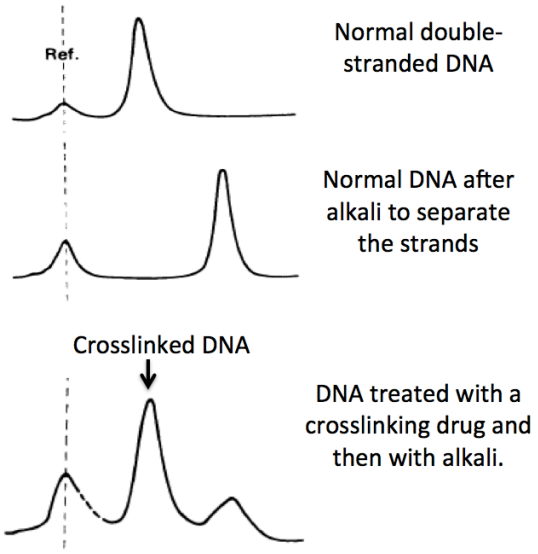

Figure 1.18. Analytical ultracentrifuge tracings showing how I detected and measured crosslinked DNA. The DNA was dissolved in a highly concentrated solution of a cesium (Cs) salt and then centrifuged at a high speed for 48 hours. The heavy Cs atoms tended to move in the direction of centrifugal field, reaching an equilibrium between centrifugal force and back-diffusion and forming a Cs salt concentration density gradient. The critical fact was that double-helical DNA banded at a lower density than single-stranded DNA. The two strands of crosslinked DNA did not separate completely when the pH was raised to 12.0, because the strands were held together at the point where there was even by a single inter-strand crosslink. When the solution was neutralized 2 minutes later, the crosslinked strands quickly found their matching base-pairs, thereby restoring the double-helix.

Together with Donald McDonald Green in the Doty lab, I showed that the nitrogen mustard-treated DNA whose base pairs had completely dissociated and then re-associated retained its gene coding ability (genetic transformation activity in bacteria) (Kohn and Green, 1966). In addition, we showed that DNA crosslinking by nitrogen mustard determined the sensitivity of bacterial cells to being killed by the drug (Kohn et al., 1965). Later studies in many laboratories established that the ability of human cells to survive treatment with nitrogen mustards and related drugs depends in large part on the cells' ability to repair DNA crosslinks.

There is however a different kind of alkylating agents that bind largely to the oxygen at guanine position 6. These drugs differ from the nitrogen mustard-like drugs, (which crosslink between guanines in the 2 strands) in that they produce crosslinks between a guanine and its base-paired cytosine. Importantly, there is a special repair enzyme that prevents those crosslinks from forming. These drugs have unique chemical and biological properties and are the subject of the next chapter.

There is yet another class of DNA crosslinking drugs, very important ones in cancer chemotherapy, which have an entirely different chemistry. They are not alkylating agents, but instead use a platinum atom to carry out analogous reactions. The fascinating story of the platinum drugs is the subject of Chapter 3.

Alkylating agents in clinical research and practice

The first clinical study of nitrogen mustard, already described above, was officially summarized in 1946 by Cornelius P. Rhoades (Rhoades, 1946). At the time of that report, 160 patients with lymphoma, leukemia, and allied conditions had been treated. Rhoades indicated that the drug was available for experimental purposes only through the National Research Council in cooperation with the Chemical Warfare Service. He summarized information about dosage, side effects, and toxicity, noting that divided doses over several days was safer than injecting a single large dose. The most frequent toxicity was suppression of white cells, anemia, and bleeding tendency due to fall in platelet count, which was to become a well-known toxicity pattern in cancer chemotherapy. It was already suspected that rapidly dividing tissues, whether normal or cancer, were particularly vulnerable to the drug.

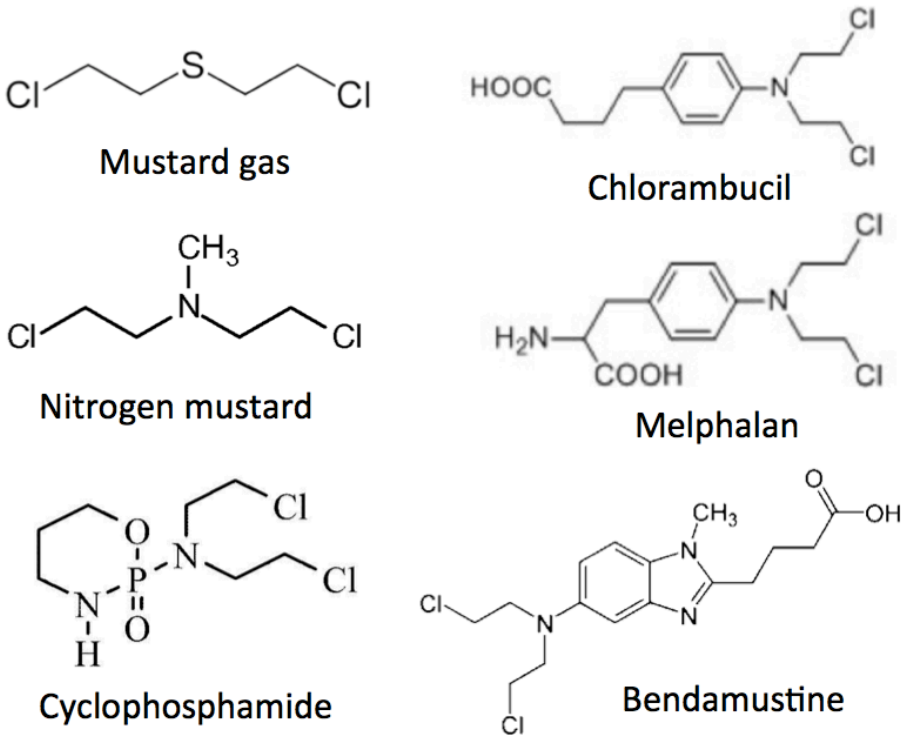

Figure 1.19. Chemical structures of nitrogen mustards in clinical use, in comparison with mustard gas (also known as sulfur mustard).

The Search for better nitrogen mustards through chemical modification

The remarkable ability of nitrogen mustard to shrink large lymphoma tumors in some of the patients treated by Goodman and his team in 1942-5 (Goodman et al., 1946) inspired medicinal chemists to prepare modified versions of the drug. The easiest modifications to make was to add various chemical groups to the methyl group, a change that would not disturb the ability of the drug to form crosslinks. Of the large number of structures prepared, a few became important in chemotherapy, in particular, chlorambucil, melphalan, and cyclophosphamide (Figure 1.19).

Chlorambucil and Melphalan were developed by Alexander Haddow in 1953 at the Chester Beatty Institute in England, and both were for decades in the mainstream of cancer chemotherapy. Chlorambucil found its place in the treatment of chronic lymphatic leukemia (CLL) and chronic myelogenous leukemia (CML), while melphalan for a time became standard treatment for multiple myeloma (Catovsky et al., 2011).

Chlorambucil

Chlorambucil was one of the first modified nitrogen mustards to become widely used in cancer therapy. Early studies indicated that it was effective, although of course not curative, in the treatment of lymphomas and chronic myelogenous leukemia, and it was thought to have less side-effects than nitrogen mustard (Gellhorn et al., 1956; Krakoff et al., 1958; Ultmann et al., 1958).

The difference in biological properties caused by the change in chemical structure on going from nitrogen mustard to chlorambucil may largely be due to the negative charge from the carboxyl group on the side chain that replaces the methyl group of nitrogen mustard (Figure 1.19), which may affect the drug's distribution in tissues and ability to enter cells.

Chlorambucil became the drug of choice for chronic lymphatic leukemia (CLL). However, the rate of complete response was increased by adding to the treatment regimen an antibody to CD20 (also known as MS4A1). CD20 is a protein that is displayed on the surface of B-type lymphocytes, which is the cell type that is over-produced in CLL; the anti-CD20 antibody helps to kill the CLL cells (Lepretre et al., 2015).

Melphalan

Melphalan (L-phenylalanine mustard) was, with chlorambucil, one of the first nitrogen mustard derivative to become a significant part of our chemotherapy armamentarium. The idea behind its synthesis in 1953 was that the L-phenylalanine part of the melphalan molecule would serve as a carrier to steer the mustard

warhead into cancer cells. L-phenylalanine is one of the amino acid building blocks that make up proteins. A cell's ability to take up this amino acid from the outside is enhanced by specific transporter channels in the surface membrane; it was hoped that cancer cells would have relatively large numbers of these active transport channels in their surface membranes through which the L-phenylalanine mustard would be taken up.[2]

The malignant tumor most susceptible to treatment with melphalan was multiple myeloma (Musto and D'Auria, 2007), a disease of antibody-producing white blood cells, plasma cells, that grow wild, invade the bone marrow, dissolve calcium from bone, make bones prone to fractures, and cause bone pain. Before the advent of melphalan, there was no effective therapy. The only available therapy was urethane, which was rarely effective (Hoogstraten et al., 1967). Melphalan, although not by itself curative, prolonged the lives of many patients. First approved for the treatment of multiple myeloma and ovarian cancer, melphalan became part of drug combinations for treatment of a variety of malignancies (Falco et al., 2007).

Early reports of melphalan as a promising treatment for multiple myeloma appeared in 1964 (Speed et al., 1964; Waldenstroem, 1964). In 1968, Raymond Alexanian, Daniel Bergsagel and their colleagues at M. D. Anderson Hospital in Huston, Texas, found that 40% of their patients with multiple myeloma responded to melphalan, which prolonged their lives by more than 2 years (Alexanian et al., 1968). The addition of prednisone increased responses to 70%, although all of the patients eventually relapsed (Alexanian et al., 1969). Until recently, when additional modalities further improved the therapy, the melphalan-prednisone combination remained standard treatment for multiple myeloma (Falco et al., 2007; Musto and D'Auria, 2007).

Bendamustine

Bendamustine was synthesized in the 1960's in the German Democratic Republic (East Germany) and was commonly used there, although not very much studied. After Germany was reunited, studies eventually showed it to be effective in breast cancer and certain lymphomas, and only partially cross-resistant to other nitrogen mustards. In some chemotherapy combinations it was used in place of cyclophosphamide, sometimes giving less toxicity and longer time before progression of the disease (Herold et al., 2006; Kalaycio, 2009; von Minckwitz et al., 2005). Unlike cyclophosphamide, it does not require activation in the liver.

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[2] The "L" in L-phenylalanine indicates which one of the two possible mirror image structures the molecule has. All natural amino acids of proteins have the L-configuration, and the active transport channels only take up this form of the amino acid or of melphalan. The "*el*" in m*el*phalan emphasizes that the drug molecule has the "L" configuration.

Cyclophosphamide

Cyclophosphamide is a modified nitrogen mustard that became one of the most commonly used chemotherapy drugs and is on the World Health Organization's List of Essential Medicines. Recently, however, its use has been declining as other drugs have begun to replace it.

The original concept behind the development of cyclophosphamide was that its phosphate moiety would tend to draw electrons away from the nitrogen of the mustard moiety and thereby prevent that nitrogen from releasing the chloride to form the 3-membered ring of an active nitrogen mustard. The drug would then remain inactive until, it was thought, the bond between the P and N would be cleaved by an enzyme thought to be present at high levels in cancer cells.

That idea, it turned out, was only partly correct. The drug is inactive and requires activation, as predicted. But this activation does not occur in the cancer cell; it occurs in the liver and does not involve cleavage of the P-N bond. A series of chemical steps (which involves removal of the 3 carbons in the ring containing the phosphorous atom (Figure 1.19)) yield the active form of the drug, called phosphoramide mustard, which then crosslinks DNA in both tumor and normal cells (Dong et al., 1995). The activation of the drug depends on liver enzymes whose activity can vary from patient to patient, which might make the drug effect delivered by a given administered dose inconsistent (Madondo et al., 2016).

Some of the first careful studies of the effects of cyclophosphamide on leukemia in mice were carried out in 1958 by Montague ("Monty") Lane, with the technical assistance of Sidney Yancey, in the former Clinical Pharmacology Service of the General Medicine Branch of the National Cancer Institute, while I was a member of that group upon coming to NIH in 1957. They found that, at the optimum dose, cyclophosphamide greatly extended the life span of the leukemic mice and was more effective than previous drugs, including nitrogen mustard (Lane, 1959).

Some of the first clinical observations on the toxicological effects of various doses of cyclophosphamide in cancer patients were reported from the University of Pennsylvania by Peter Coggins and his coworkers. The drug seemed to be less toxic than nitrogen mustard and produced partial regression of tumors in many of the 130 patients with measurable tumors of various kinds in the study. Although it was a preliminary uncontrolled study, the investigators felt that the drug produced better results than what was previously available (Kovacs et al., 1960).

Early experience put cyclophosphamide on the road to becoming one of the most commonly used drugs in cancer chemotherapy. A lingering question however was the role of the liver activation that the drug required. In what way was that helpful, or did it produce variability among patients, depending on the activity of their liver enzymes? An important advantage however was that the drug could be given orally;

once absorbed from the intestinal tract, it passed directly to the liver, where it was activated.

Chemical activation of cyclophosphamide, however, produced a toxic by-product: acrolein (Madondo et al., 2016). This situation of a drug that, when activated, generates two different reactive compounds, one of which only adds to toxicity, was seen also with the nitrosoureas, which will come up in the next chapter.

An intriguing result of recent investigations is that cyclophosphamide may potentiate the anti-cancer immune system. The immune system's cytotoxic T-cells are part of a surveillance system that can eliminate small nests of cancer cells before they grow into tumors. They also attack developed tumors but are held in check by so-called Treg cells that normally function to prevent cytotoxic T-cells from attacking normal tissues. Tumors can stimulate the proliferation of Treg cells in their neighborhood, which reduces the ability of the cytotoxic T-cells to attack the tumor. The exciting new findings are that regular treatments with low non-toxic doses of cyclophosphamide can directly or indirectly inhibit Treg cells, which would free the immune system to mount a stronger attack on the cancer (Madondo et al., 2016).

The Mitomycin C story

Mitomycin C is produced by certain microorganisms for the purpose of biochemical warfare in nature. It crosslinks DNA by way of a much more complicated chemistry than nitrogen mustards (Figure 1.20). Although not in the nitrogen mustard class, it alkylates DNA guanines at the 7 position and goes on the form inter-strand crosslinks. I once heard Waclaw Szybalski, who discovered the DNA crosslinking activity of mitomycin C (Iyer and Szybalski, 1963) (Iyer and Szybalski, 1964), aptly describe the molecule as "bristling with reactive groups," a phrase that was especially effective when delivered with his sharp Polish accent (you may not think of Polish as being "sharp," but the way he rolled his r's for emphasis in that phrase was striking).

He told me the story of the discovery this way: He was using the analytical ultracentrifuge to study the breakage of DNA in bacteria when they are deprived of the essential DNA building block, thymine. V. N. Iyer had just joined the lab, and Szybalski asked him to do a simple control experiment to get some experience with the analytical ultracentrifuge. A control experiment was needed to check whether the DNA breakdown was merely a consequence of the DNA synthesis inhibition caused by thymine deprivation. So, he looked around the lab to see what DNA synthesis inhibitor he happened to have on the shelf and found a vial of the known DNA synthesis inhibitor, mitomycin C.

Now, in order check on DNA strand breakage, it was necessary first to separate the DNA strands, because the intact DNA helix would hold the whole structure together and hide the breaks. They separated the DNA strands by heating the solution to near boiling (as described above in the context of our findings in Paul Doty's lab). The strands then normally stay separated after quick cooling, because the complementary strands then cannot find each other again.

The result of the first experiment, however, was strange: the strands of the heated DNA did not separate. Szybalski thought, well, Iyer must not have heated the solution to a high enough temperature. But repeated careful experiments always gave the same result: mitomycin prevented the strands from separating. Then the light dawned: mitomycin prevented the DNA strands from separating, because it produced crosslinks between them!

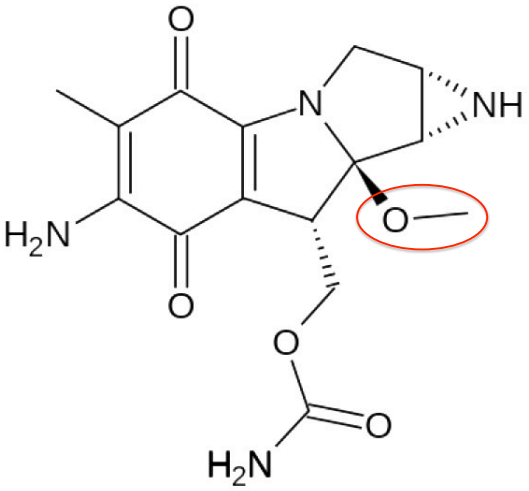

Figure 1.20. Mitomycin C, an alkylating agent and DNA crosslinker unrelated to the nitrogen mustards. Its chemistry is complicated. It is activated in the cell by reduction of the quinone moiety (adding a hydrogen atom to each to the double-bonded oxygens on the 6-membered ring). That allows the methoxy group (red encircled) to come off and create an alkylating center. A key to this reaction is the N that connects between the two 5-membered rings; its unshared electron pair forms a double-bond that allows the methoxy group to leave. Reducing the quinone allows enough negative charge to flow to the N, so that its unshared electron pair can form the double-bond. A second alkylating groups is the 3-membered ring consisting of an N and 2 C's in the upper right, which is analogous to the alkylating group in activated nitrogen mustard. Thus 2 alkylating groups are generated, which together form DNA inter-strand crosslinks.

The Psoralen story.

Psoralen is, like mitomycin, another natural product capable of forming inter-strand crosslinks in DNA. It is produced by many plants, but it can react with DNA only when activated by ultraviolet light (UV). The activation is an electronic excitation that has a brief life-time; therefore, the UV exposure has to be while psoralen is at the site where it is to react.

The psoralen story traces back to the treatment of vitiligo (unpigmented patches of skin) using plants that happened to contain psoralen-like compounds. In Egypt about 4000 years ago, the juice of *Ammi majus* (Figure 1.21) was rubbed on patches of vitiligo, after which, patients were to go out into the sun. The ancient Egyptians apparently had already noted the combined effect of the plant material and sunlight. Then, in the 13th century, ground seeds of the plant were used to treat vitiligo (Sidi and Bourgeois-Gavardin, 1952) (Lerner et al., 1953). Psoralen derives its name from *Psoralea corylifolia* (Figure 1.22), whose seeds contain psoralen among several related compounds; it was included in the Chinese system of traditional medicine. Psoralen is also found in figs, limes, celery, and parsnips.

Ammi majus was tested in 1951 by dermatologists in Paris and found to have some benefit; they swabbed the vitiligo areas with solutions of compounds derived from the plant and then exposed the areas to ultraviolet light (Sidi and Bourgeois-Gavardin, 1952). They had already determined that *Ammi majus* contains compounds related to psoralen (Fahmy et al., 1947).

In 1953, Aaron Lerner and colleagues at the University of Michigan Medical School reported a detailed study of the chemical properties of 8-methoxypsoralen and its use for treatment of vitiligo (Lerner et al., 1953). They gave this psoralen derivative to patients orally and found it to be non-toxic. They then exposed the vitiligo areas of skin to ultraviolet light, in order to cause the white areas to become pigmented. The effectiveness of this treatment was dramatically shown when a laboratory worker accidentally exposed an area of arm to an alcoholic solution of 8-methoxypsoralen, followed by ultraviolet light (Figure 1.23).

So, what does treatment of vitiligo have to do with cancer treatment? There are two parts to the answer. First, psoralen is a flat molecule having the size and shape suitable for binding to DNA by intercalation (Figure 1.24). UV-activated psoralen produces DNA inter-strand crosslinks (Figure 1.25) (Cole, 1970) (Gasparro et al., 1985). The double-bond pattern of the psoralen molecule allows the molecule to absorb a quantum of UV light that elevates an electron orbital to an excited state that makes the molecule reactive.

Second, psoralen, together with long-wavelength ultraviolet light (UVA), was found useful for treatment of mycosis fungoides, a malignant lymphoma that is localized, in its early stages, to skin (Gilchrest et al., 1976) (Abel et al., 1981). This was obviously a logical treatment because skin can easily be exposed to ultraviolet light. The treatment was called PUVA for psoralen plus UVA light. The long-wavelength UVA was by itself less damaging than shorter wavelengths of ultraviolet light, or of

sunlight. Psoralen effectively absorbs UVA, thereby becoming reactive and able to produce DNA inter-strand crosslinks that kill the malignant lymphoma cells in the skin.

In later years, the malignancy was found to be of T-lymphocytes, and the term "mycosis fungoides" was dropped in favor of "cutaneous T-cell lymphoma." Most studies of PUVA treatment of the disease reported complete disappearance of tumor in over 80% of patients (Gasparro et al., 1985). But it was still difficult to eliminate all of the malignant cells, and the disease usually recurred within a few years. Treatment usually failed if malignant cells had grown deeper below the skin or metastasized to lymph nodes or other tissues.

Figure 1.21. *Ammi majus* Linn.

Figure 1.22. *Psoralea corylifolia*, whose seeds contain psoralen, among several related compounds; it is included in the Chinese system of traditional medicine.

Psoralen is also found in figs, limes, celery, and parsnips. Psoralen's flat shape and double bonds allow the molecule to bind DNA by intercalation (the subject of Chapter 4). When activated by ultraviolet light, intercalated psoralen can react with and bind to thymines in DNA and form inter-strand crosslinks.

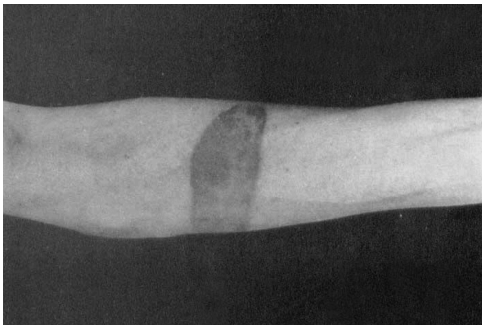

Figure 1.23. Hyperpigmented area of the arm of a laboratory worker, whose arm was accidentally exposed to an alcoholic solution of 8-mehtoxypsoralen and then to ultraviolet light (Lerner et al., 1953).

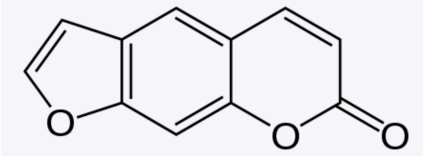

Figure 1.24 Chemical structure of psoralen. Its flat shape allows it to bind DNA by intercalation (see Chapter 4). Upon activation by ultraviolet light, the intercalated molecule can react with thymines in the DNA and form inter-strand crosslinks.

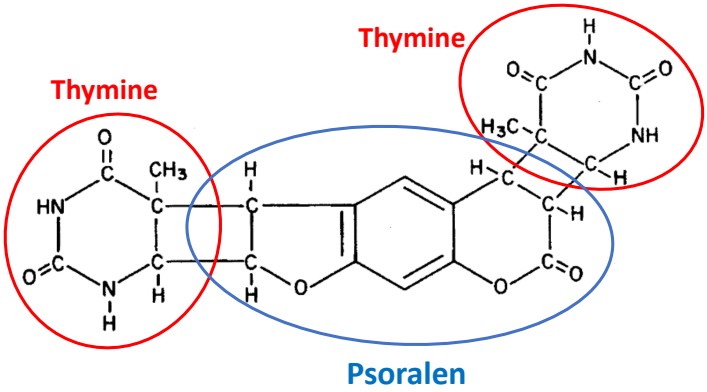

Figure 1.25. Psoralen with crosslink between two thymines, as occurs in DNA after treatment with ultraviolet light (UVA, long wavelength UV). UV excites psoralen to activate a double bond to react with the thymine double bond, forming a cyclobutene connection between the two molecules (4-membered rings in the structure). This can occur at double bonds at both ends of the psoralen molecule (shown at the intersection between the blue and red ovals). Inter-strand crosslinks are produced when the psoralen molecule can reach a thymine on each of the two DNA strands (Gasparro et al., 1985).

Synopsis

It was a long road from the mustard gas war tragedies to the current application of DNA crosslinking drugs in cancer therapy. There was hope, disappointment, and some surprises. Along with those developments, detailed knowledge of the chemistry and molecular biology of these drugs emerged and has continued to grow. This chapter has been about anti-cancer drugs that produce DNA inter-strand crosslinks. Except for the natural product, psoralen, they are all alkylating agents that attack DNA at the guanine-N7 positions. The next chapter will be about alkylating agents that attack DNA at the guanine-O6 position. The chapter following that will be about platinum complexes: drugs that crosslink DNA, but that are not alkylating agents; starting with a surprising discovery, they became some of the most useful DNA drugs for cancer therapy.

References

Abel, E.A., Deneau, D.G., Farber, E.M., Price, N.M., and Hoppe, R.T. (1981). PUVA treatment of erythrodermic and plaque type mycosis fungoides. J Am Acad Dermatol *4*, 423-429.

Alexanian, R., Bergsagel, D.E., Migliore, P.J., Vaughn, W.K., and Howe, C.D. (1968). Melphalan therapy for plasma cell myeloma. Blood *31*, 1-10.

Alexanian, R., Haut, A., Khan, A.U., Lane, M., McKelvey, E.M., Migliore, P.J., Stuckey, W.J., Jr., and Wilson, H.E. (1969). Treatment for multiple myeloma. Combination chemotherapy with different melphalan dose regimens. Jama *208*, 1680-1685.

Brookes, P., and Lawley, P.D. (1960). The reaction of mustard gas with nucleic acids in vitro and in vivo. The Biochemical journal *77*, 478-484.

Catovsky, D., Else, M., and Richards, S. (2011). Chlorambucil--still not bad: a reappraisal. Clinical lymphoma, myeloma & leukemia *11 Suppl 1*, S2-6.

Cole, R.S. (1970). Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Studies with phage lambda, Escherichia coli, and mouse leukemia cells. Biochimica et biophysica acta *217*, 30-39.

Conant, J. (2020). The Great Secret. The classified World War II disaster that launched the war on cancer., First Edition edn (New York and London: W. W, Norton & Company).

Correia, J.J., Johnson, M.L., Laue, T., Stafford, W.F., and Williams, R.C. (2004). Spinning with Dave: David Yphantis's contributions to ultracentrifugation. Biophysical chemistry *108*, 23-42.

DeVita Jr., V.T. (2015). The Death of Cancer (New York: Farrar, Straus and Giroux).

Dong, Q., Barsky, D., Colvin, M.E., Melius, C.F., Ludeman, S.M., Moravek, J.F., Colvin, O.M., Bigner, D.D., Modrich, P., and Friedman, H.S. (1995). A structural basis for a phosphoramide mustard-induced DNA interstrand cross-link at 5'-d(GAC). Proceedings of the National Academy of Sciences of the United States of America *92*, 12170-12174.

Dunikowska, M., and Turko, L. (2011). Fritz Haber: the damned scientist. Angew Chem Int Ed Engl *50*, 10050-10062.

Fahmy, I.R., Abushady, H., and et al. (1947). A crystalline principle from Ammi majus L. Nature *160*, 468.

Falco, P., Bringhen, S., Avonto, I., Gay, F., Morabito, F., Boccadoro, M., and Palumbo, A. (2007). Melphalan and its role in the management of patients with multiple myeloma. Expert review of anticancer therapy *7*, 945-957.

Friedrich, B., and Hoffmann, D. (2016). Clara Haber, nee Immerwahr (1870-1915): Life, Work and Legacy. Z Anorg Allg Chem *642*, 437-448.

Gasparro, F.P., Chan, G., and Edelson, R.L. (1985). Phototherapy and photopharmacology. Yale J Biol Med *58*, 519-534.

Gellhorn, A., Hyman, G.A., and Ultmann, J.E. (1956). Chlorambucil in treatment of chronic lymphocytic leukemia and certain lymphomas. J Am Med Assoc *162*, 178-183.

Gilchrest, B.A., Parrish, J.A., Tanenbaum, L., Haynes, H.A., and Fitzpatrick, T.B. (1976). Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer *38*, 683-689.

Gilman, A. (1963). The initial clinical trial of nitrogen mustard. American journal of surgery *105*, 574-578.

Gilman, A., and Philips, F.S. (1946). The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. Science *103*, 409-415.

Goldacre, R.J., Loveless, A., and Ross, W.C. (1949). Mode of production of chromosome abnormalities by the nitrogen mustards; the possible role of cross-linking. Nature *163*, 667-669.

Goodman, L.S., Wintrobe, M.M., Dameshek, W., Goodman, M.J., Gilman, A., and McLennan, M.T. (1946). Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. J Am Med Assoc *132*, 126-132.

Goodman, L.S., Wintrobe, M.M., Dameshek, W., Goodman, M.J., Gilman, A., and McLennan, M.T. (1984). Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan. Jama *251*, 2255-2261.

Herold, M., Schulze, A., Niederwieser, D., Franke, A., Fricke, H.J., Richter, P., Freund, M., Ismer, B., Dachselt, K., Boewer, C., *et al.* (2006). Bendamustine, vincristine and

prednisone (BOP) versus cyclophosphamide, vincristine and prednisone (COP) in advanced indolent non-Hodgkin's lymphoma and mantle cell lymphoma: results of a randomised phase III trial (OSHO# 19). Journal of cancer research and clinical oncology *132*, 105-112.

Hoogstraten, B., Sheehe, P.R., Cuttner, J., Cooper, T., Kyle, R.A., Oberfield, R.A., Townsend, S.R., Harley, J.B., Hayes, D.M., Costa, G.*, et al.* (1967). Melphalan in multiple myeloma. Blood *30*, 74-83.

Infield, G.B. (1971). Disaster at Bari (New York: Macmillan).

Iyer, V.N., and Szybalski, W. (1963). A Molecular Mechanism of Mitomycin Action: Linking of Complementary DNA Strands. Proceedings of the National Academy of Sciences of the United States of America *50*, 355-362.

Iyer, V.N., and Szybalski, W. (1964). Mitomycins and Porfiromycin: Chemical Mechanism of Activation and Cross-Linking of DNA. Science *145*, 55-58.

Kalaycio, M. (2009). Bendamustine: a new look at an old drug. Cancer *115*, 473-479.

Kohn, K.W., and Green, D.M. (1966). Transforming activity of nitrogen mustard-crosslinked DNA. Journal of molecular biology *19*, 289-302.

Kohn, K.W., Spears, C.L., and Doty, P. (1966). Inter-strand crosslinking of DNA by nitrogen mustard. Journal of molecular biology *19*, 266-288.

Kohn, K.W., Steigbigel, N.H., and Spears, C.L. (1965). Cross-linking and repair of DNA in sensitive and resistant strains of E. coli treated with nitrogen mustard. Proceedings of the National Academy of Sciences of the United States of America *53*, 1154-1161.

Kovacs, E.T., Ohno, S., and Kinosita, R. (1960). Studies on cyclophosphamide, antitumor alkylating compound. I. Effect on mouse leukemias. Journal of the National Cancer Institute *24*, 759-768.

Krakoff, I.H., Karnofsky, D.A., and Burchenal, J.H. (1958). Remissions induced by chlorambucil in chronic granulocytic leukemia. J Am Med Assoc *166*, 629-631.

Lane, M. (1959). Some effects of cyclophosphamide (cytoxan) on normal mice and mice with L1210 leukemia. Journal of the National Cancer Institute *23*, 1347-1359.

Lepretre, S., Dartigeas, C., Feugier, P., Marty, M., and Salles, G. (2015). Systematic review of the recent evidence for the efficacy and safety of chlorambucil in the treatment of B-cell malignancies. Leukemia & lymphoma, 1-14.

Lerner, A.B., Denton, C.R., and Fitzpatrick, T.B. (1953). Clinical and experimental studies with 8-methoxypsoralen in vitiligo. J Invest Dermatol *20*, 299-314.

Loveless, A., and Revell, S. (1949). New evidence on the mode of action of mitotic poisons. Nature *164*, 938-944.

Madondo, M.T., Quinn, M., and Plebanski, M. (2016). Low dose cyclophosphamide: Mechanisms of T cell modulation. Cancer treatment reviews *42*, 3-9.

Musto, P., and D'Auria, F. (2007). Melphalan: old and new uses of a still master drug for multiple myeloma. Expert opinion on investigational drugs *16*, 1467-1487.

Panahi, Y., Gholami, N., Ghojazadeh, M., Moslemi, F., Naghavi-Behzad, M., Azami-Aghdash, S., Ghaffari, A., and Piri, R. (2015). Complications and Carcinogenic Effects of Mustard Gas - a Systematic Review and Meta-Analysis in Iran. Asian Pacific journal of cancer prevention : APJCP *16*, 7567-7573.

Reminick, G. (2001). Nightmare at Bari (Glencannon Press).

Rhoades, C.P. (1946). Nitrogen mustards in the treatment of neoplastic disease. J Am Med Assoc *131*, 656-658.

Shapiro, D.M., Goldin, A., and et al. (1949). Cancer chemotherapy; analysis of results of different screening techniques with nitrogen-mustard analogues. Cancer *2*, 100-112.

Sidi, E., and Bourgeois-Gavardin, J. (1952). The treatment of vitiligo with Ammi Majus Linn; a preliminary note. J Invest Dermatol *18*, 391-395.

Speed, D.E., Galton, D.A., and Swan, A. (1964). Melphalan in the Treatment of Myelomatosis. British medical journal *1*, 1664-1669.

Stern, F. (2012). Fritz Haber: flawed greatness of person and country. Angew Chem Int Ed Engl *51*, 50-56.

Ultmann, J.E., Hyman, G.A., and Gellhorn, A. (1958). Chlorambucil and triethylene thiophosphoramide in the treatment of neoplastic disease. Annals of the New York Academy of Sciences *68*, 1007-1016.

von Minckwitz, G., Chernozemsky, I., Sirakova, L., Chilingirov, P., Souchon, R., Marschner, N., Kleeberg, U., Tsekov, C., Fritze, D., Thomssen, C.*, et al.* (2005). Bendamustine prolongs progression-free survival in metastatic breast cancer (MBC): a phase III prospective, randomized, multicenter trial of bendamustine hydrochloride, methotrexate and 5-fluorouracil (BMF) versus cyclophosphamide, methotrexate and 5-fluorouracil (CMF) as first-line treatment of MBC. Anti-cancer drugs *16*, 871-877.

Waldenstroem, J. (1964). Melphalan Therapy in Myelomatosis. British medical journal *1*, 859-865.