Chapter 5. The methotrexate story 220719dj3

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

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CHAPTER 5

The methotrexate story: folic acid analogs.

Discovery of methotrexate as an anti-leukemia drug

Acute leukemia in the 1940's was relentless and invariably fatal, and there was no way of even slowing down the disease. That terrible disease, often of children, was caused by abnormal white blood cells growing unchecked: they overgrew the bone marrow and blocked normal blood cell production there. The result was depletion of red blood cells with consequent anemia, dearth of normal white blood cells that were needed to fight infections, and reduction in platelets needed to prevent bleeding.

In June 1948, just 2 years after Goodman, Gilman and their coworkers reported the lymphoma tumor-melting effect of nitrogen mustard (Goodman et al., 1946) (see Chapter 1), Sidney Farber and his coworkers at Harvard Medical School and The Children's Hospital in Boston reported that aminopterin, an analog and antagonist of folic acid, was able to slow the progress of childhood leukemia (Farber and Diamond, 1948) (Figures 5.1). That was the second breakthrough, after nitrogen mustard, that hastened the era of cancer chemotherapy. Although it was not a cure, it did set the stage for a cure.

Aminopterin was a chemically modified folic acid that was known to inhibit the actions of folic acid. This inhibition impaired the production of building blocks for the synthesis of DNA and RNA. Consequently, the drug impaired the ability of cells to grow and divide.

Farber had followed up on a report in 1947 from Lederle Laboratories in Pearl River, New York that folic acid antagonists suppressed white blood cell counts in rats. A modest stretch of the imagination suggested that such anti-folate drugs might impair the growth of leukemic blood cells (Franklin et al., 1947).

Farber began cautiously treating children in the last stages of the disease with modified forms of folic acid. After some encouraging results, Farber selected aminopterin for the further studies, because it was the most potent folic acid antagonist available. To the investigators' surprise and delight, some of the children had a remarkable response: their symptoms improved and their leukemia cells disappeared (Farber and Diamond, 1948). For a short time, it even seemed as if they might be cured. But within a few months, leukemia cells began to grow again, and those newly growing leukemia cells did not respond to the drug. Similar temporary responses were soon reported also in adult patients with acute leukemia (Dameshek, 1949). In addition to aminopterin, the latter trials used another folate antagonist, amethopterin, which came to be called methotrexate and was to become a mainstay of cancer therapy.

Dameshek likened acute leukemia to a wildfire, which, although dampened by aminopterin, continued to smolder and could suddenly light up again (Dameshek, 1949). The temporary responses of childhood acute leukemia to the "antifols" were impressive and beyond previous experience. However, aminopterin or methotrexate, used by itself, was far from a cure.

The roots of the antifol discovery however can be traced further back to the 1930's and early 1940's, when researchers found that a folic acid deficiency often caused anemia (Hoffbrand and Weir, 2001). The bone marrow of some of the anemic patients contained unusual enlarged cells that they thought resembled leukemia cells. The researchers therefore thought that leukemia might result from a folic acid deficiency. This was incorrect, however, because those enlarged cells were abnormal precursors of red blood cells, not leukemic white blood cells.

Even though the conjecture was wrong, it led to a major break-through. Following up on that erroneous idea, Henle and Welch treated a leukemia patient with folic acid, thinking that the leukemia was caused by a folic acid deficiency. Instead of slowing the disease, however, folic acid caused it to progress even faster. Well, they thought, if folic acid speeded up the disease, maybe folic acid deficiency would slow it down. Indeed, when they treated another leukemia patient with a crude folic acid antagonist, there was a dramatic reduction in the number of leukemia cells in the blood. Henle and Welch published this observation in a very brief report in 1948 (Heinle and Welch, 1948). It was the first clue that folic acid antagonists could suppress the progress of leukemia.

That brief report, spurred chemists at Lederle Laboratories to synthesize new folic acid antagonist. The most potent of these was aminopterin, which was the drug Sidney Farber used in his landmark findings in the treatment of childhood leukemia

-- which was also published in 1948, showing how quickly a preliminary observation led to a substantial clinical result.

It was not yet a cure, however, because the patients inevitably relapsed, and their leukemia then no longer responded to the drug. This experience however provided a foundation for the eventual cure of acute leukemia in children.

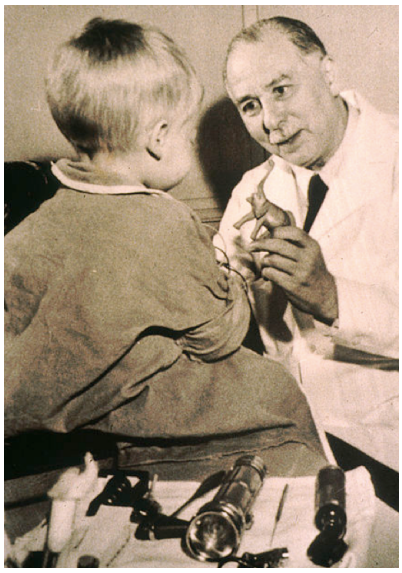


Figure 5.1. Sidney Farber (1903-1973), discoverer of aminopterin and methotrexate as effective drugs for the treatment of acute leukemia in children. Although they did not cure, the drugs did temporarily shut down the disease and prolonged life.

Aminopterin's action against childhood leukemia was soon confirmed and extended to leukemia in adults, as well as solid tumors, such as breast cancer (reviewed by Farber and by Dameshek in 1949 (Farber, 1949) (Dameshek, 1949)). The speed of this progress in discovery and clinical application is notable, especially when compared with the delays and difficulties that new therapies now often encounter (DeVita Jr., 2015). Still, temporary remissions in those early studies were achieved in only a fraction of patients, and at the cost of sometimes severe toxicity.

At about the same time, Chester Stock and Abraham Goldin and their colleagues showed that aminopterin inhibited the growth of malignant tumors in mice (Schoenbach et al., 1949; Sugiura et al., 1949) (Figure 5.2). Moreover, the effect of the drug was prevented by folic acid, which supported the idea that aminopterin did in fact inhibit the tumor by competing with folic acid (Goldin et al., 1949).

Aminopterin differed from folic acid only in that an oxygen atom was replaced by an amino group (Figure 5.3). It is now known that aminopterin or methotrexate

compete with an active form of folic acid for binding to two critical enzymes, as will be explained later in this chapter.

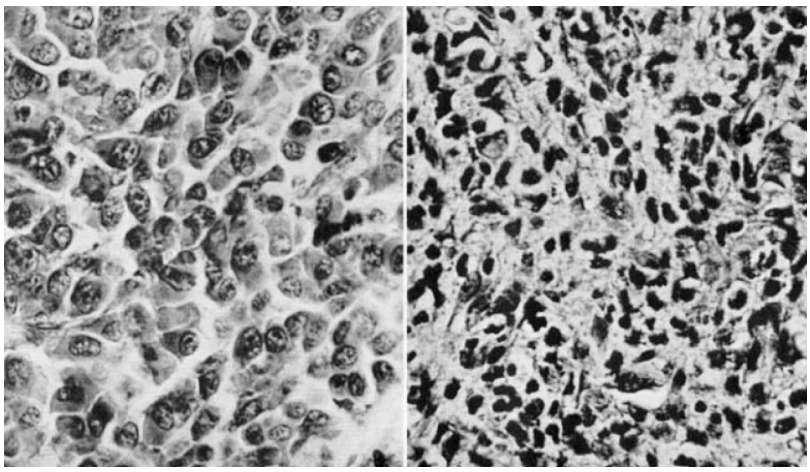


Figure 5.2. Effect of aminopterin on a mouse tumor (sarcoma 180). *Left*, before treatment; *right*, after treatment. After treatment, the tumor cells were dying and disintegrating (240X) (Sugiura et al., 1949).

Abraham Goldin and his coworkers at NCI found that amethopterin (methotrexate) had a better therapy-versus-toxicity ratio than aminopterin. Therefore, in 1956, methotrexate replaced aminopterin in treatment of patients. Aminopterin and methotrexate are chemically and pharmacologically very similar; however it seems that the two drugs may never have been compared head-to-head in human patients (Bertino, 1993). Interestingly, methotrexate had better antitumor properties (in animals), despite being much less potent (a higher dose was needed) than aminopterin (Ferguson et al., 1950)).

Both aminopterin and methotrexate killed most of the leukemia cells, but also depleted the bone marrow of the normal red blood cells, white blood cells, and platelets needed to prevent anemia, fight infection, and prevent bleeding (Thiersch, 1949). Therefore, the normal bone marrow was given time to recover between doses of the drug, which was a major advance in effectiveness of the drugs. Another major step toward the eventual cure of childhood leukemia was the development of platelet transfusion, which prevented bleeding during the time required for the bone marrow to recover. This critical development was spearheaded by Emil J Freireich at the National Cancer Institute.

Methotrexate by itself produced remissions that only lasted several months to about a year. Life was prolonged, but the leukemia invariably recurred and no longer responded to the drug. Cancer researchers however were relentless in their quest to

cure the disease; it was a long struggle, but over the next three decades they succeeded in doing so. Folic acid antagonists were an essential part of the story, but eventual success required the careful design of therapy using multi-drug combinations, as well as platelet transfusions and bone marrow implants (DeVita Jr., 2015; Laszlo, 1995).

The road to the cure of childhood leukemia was a long and difficult struggle. Some clinicians in the 1940's and 1950's felt that the children should be allowed to die in peace, rather than being subjected to the additional discomforts of drug toxicities and the pain of bone marrow aspirations that were needed to gage the effects of the drugs. Even in 1957, when I arrived at NCI and served on the childhood leukemia ward, some of my fellow Clinical Associates felt that way and at least one of my close friends refused to serve on the cancer wards, because he felt that some of the research was unethical. However, if left untreated, these children were all fated soon to die of their disease, and many parents felt that anything was worth a try. We did succeed in temporarily suppressing the disease with methotrexate, as Farber had described, as well as with other drugs that were being tried. My clinical associate colleagues on the NCI cancer wards in the late 1950's however would have been surprised, as I myself was, that the clinical studies of those early days were the beginning of a path that really did lead to a cure.

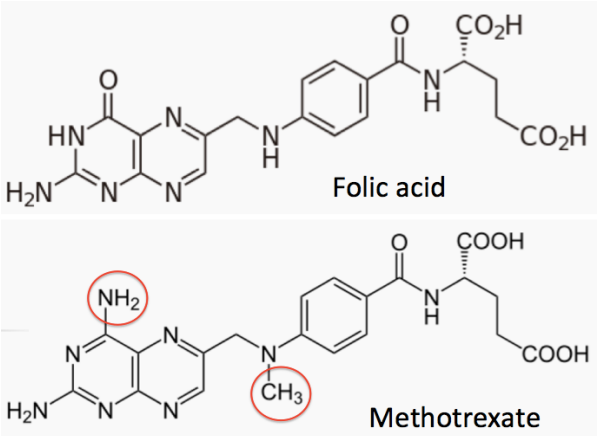


Figure 5.3. Simple modifications of folic acid yielded the anticancer drugs aminopterin and amethopterin (now called methotrexate). Placing an amino group (NH_2) in place of the oxygen on the pteridine ring of folic acid yielded aminopterin; further addition of the methyl (CH_3) group (encircled red) yielded methotrexate. The replacement of the pteridine oxygen by an NH_2 group, caused the molecule to become an antagonist of folic acid: it inhibited the actions of folic acid and put a monkey wrench (the English might say “spanner”) into the mechanisms where folic acid was critical.

Methotrexate cures choriocarcinoma.

Only 2 years after Abraham Goldin's discovery of the superior effectiveness of methotrexate, Roy Hertz and his colleagues at NCI reported that methotrexate was remarkably effective against choriocarcinoma, a rapidly fatal cancer arising from embryonic tissues of the placenta in pregnant women (Figure 5.4) (Hertz et al., 1961; Hertz et al., 1956; Li et al., 1958). Methotrexate's dramatic cure of many cases of choriocarcinoma was soon confirmed by James Holland at the Roswell Park Memorial Institute in Buffalo, New York (Holland, 1958). Methotrexate, given in the appropriate dose schedule, cured most of the patients, even if the tumor had already metastasized (Hertz et al., 1964). The reported cure of a metastatic cancer astounded many cancer researchers who at first found it hard to believe. Choriocarcinoma was the first malignant tumor to be cured by chemotherapy, and, most remarkably, it could be cured with the administration of just a single drug, an antifol such as methotrexate.

Chemotherapy worked so well against choriocarcinoma, because the cells derive from the embryo, which is a foreign tissue, as far as the patient's immune system is concerned. After methotrexate killed most of the rapidly dividing cancer cells, the remainder were often mopped up by the patient's immune system reacting against the choriocarcinoma cells that are genetically derived from the embryo. The immune system sees this cancer as foreign tissue, because mother and child are not genetically identical: half of the embryo's genes come from the father.

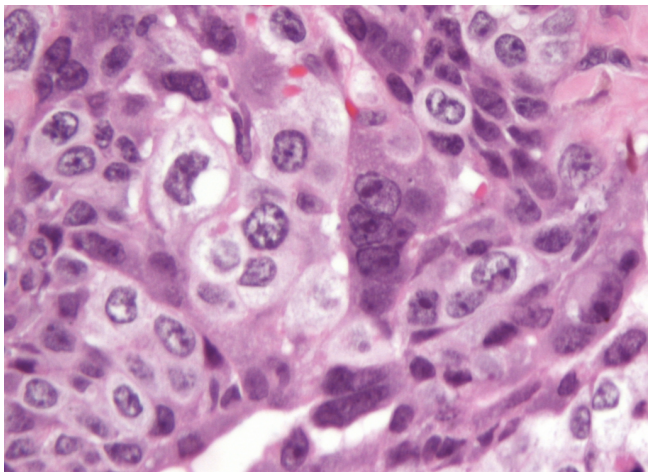


Figure 5.4. Choriocarcinoma, a malignant tumor that methotrexate cured. It usually arose in the placenta of pregnant women and was made up of wildly growing cells of various sizes and shapes. Some, such as the large dark one in near the center, have several nuclei. These were the cells that produced chorionic gonadotropin (HCG) in

the placenta, as well as in the tumor. When the HCG hormone in the blood declined to undetectable levels, it was a sign of response and eventual cure.

How methotrexate works -- Overview

Skipping the details for now, the essential point is that methotrexate inhibits the synthesis of DNA: it prevents the chromosomes from being duplicated for cell division. In other words, it blocks the step in the cell division cycle where DNA has to be duplicated. True, it is good to block the division of leukemia or tumor cells, but normal cells in some important tissues also have to duplicate at a high rate and inhibiting those cells often caused major problems. The normal tissues most sensitive to blockage of cell division by methotrexate were the rapidly dividing blood-forming cells in the bone marrow and in the lining ("mucosa") of the intestines.

In some malignancies, particularly leukemias, tumor cells can enter the brain, where methotrexate is kept out by the blood-brain barrier. The drug was therefore also injected into the cerebrospinal fluid by way of a spinal tap, in order to kill tumor cells that may be lurking in the central nervous system (Whiteside et al., 1958).

Folic acid was needed to produce the chemical building-blocks required to make DNA. To do so, however, the folic acid molecule had to be altered, first by addition of two hydrogen atoms to produce dihydrofolate, and then addition of two more hydrogen atoms to produce tetrahydrofolate. The latter is the reaction step that methotrexate blocks (Figure 5.5). The enzyme that carries out this reaction is dihydrofolate reductase (DHFR), and it is this enzyme that methotrexate bound and blocked.

Methotrexate usually had to be combined with other anticancer drugs to have lasting benefit. There was one type of cancer however that was cured by methotrexate alone, and that was choriocarcinoma. This rare cancer, as already mentioned, occurs during pregnancy from cells in the placenta of the embryo. The cells of this form of cancer divide rapidly, which is one reason that this cancer responds so well to a DNA synthesis inhibitor such as methotrexate. Before methotrexate, metastatic choriocarcinoma was fatal in 90% of cases (Yarris and Hunter, 2003).

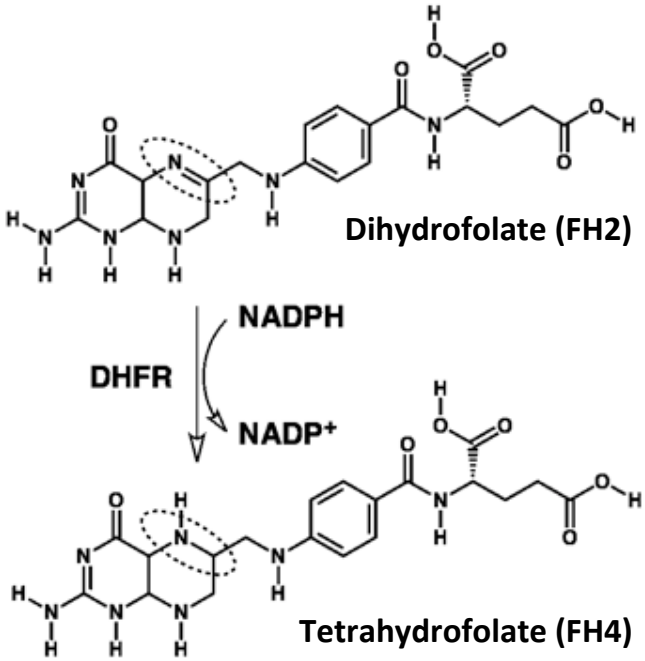


Figure 5.5. An essential reaction that methotrexate inhibits. The reaction is carried out by the enzyme dihydrofolate reductase (DHFR), which methotrexate binds and blocks. What DHFR does is to convert the double-bond enclosed by the dashed oval (upper structure) to a single bond (lower structure). This involves adding 2 hydrogens (not shown). The product, tetrahydrofolate, is required for the manufacture of building-blocks for DNA.

How methotrexate kills cancer cells.

Methotrexate, like folic acid, enters the cell by way of channels through the cell surface membrane. Cancer cells that have too few of those channels don't let much methotrexate in and therefore do not respond well to the drug (Chen et al., 2013).

Once inside the cell, an enzyme adds several more glutamates to the end of the methotrexate molecule, and this polyglutamated form cannot exit from the cell, because the glutamates bear negative charges, and electrical charge impairs the ability of molecules to pass through the cell surface membrane (Figure 5.6). Moreover, molecular pumps that pump many drugs out of the cell do not work with the polyglutamated form of methotrexate (Chen et al., 2003). That was important because drug resistance was often caused by increased quantities of those drug efflux pumps, and this resistance mechanism would not work to remove the polyglutamated methotrexate from the cell. Thus, the polyglutamate avoided this common mechanism of resistance (Szabo et al., 2016).

As usual, however, the real-life situation was more complicated: there were enzymes in the cell that removed the extra glutamates; folic acid competes with methotrexate for the polyglutamating enzyme; methotrexate polyglutamate drugs cannot be gotten directly into the cell, because they will not pass through the cell membrane (Szabo et al., 2016). Also, when methotrexate reduces the amount of thymidylate in the cell, a feedback signal initiates an attempt to compensate by making more dihydrofolate reductase enzyme (Rushworth et al., 2015). Strategies were being developed to overcome these problems.

Methotrexate then binds and inhibits the key target enzyme, dihydrofolate reductase (Volpato and Pelletier, 2009). The cell needs these enzymes to produce components required for DNA synthesis, particularly thymine, adenine, and guanine.

In a little more detail, here are the steps that were found to be relevant for the action of methotrexate:

• In the cell, folic acid (folate) readily picks up 2 hydrogen atoms to become dihydrofolate (FH2).

• Dihydrofolate reductase (DHFR) then adds 2 more hydrogens to FH2 to form tetrahydrofolate (FH4) (Figure 5.5). Methotrexate binds to and inhibits DHFR and therefore blocks this reaction. What happens is that the drug binds to the folate binding site on the enzyme and prevents normal folate from coming in and binding there (Volpato and Pelletier, 2009).

• Another enzyme in the cell adds a methyl group to FH4 to form methylene-tetrahydrofolate (meFH4), a very important molecule that makes methyl groups available for the syntheses of thymine, adenine, and guanine.

• meFH4 provides a methyl group for the enzyme thymidylate synthase to make thymine from uracil (the enzyme converts deoxyuridine phosphate to thymidine phosphate). Since methotrexate inhibits dihydrofolate reductase, the production of FH4 needed to make meFH4 is blocked. Without meFH4, thymidylate synthase function and the production thymine components for DNA are impaired. Moreover, methotrexate also blocks thymidylate synthase directly, which more completely inhibits thymidylate production.

Result: by inhibiting dihydrofolate reductase and thymidylate synthase, methotrexate blocks the production of thymine, adenine, and guanine components for DNA synthesis (Fang et al., 2016).

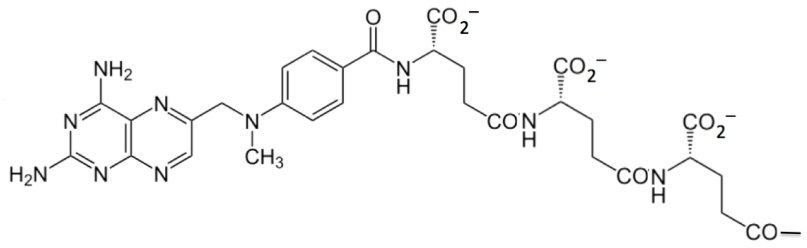


Figure 5.6. Methotrexate polyglutamate. Three glutamates are shown, but there can be as many as 8. Note the negative charges on the -CO_2's, which prevent the molecule from passing through the cell's surface membrane. When the glutamates are added inside the cell, the polyglutamate methotrexate cannot exit from the cell.

How cells become resistant to methotrexate.

Drug resistance, either intrinsic to the tumor, or acquired through selective proliferation of resistant cells, was the major bugaboo of chemotherapy. Resistance to methotrexate was found to be due to any of several factors; some of the best understood were the following (Walling, 2006):

• Methotrexate uptake channels that are too few in number or that have a reduced binding affinity for the drug; the drug then cannot enter the cell (Sirotnak et al., 1968).

• Reduced addition of glutamates to the methotrexate molecule, thereby reducing the retention of the drug in the cell (Chen et al., 2003).

• Increased activity of the enzyme that removes the extra glutamates from methotrexate polyglutamates inside the cell. Without the extra glutamates, the drug can escape from the cell.

• Reduced binding affinity by mutation of the dihyrofolate reductase enzyme for methotrexate (Volpato and Pelletier, 2009). Methotrexate would then be unable to inhibit the enzyme.

• Overproduction of dihydrofolate reductase (DHFR) by amplification of the gene, i.e., by an increase in the number of copies of the gene in the cell's chromosomes (Flintoff et al., 1982). The methotrexate would then be unable to block all of the increased amount of DHFR inside the cell.

This shows how complex the problem of overcoming drug resistance can be. Most of the changes causing drug resistance were due to mutations, which were much more frequent in cancer cells than in normal cells. Only the more resistant tumor cells survived, but these could keep on dividing to form cancers that did not respond to the drug.

Amplification of the DHFR gene in homogeneously staining regions (HSR) of chromosomes.

A striking and unexpected observation was made in 1976, by June Biedler and Barbara Spengler at Memorial Sloan-Kettering Cancer Center in New York. They were examining the chromosomes of cells that had been made resistant to methotrexate or other anti-folate drugs. I imagine that it might have been a surprise, or perhaps even a shock, to see that among the cell's chromosomes there was one that was greatly elongated. The reason for its greater length appeared to be that the chromosome had an insertion of a long region that was devoid of the usual banding pattern, a region that they therefore dubbed "homogeneously staining region" (HSR) (Figure 5.7). They surmised correctly that the HSR contained or was made up of a huge number of DHFR genes – which was the cause of the cell's drug resistance (Biedler and Spengler, 1976). The story was confirmed by Jack Nunberg and coworkers at Columbia University in 1978 (Figure 5.8) (Nunberg et al., 1978). HSR's have since been found in chromosomes of many cancers, generally associated with drug resistance attributable to a gene amplified in the HSR.

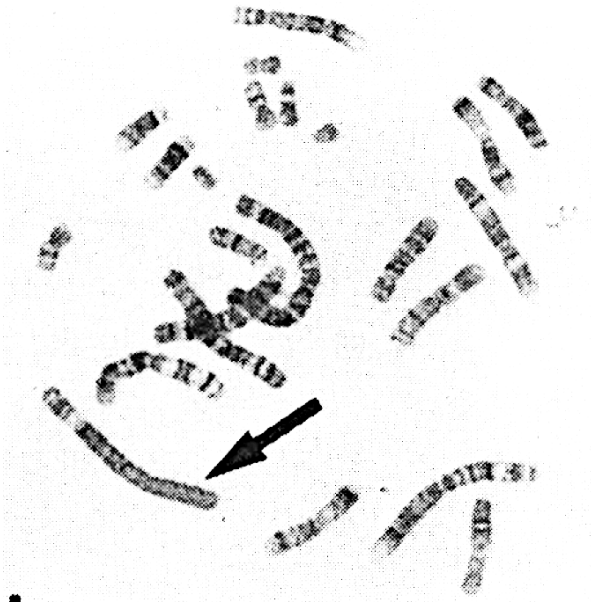


Figure 5.7. An example of a homogeneously staining region (HSR) (*arrow*) in a chromosome of a cancer cell observed by Biedler and Spengler in 1976. An HSR was presumed to be an amplification of a gene, resulting in drug resistance (Biedler and Spengler, 1976).

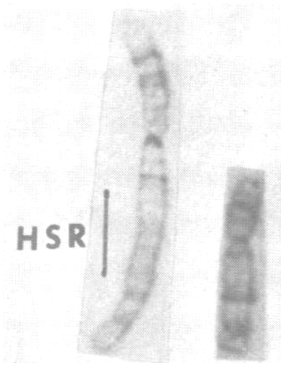


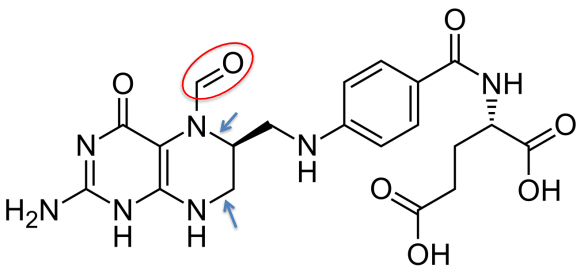
Figure 5.8. Amplification of the DHFR gene in a homogeneously staining region (HSR) in a chromosome of a methotrexate-resistant Chinese hamster cell reported by Nunberg and coworkers in 1978. The HSR contained a huge number of DHFR genes, which greatly extended the length of the chromosome and caused the resistance to the DHFR-inhibitor drug. The corresponding normal chromosome is on the right (Nunberg et al., 1978).

Leucovorin comes to the rescue.

Chemotherapy with methotrexate often required high dosage that produced troubling toxicity, especially to the bone marrow and gastrointestinal tract. Fortunately, an antidote was available: leucovorin (also known as folinic acid or citrovorum factor) (Flombaum and Meyers, 1999; Schoenbach et al., 1950) (Figure 5.9). Patients could tolerate up to 50-fold higher methotrexate doses if leucovorin was administered within 24-48 hours (Frei et al., 1980). This so called “high-dose methotrexate/leucovorin rescue” regimen given at weekly intervals was found effective, especially in cancers that do not take up methotrexate well; the high dose helps to push the drug into the cells. How much better this regimen was than methotrexate by itself, however, remained uncertain (Frei et al., 1980; Zelcer et al., 2008).

Methotrexate inhibited DNA synthesis by blocking both dihydrofolate reductase and thymidylate synthase. These inhibitions could be reversed by administering leucovorin (Schoenbach et al., 1950).

Therefore, when high doses of methotrexate were needed for effective anticancer treatment, leucovorin successfully countered methotrexate's major toxic effects on the bone marrow, gastrointestinal tract and kidney, as well as toxicity to the brain and spinal cord, particularly if the drug was administered into the spinal fluid to kill cancer cells lurking in the central nervous system (Whiteside et al., 1958). Leucovorin provided excess dihydrofolate (FH2), which circumvented the methotrexate-blocked reaction steps (Howard et al., 2016).



Leucovorin (Folinic acid)

Figure 5.9. Leucovorin (folinic acid, also known as citrovorum factor) is a natural active derivative of folic acid. It differs from folic acid in having 4 hydrogens added to make single-bonds (arrows) from the double-bonds in folic acid. There is also a C=O addition (red oval), which makes a methyl group available for the synthesis of thymine, adenine, and guanine (in a manner similar to the case of meFH4 described in Chapter 6).

Platelet transfusion to control bleeding becomes essential in the search for a cure.

In the advanced stages of acute leukemia, the normal bone marrow cells become replaced by leukemic cells. A life-threatening consequence was that not enough platelets were made to control bleeding, and the patient was in danger of bleeding to death. That danger limited the amount of drug that could be safely administered.

When the problem of how to transfuse fresh platelets was solved, the amount of drug that could be safely administered was increased. Platelet transfusion was essential for patient to survive the dosage of the drug combinations that were needed for cure.

Here is how platelet transfusion became possible:

Much of the credit goes to Emil J Freireich ("Jay"), whose personality, determination, and thinking outside the box is entwined in the story. Freireich's remarkable career and accomplishments was described in poignant detail by John Laszlo (Laszlo, 1995).

Freireich came to the National Cancer Institute shortly after the NIH Clinical Center was opened in 1953. Since he had trained in hematology, Gordon Zubrod asked him to start a Leukemia program. Emil J Freireich immediately met Emil ("Tom") Frei III, who directed the NCI's clinical program and whose office was next door. The

remarkable coincidence of the similarity of their names caused some confusion. However, Tom was precise and systematic in contrast to Jay's predilection for "wild" ideas, which he pursued relentlessly, and which often worked out. Their names were always "Tom" and "Jay"; their common name "Emil" was never used. They complemented each other and their collaboration worked extraordinarily well. Their different personalities and ways of thinking were very evident when I served as a Clinical Associate on the Childhood Leukemia Service in 1957. Tom Frei impressed me in the scope of his knowledge. He always had a stack of punched cards in his long white coat and answered my questions with reference to published evidence. Jay had some extraordinary idea that I had difficulty accepting; some of them however led to important breakthroughs, such as the way concentrated blood platelets could be stored for transfusion.

Combination chemotherapy including methotrexate cures childhood leukemia.

"Full speed ahead and damn the torpedoes."

Freireich was full of 'crazy ideas' for new treatments to try. Gordon Zubrod as head of the Medicine Branch was often skeptical, but nevertheless often supported him, because the outlook for the children was so bleak. Zubrod's instincts bore fruit as Emil J Freireich ("Jay") was to deserve much of the credit for the first cures of childhood leukemia.

The details of how childhood leukemia was eventually cured is told in the book by John Lazlo, which also describes the personalities who made it possible (Laszlo, 1995). Methotrexate was a key part of the drug combination that enabled the cures. The stories of the other anti-cancer drugs that made up the first successful combination are told in their respective chapters: vincristine, Chapter 10; amethopterin (methotrexate), this chapter; 6-mercaptopurine, Chapter 7; and prednisone. The therapy was named VAMP, a combination of the first letters of the aforementioned drug names.

By 1962, Jay Freireich and Tom Frei (Emil Frei) had decided that it made sense to combine some of the drugs that individually had shown some activity. They eventually sought to combine 4 drugs with different mechanisms of action, but in addition avoiding multiple drugs having the same toxicity. The idea was to attack the leukemic cells from different directions, while avoiding synergistic toxicity. The idea nevertheless struck some clinicians as far-fetched or even crazy (Laszlo, 1995). Some thought the studies were needlessly poisoning the very sick children. Nevertheless, Jay Freireich's dogged determination, much to the surprise of many, cured some of the children.

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