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THE PURINE PATH TO CHEMOTHERAPY

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When I joined the Wellcome Research Laboratories in 1944, World War II was in progress. Since the work I am about to discuss covers a period of some 40 years, it may be pertinent to consider the "state of the art" at that time. Our ultraviolet absorption spectra were measured with a Bausch and Lomb spectograph which had a carbon arc as the light source and photographic plates for recording the amount of light absorbed at each wavelength. There was no paper or ion-exchange chromatography, and purines were isolated and separated as copper and silver salts, or picrates, by fractional crystallization. Tritium and $^3$H were available, but no $^{14}$C or $^{32}$P.  Geiger counters were used for counting radioactivity; scintillation counters came much later. Some heavy isotopes, e.g., $^{15}$N and $^{18}$O were obtainable but required the use of a mass spectrometer, which few laboratories had. The state of knowledge of nucleic acids was rather rudimentary.  We knew they contained purines and pyrimidines but the sequences were not known. The prevailing theory was that there were two purines and two pyrimidines in each tetranucleotide and that these tetranucleotides were strung together in some fashion. However, the nature of the internucleotide linkage had not been established and the helical structure of DNA had not yet been proposed.

In 1940 Woods and Fildes (1,2) had put forth the antimetabolite theory to explain the action of sulfonamides on bacteria, suggesting that the sulfonamides interfered with the utilization of a necessary nutrient, para-aminobenzoic acid. Hitchings theorized that, since all cells required nucleic acids, it might be possible to stop the growth of rapidly dividing cells (e.g., bacteria, tumors, protozoa) with antagonists of the nucleic acid bases. One might hope to take advantage of the faster rate of multiplication of these cells compared with normal mammalian cells and essentially sort out the biochemical differences between various types of cells by the way they responded to these antimetabolites (3,4). It was my assignment to work on purines, pteridines, and some other condensed pyrimidine systems.

It was, of course, necessary to have some biological systems to determine the potential activities of the new compounds. Essentially nothing was known at that time about the anabolic pathways leading to the utilization of purines for nucleic acid synthesis. A number of catabolic enzymes were known: nucleases, nucleotidases, nucleosidases, deaminases (for guanine, adenine, adenosine and adenyl acid), xanthine oxidase and uricase. In 1947 Kalckar described the reversibility of nucleoside phosphoribosyltransferase (5). The enzymes guanase and xanthine oxidase were useful in our laboratory to examine the purines as substrates or inhibitors of these enzymes (6,7). However, it was the microorganism *Lactobacillus casei* upon which we mainly relied. It could grow on adenine, guanine, hypoxanthine or xanthine, provided the pyrimidine thymine was added. It could also synthesize purines and thymine, if given a source of folinic acid in the form of liver powder. [The structure of folic acid was not elucidated until 1946 by the Lederle group (8)].  Hitchings and Falco had devised a screening test in which it was possible to determine whether a compound could substitute for thymine (9) or a natural purine (4,10) or inhibit its utilization, and could also determine whether a compound was a folic acid antagonist (11,12).

Few chemists were interested in the synthesis of purines in those days and I relied mainly on methods in the old German literature. The transformation reactions were carried out mainly by the methods of Emil Fisher and the syntheses from pyrimidine intermediates by the methods of Traube. The direct replacement of oxygen by sulfur by the method of Carrington (13) also proved to be exceedingly useful for synthesizing the mercaptouridines (14,15).

In 1948 we found that 2,6-diaminopurine inhibited the growth of *L. casei* very strongly and that the inhibition was reversed specifically by adenine, but not by the other natural purines (4,16). However, low concentrations of diaminopurine could also be reversed by folic acid, an attribute which diaminopurine had in common with other diaminopyrimidines and diaminopyrimidine condensed systems (10).  Studies on a diaminopurine-resistant strain of *L. casei* revealed that it grew poorly on adenine as a source of purine. We deduced that adenine and 2,6-diaminopurine must be anabolized by the same enzyme, and that the product of diaminopurine anabolism interfered with purine interconversion (17). That enzyme was reported by Kornberg in 1955 to be adenylyl pyrophosphorylase (adenine phosphoribosyltransferase) (18). When tested on mouse tumors and the AKR mouse leukemia (19) or tumor cells in tissue culture (20) diaminopurine proved to be strongly inhibitory. It

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produced two good clinical remissions in chronic granulocytic leukemia in adults but produced severe nausea and vomiting, as well as severe bone marrow depression in two other patients (21). Interestingly, diaminopurine showed activity against vaccinia virus, a DNA virus, in vitro (22), but its toxicity in animals led us to abandon that possible utility.

**ANTILEUKEMIC DRUGS**

By 1951 we had made and tested over 100 purines in the *L. casei* screen (23) and discovered that the substitution of oxygen by sulfur at the 6-position of guanine and hypoxanthine produced inhibitors of purine utilization. 6-Mercaptopurine (6-MP) and 6-thioguanine (TG) were tested at the Sloan-Kettering Institute, with whom we had established a collaboration, and were found to be active against a wide spectrum of rodent tumors and leukemias. Of special interest was the finding by Clarke (24) that 6-MP-treated tumors, although they had not regressed completely in the host mouse, were not transplantable into other mice. After some animal toxicology studies by Philips et al. (25), Burchenal proceeded rapidly to a clinical trial with 6-mercaptopurine (6-MP) in children with acute leukemia (26). At that time the only drugs available for the treatment of these terminally ill children were methotrexate and steroids and the median life expectancy was between 3 and 4 months; only 30% lived for as long as one year. The findings that 6-MP could produce complete remissions of acute leukemia in these children, although most of them relapsed at various intervals thereafter, led the Food and Drug Administration to approve the drug for this use in 1953, a little more than two years after its synthesis and microbiological investigation. A symposium on 6-MP was held at the New York Academy of Sciences in 1954 (27). The addition of 6-MP to the antileukemia armamentarium increased the median survival time to 12 months in these children, and a few remained in remission for years with 6-MP and steroids. This convinced us, as well as many other investigators in the cancer field, that antimetabolites of nucleic acid bases were fruitful leads to follow. Today 6-MP remains one of the dozen or more drugs found useful in the treatment of acute leukemia. With the use of combination chemotherapy with three or four drugs to produce and consolidate remission, plus several years of maintenance therapy with 6-MP and methotrexate, almost 80% of children with acute leukemia can now be cured.

Although we felt we were on the right track in 1952, there were still many unanswered questions. How did 6-MP work? What was the reason for its differential effect on neoplastic cells? How could one improve this differential effect? Reversal studies with 6-MP in *L. casei* did not pinpoint antagonism for any single purine. The inhibition was reversed by hypoxanthine, adenine, guanine and xanthine (28). However, studies with a 6-mercaptopurine-resistant strain of *L. casei* revealed that 6-MP was unable to utilize hypoxanthine for growth (29). Again, as with the earlier studies with 2,6-diaminopurine, we concluded that 6-MP and hypoxanthine were anabolized by the same enzyme and that interference with purine interconversions at the nucleotide level were involved (30). In 1955, (two years after the introduction of 6-MP into clinical use) the enzyme which converts hypoxanthine and 6-MP to their respective nucleotides was identified as hypoxanthine phosphoribosyltransferase (HGPRT) (18). Also in the mid-fifties the pioneering work of Greenberg (31) and of Buchanan (32,33) revealed the pathways of the biosynthesis of purines and the importance of hypoxanthine ribonucleotide (inosinic acid, IMP) as the first purine nucleotide formed in this biosynthetic pathway. It took a number of investigators and a period of years to unravel all of the pathways in which the nucleotide of 6-MP, thioguanosinic acid (TIMP), and nucleotides derived from TIMP participated as substrates and inhibitors (34). These are summarized in Figure 1. Based on our current knowledge of enzyme inhibition constants and the concentrations of the various nucleotides achieved with the therapeutic regimens, the principal sites of action appear to be feedback inhibition of *de novo* purine synthesis (particularly with methylthioinosinic acid), inhibition of inosinate dehydrogenase, and incorporation into DNA in the form of thioguanine (35). Selectivity for neoplastic cells probably depends on the levels of the individual anabolic and catabolic enzymes. Catabolic enzyme levels are generally much lower in tumor cells than in normal cells. In addition, mitotic rate, drug transport and metabolite pool sizes can be responsible for selective toxicity.

While these biochemical studies were going on, we studied the metabolic fate of 6-MP, first in mice (36) and then in humans (37). Pharmacokinetic and metabolic studies were then in their infancy, perhaps because the methodology for separation of metabolites and the counting of radioactive samples as thin films in a flow Geiger counter were both very tedious and time-consuming. Nevertheless, using Dowex-1 and Dowex-50 ion-exchange columns and paper chromatography, we investigated the fate of 6-MP in *vivo* and attempted to discover whether it was possible to modify this metabolism and thus improve the efficacy of 6-MP. We continued to synthesize derivatives of 6-MP and thioguanine and investigate structure-activity relationships (38-40). Thioguanine, which we had synthesized earlier than 6-MP, was more active but also more toxic (25). It was also more difficult to synthesize and, since its mechanism of action appeared to be similar to that of 6-MP, its metabolic fate and clinical activity was explored somewhat later (41-43). Thioguanine later found its main utility in the treatment of acute myelocytic leukemia in adults, in combination with cytosine arabinoside.

Studies of the urinary metabolites of 6-MP revealed that extensize metabolic transformations occurred in *vivo* (36,37,44-46). The single product present in highest amount was 6-thiouric acid, formed by the action of xanthine oxidase on 6-MP. In addition, there were various substances in which the sulfur had been methylated, and the methylthio derivative had been oxidized on the sulfur or on the purine ring. A considerable amount of the sulfur had been removed and converted by oxidation to