Feature Articles

Chemistry, Cyclophosphamide, Cancer Chemotherapy, and Serendipity: Sixty Years On

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Abstract. Cambridge Dictionary: serendipity | noun | the phenomenon of finding interesting or valuable things by chance.

The year 2019 marked the 60th anniversary of the approval of cyclophosphamide (CP) as an anticancer drug by the U.S. Food & Drug Administration in 1959 for the treatment of lymphoma. Between 1959 and 2019 there were ~50,000 publications listed in PubMed that have CP in the title and/or abstract, with these annual numbers showing a continual increase, and over 1,800 such articles in 2019 alone. The discovery of CP is a prime example of serendipity in science, which also applies to key elements of the metabolism and pharmacological basis for the specificity of the cytotoxicity of CP toward cancer cells. Phosphoramid mustard (PM), HO(H2N)P(O)N(CH2CH2Cl)2, the principal metabolite of CP with DNA alkylating activity, was synthesized and reported by Friedman and Seligman in 1954 prior to the discovery of CP. Interestingly, the original drug design premise for synthesizing PM, which was based on elevated phosphamidase enzyme activity in cancer cells proved to be incorrect. While this wrong premise also led to the synthesis of CP, as a six-membered ring cyclic phosphamidase-activated precursor of PM, the actual metabolic conversion of CP to PM was subsequently found to involve a surprisingly complex array of metabolites and metabolic pathways, all completely unrelated to phosphamidase. Although the molecular structure of CP has an asymmetrically substituted, i.e. chiral phosphorus center, the racemic mixture of the Rp and Sp enantiomers of CP was used throughout its initial investigations and subsequent clinical trials despite the involvement of an initial enzyme-mediated metabolic activation step, which could, in principle, be stereoselective for only one of the enantiomers of CP. Stereochemical investigations along those lines were eventually carried out, but the results did not warrant replacement of racemic CP with either enantiomer in the clinic. Amazingly, there are now ~4,000 structural congeners of PM listed in Chemical Abstracts, but none have led to an anticancer drug superior to CP. This account provides a synopsis of the key chemistry and stereochemistry investigations that comprise this story of CP, as a remarkable instance of serendipity in science, and my chance involvement in the unfolding of this fascinating story.

Keywords: cyclophosphamide, cancer, metabolism, synthesis, stereochemistry.

INTRODUCTION

As part of U.S. President Richard M. Nixon’s pledge in 1971 to launch an intensive campaign to find a cure for cancer, the National Cancer Act,
popularity referred to as "The War on Cancer," gave the National Cancer Institute (NCI) unique autonomy and, importantly, budgetary discretion to increase its efforts to acquire new compounds for testing. Knowing this and, as a newly hired Assistant Professor of organic chemistry at the Catholic University of America (CUA) in need of obtaining my first research grant, I began extensive reading about then existing anticancer drugs. Late one evening in the CUA chemistry department library, I came across a listing of cyclophosphamide (CP) together with its structure (Figure 1), the chirality of which at phosphorus immediately caught my eye, having recently trained in mechanistic organophosphorus stereochemistry as a predoctoral student with Prof. Kurt Mislow at Princeton University just several years earlier.

Being chiral, the instantaneous question in my mind was whether CP was administered to cancer patients in enantiomerically pure form [i.e. optically pure (+)- or (-)-CP] or as a racemic mixture of both enantiomers [i.e. (±)-CP]. The later was the case, which was intriguing to me in view of the then known need for enzyme-mediated metabolic "activation" of CP in vivo, and the fact that many different types of enzymes were known to be stereoselective for one enantiomer over the other. My second question was sparked by the structural simplicity of CP compared to most of the other U.S. FDA-approved cancer drugs at the time, namely, methotrexate (1959), vincristine (1963), actinomycin (1964), and vinblastine (1965). What was so special about the structure of CP based on the then available literature?

Investigating these basic questions about CP led to my subsequent participation in The War on Cancer as an active "chemist combatant," so to speak, during the next ~15 years via several NCI-funded research grants focused on the synthesis of CP enantiomers for mechanistic and kinetic studies of CP and its metabolites using Fourier-transform nuclear magnetic resonance (NMR), which was then a relatively new and powerful analytical tool. Fortunately, this was especially true for organophosphorus compounds, such as CP, since ${}^{31}$P, an NMR-active isotope of phosphorus, comprises 100% of the natural abundance of phosphorus. Before providing key findings from that work, and findings by others, the next section provides a brief synopsis of some of the history of CP prior to the 1970s. Another first-hand account of the earlier history of CP with a different perspective was published on CP’s 30th anniversary in 1989 by Norbert Brock, who discovered CP, as discussed in the next section.

EARLY HISTORY OF CP

The history of CP can be traced back to the December 2, 1943, bombing of Allied ships in the harbor of the Italian town of Bari (Figure 2) that led to a massive explosion of the SS John Harvey, a U.S. World War II Liberty ship, which released 130,000 pounds of “mustard gas,” S(CH$_2$CH$_2$Cl)$_2$. A total of 628 military victims were hospitalized with mustard gas symptoms, and by the end of the month, 83 of them had died. The number of civilian casualties, thought to have been even greater, could not be accurately gauged since most had evacuated the city to seek shelter with relatives. This horrific tragedy led to lengthy investigations as to the source of this warfare agent and its biological effects by Lt. Col. Stewart Alexander, a medical officer attached to the staff of Gen. Dwight D. Eisenhower, and by Col. Cornelius P. Rhoads, chief of the Medical Division of the Chemical Warfare Service.

The secret shipment had most likely been destined for a chemical stockpile at Foggia, 75 miles away, in order to provide the Allied forces with the capability to retaliate against a German chemical attack. Armed with the Bari report by Alexander, and the results of a top-secret Yale University study that demonstrated for the first time that a careful regimen of intravenous administration of N-methyl nitrogen mustard, CH$_3$N(CH$_2$CH$_2$Cl)$_2$, could result in human tumor regression, Rhoads went in search of funding to develop this experimental treatment known today as chemotherapy. He persuaded Alfred P. Sloan Jr., the chairman of the General Motors company, along with the company’s chief engineer, Charles F. Kettering, to endow a new institute that would bring together leading scientists and physicians to make a concerted attack on cancer.

On August 7, 1945, ironically the same day an atom bomb was dropped on Japan, they announced their plans for the Sloan Kettering Institute for Cancer Research—the "World War II was over, but the war on cancer had just been launched," and relaunched, as it were, by President Nixon in 1971.

The above-mentioned beneficial structural change from S(CH$_2$CH$_2$Cl)$_2$, a chemical warfare agent, to CH$_3$N(CH$_2$CH$_2$Cl)$_2$, an anticancer agent, led to the con-
CEPT of analogous RN(CH₂CH₂Cl)₂ structures wherein the chemical nature of R could be varied to modulate CH₂CH₂Cl alkylating activity, i.e. CH₂CH₂Cl → CH₂CH₂Nuc, Nuc = nucleophile, e.g. N or O in DNA. The use of phosphorus attached to nitrogen as a possible R group in RN(CH₂CH₂Cl)₂ for modulating DNA alkylating activity, and hence DNA crosslinking potential by virtue of having two CH₂CH₂Cl alkylating moieties, was rationalized as follows.

In a 1954 publication in *J Amer Chem Soc*, Orrie M. Friedman (Brandeis University) and Arnold M. Seligman (Harvard Medical School) stated⁶ that they “considered it worthwhile to re-examine the question of the abundance of phosphamidase activity in malignant tissue as compared to normal tissues, with substrates which could be used as possible chemotherapeutic agents,” should earlier reports of higher enzymatic activity “represent the true state of affairs.” They reasoned that “phosphorylated nitrogen mustards would be expected to be devoid of mustard action as a consequence of loss of basicity of the nitrogen atom in the phosphamide bond, [and] enzymatic hydrolysis of this bond would liberate nitrogen mustard within cells in proportion to their phosphamidase activity.” In this envisioned phosphorylated version of RN(CH₂CH₂Cl)₂, R = P(O)XY with X and Y being variable substituents. Consequently, they further reasoned, “[i]f malignant cells were, indeed, rich in phosphamidase activity, more nitrogen mustard could be delivered to them by the intravenous injection of a suitable N-phosphorylated nitrogen mustard than by injection of a tolerated dose of nitrogen mustard itself.”⁷ Of the synthesized phosphorylated bis-(2-chloroethyl)amines in which at least one of the groups on phosphorus is either amino or hydroxyl, there was extensive spontaneous hydrolysis of the reported analogs of PM (Figure 1) over the physiologically relevant pH range 4.5–8.5.⁶ Later in 1954, Friedman and coworkers reported that N-phosphorylated secondary nitrogen mustards, including PM, were “capable of intramolecular cyclization to potent tertiary amine mustards,” (i.e. highly reactive 3-membered ring aziridinium ions to be discussed in another section) and “were much less toxic” than nitrogen mustard.⁸ They concluded by suggesting that “[s]uitable phosphoryl derivatives of the new secondary nitrogen mustards may turn out to be chemotherapeutic agents for tumors with high phosphamidase activity.”⁸

More or less concurrent with the above synthetic work and suggestive reasoning in the U.S.,⁶,⁸ Norbert Brock and coworkers at Asta-Werke Aktiengesellschaft Chemische Fabrik, Brackwede, Germany, published⁹ a 1958 paper in German titled (when translated to Eng-
Novel cancer chemotherapeutic agents from the group of cyclic nitrogen mustard phosphamide esters, wherein results for CP were described. Brock has said that he and his team had synthesized and screened more than 1,000 of these candidate oxazaphosphorine compounds. Synthetic details for CP and other cyclic analogs were later available in a U.S. issued patent (3,018,302) published in 1962 by Arnold et al. at Asta-Werke (Figure 3). In this patent, exemplary data are given which show CP as having the most promising therapeutic index (TI) in vivo in several model tumor systems, commonly used to measure a drug’s safety. This index, with the formula $T I = \frac{T D_{50}}{E D_{50}}$ was (and still is) commonly used to measure a drug’s safety “window,” as $T D_{50}$ is the median toxic dose while $E D_{50}$ is the median effective dose.

This patented discovery of promising in vivo antitumor activity for CP in animal models described by Arnold et al. led to early clinical trials in Germany and the U.S. A noteworthy historical newspaper account of the latter, which was published on April 13, 1959, is reproduced in Figure 4. This newspaper article reports on “encouraging results in the first American clinical trials of a German-developed compound called ‘cyclophosphamide’,” which were described by Dr. Robert G. Ravdin (Department of Surgical Research, University of Pennsylvania Schools of Medicine) at the 50th annual meeting of the American Association for Cancer Research held in Atlantic City, New Jersey. The newspaper article goes on to say that this trial involved administration of CP to 45 patients with a wide variety of...
advanced cancers, and that "Ravdin said CP proved less toxic to healthy tissue, and apparently was also somewhat more effective in temporarily checking, or reducing cancer than any of the previously-known nitrogen-mustard drugs." Ravdin added that CP's "apparent action is roughly analogous to the way the ancient Greeks sneaked soldiers inside the walls of Troy by hiding them in a wooden 'gift' horse. The intent with the drug 'Trojan horse,' however, is to minimize undesirable toxic effects on healthy tissue while bringing as much punch as possible to bear on the cancer. Ravdin's formal report to the meeting was co-authored by Doctors Peter R. Coggins and Sylvan H. Eisman of the Harrison Department of Surgical Research, University of Pennsylvania Schools of Medicine.

Reporting on use of the material in 45 advanced cases, including a wide variety of cancers, Ravdin said it proved less toxic to healthy tissue. It apparently was also somewhat more effective in temporarily checking, or reducing cancer than any of the previously-known nitrogen-mustard drugs, he said.

The nitroen-mustards as a class are the most versatile of all the anti-cancer drugs now in use, though none of them can actually cure a cancer. In 15 of the 45 cases tested, he said, there was evidence that the growths had temporarily been made smaller, though the longest such regression so far has been only two months. Ravdin stressed that the drug is still experimental and can by no means be considered a curative substance.

As will be evident from the next section on metabolism of CP, the 'Trojan horse' analogy attributed to Ravdin in the newspaper article in Figure 4 turned out to be conceptually correct, in a general sense, but actually involves chemistry and enzymes quite different from those proposed in the original ideas about activation of CP by a phosphamidase in cancer cells. In any event, these and additional early clinical findings led to the above-mentioned approval of CP for treatment of lymphoma by the U.S. FDA in 1959.

As a final comment to this early historical background, Ravdin's 1959 use of the 'Trojan horse' analogy (Figure 4) for release of PM in cancer cells was quite unique in that the author (G. Z.) was unable to find any articles in Google Scholar published between 1940-1959 with the term 'Trojan horse' associated with drug design, delivery, or similar terms. Interestingly, 1959 was also the year in which the term "prodrug" was first introduced by the Parke-Davis company for chemical modification of the chloramphenicol structure in order to improve this antibiotic's bitter taste and poor solubility in water. While the term prodrug is now universally applicable in modern medicinal chemistry, Ravdin's 'Trojan horse' descriptor is historically appealing.

METABOLISM OF CP

Mean rate of absorption of CP after oral administration from studies in which the drug was given in a high dose both orally and intravenously to the same tumor patients (n = 18) is very high (~88%), which indicates that CP may be administered orally with good bioavailability at that high dose. The pharmacokinetics of CP in patients (n = 7) with various types of cancer has been characterized by a two-compartment model wherein the half-life of the elimination phase of CP ranged between 3 and 11 hours. The calculated fraction of the dose of CP which was metabolized averaged 88%.

According to Brock, CP and other oxazaphosphorine cytostatics differ from directly alkylating compounds in that "they must undergo biotransformation
before they can exert their alkylating oncocidal action.” Deciphering the metabolic pathway for this “activation” of CP \textit{in vivo} and identifying CP metabolites (Scheme 1) evolved into subjects of considerable clinical interest and extensive investigations by many research groups. In 1970, Hill et al.\textsuperscript{14} reported the isolation and identification of 4-ketocyclophosphamide (keto-CP, Scheme 1) from urine of dogs given CP as “a possible active form” of CP. One year later in 1971, Bakke et al.\textsuperscript{15} similarly analyzed sheep urine and identified a ring-opened carboxylated metabolite (carboxy-CP, Scheme 1) in addition to keto-CP. Shortly thereafter, in 1973, Colvin et al.\textsuperscript{16} reported the isolation of PM from incubations of CP with mouse liver microsomes, using mass spectrometry (MS) for identification, and suggested that “[t]his compound may play a major role in the biological activities” of CP.

The following year in 1974, Connors et al.\textsuperscript{17} at the Chester Beatty Research Institute, Royal Cancer Hospital, London, England, reported enlightening findings from a comprehensive study of microsomal metabolism of CP in the liver wherein CP is first converted, “presumably by the mixed-function oxidases,” into 4-hydroxycyclophosphamide (4-HO-CP, Scheme 1), “which may then break down by elimination of acrolein [H\textsubscript{2}C=CHC(O)H, Scheme 1] from its tautomeric form, aldophosphamide” (AP, Scheme 1) to yield PM. In competition with this process is the enzymic conversion of 4-HO-CP (by dehydrogenation) and AP (by oxidation) into the known \textit{in vivo} metabolites of CP, keto-

\[ M = \text{Ni(CH}_2\text{CH}_2\text{Cl)}_2 \]

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CP and carboxy-CP, respectively, "each of which has low cytotoxicity." Connors et al.\(^\text{17}\) noted that 4-HO-CP, "which was too unstable to allow identification directly by conventional procedures, was trapped by reaction with ethanol." Importantly, however, these investigators found that the resulting two, apparently isomeric, ethyl derivatives, (1) were amenable to MS, (2) yielded acrolein 2,4-dinitrophenylhydrazone on treatment with acidic 2,4-dinitrophenylhydrazine, (3) were hydrolyzed in water (pH 4.3), each isomer apparently regenerating 4-HO-CP, and (4) were highly toxic to Walker tumor cells in culture. PM was also isolated after \textit{in vitro} metabolism of CP, and on the basis of a bioassay involving Walker tumor cells in whole animals it was found that, of the known metabolites of CP, only PM "possesses the cytotoxicity and biological half-life appropriate to the active antitumor metabolite."\(^\text{17}\)

Later, in 1977, Fenselau et al.\(^\text{18}\) provided additional MS data for AP as a transient intermediate in the metabolism of CP by means of the isolation and characterization of the cyanohydrin derivative of AP from incubation of CP with mouse liver microsomes in the presence of appropriate aldehyde trapping reagents, namely, sodium bisulfite followed by sodium cyanide. Moreover, AP was also identified in the plasma of a patient receiving CP, after treatment of the plasma with these trapping reagents. As a cautionary final comment, Fenselau et al.\(^\text{18}\) stated that the ethanol trapping data of Connors et al.,\(^\text{17}\) mentioned above, "might alternatively arise from addition of ethanol across the double bond" of a putative iminocyclophosphamide (imino-CP, Scheme 1) intermediate, which was apparently the first mention of this possibly new metabolite that is uniquely characterized by a presumably reactive C=N double bond conjugated with a phosphoryl (P=O) moiety, i.e. [C=N-P=O ↔ ^3\text{C}-N-P=O].

Better understanding of the basic biochemistry and interrelationships of all of the above-mentioned CP metabolites, especially the postulated reactive imino-CP metabolite, are discussed in the following sections, which involve use of highly informative multinuclear NMR methodology.

NMR SPECTROSCOPIC ELUCIDATION OF CP METABOLITES AND KINETICS

Application of multinuclear (^31\text{P}, ^13\text{C}, ^2\text{H} and ^1\text{H}) Fourier-transform NMR spectroscopy was integral to my various NCI-funded research projects related to CP and its metabolism, which had been previously studied by others largely using MS. Important advantages of NMR over MS include (1) data-rich structural details (e.g. chemical shift, coupling constants, and spin decoupling), (2) real-time data acquisition, and (3) inherent molar quantification, all of which combine to provide (4) unambiguous molecular identification, while (5) temperature control enables (6) direct measurement of molecular dynamic processes and/or (7) kinetics.

Initially, ^31\text{P}-NMR was used to evaluate the influence of pH on the rate of intramolecular cyclization of a N\text{CH}_2\text{CH}_2\text{Cl} moiety to a 3-membered ring aziridinium ion and the hydrolysis of this reactive alkylator. While an influence of pH on the alkylating activity of PM is expected based on first-principles of organic chemistry, no data was available at the time. Briefly, as we reported in 1979 (Engle et al.\(^\text{19}\)), the ^31\text{P}- NMR kinetic data for PM demonstrated that the half-life of this metabolite of CP exhibits appreciable variation (~5-fold decrease) over the physiologically relevant pH range of 6-8, and that the anionic conjugate base of PM (PM-) is the required precursor to its intramolecularly cyclized aziridinium ion species (Scheme 2). The relative concentration of this aziridinium ion reached a peak that was also dependent on pH, increasing with lower pH. It was therefore suggested\(^\text{19}\) that pH control over the concentration of PM- and the rate of its intramolecular cyclization, as well as the rates of reaction of the cyclized aziridinium ions with nucleophiles, "provides a chemical basis for rationalizing at least part of the oncostatic specificity" of CP.

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\(^{1}\) Adapted with permission from \textit{J Med Chem} \textbf{1979}, 22, 897-899. Copyright 1979 American Chemical Society.
The rate for cyclization of PM to its aziridinium ion would be ~50% slower in tumor cells that may be more acidic than normal cells (pH 6.9 vs. 7.4)\textsuperscript{20} and, after the active aziridinium alkylator is generated, it will have a longer lifetime under relatively acidic conditions, due to less frequent interception by hydroxide ion. Whether alone or in concert, these circumstances provide for greater probability of encountering and alkylating DNA and, in effect, represent a form of selective (i.e., pH-dependent) cross-linking. A full paper on this subject with additional experiments was published by Engle et al. \textsuperscript{1982,\textsuperscript{21}}

During our NMR investigations in the late 1970s and early 1980s, a detailed understanding of the multifaceted mechanism of selective cytotoxicity had to contend with a formidable array of intervening chemistry (Scheme 1) that was poorly understood for the presumed interconversion of \textit{cis}-4-HO-CP, AP, and \textit{trans}-4-HO-CP, and had several open questions. For example, the kinetics and thermodynamics of these presumed equilibria had not been measured, although circumstantial evidence had led to the suggestions\textsuperscript{22} that AP is much less stable than 4-HO-CP and that no equilibrium exists between 4-HO-CP and AP at room temperature or above. Similarly, there was no conclusive information regarding possible equilibria between the aldehyde moiety in AP, its hydrate\textsuperscript{22} and its enol\textsuperscript{23} or equilibrium between \textit{cis}-4-HO-CP and \textit{trans}-4-HO-CP. A possible equilibrium between AP and 5 was more than academically interesting when one considers that \textit{in vivo} factors that might influence the kinetics and thermodynamics of the AP hydration to 5 could modulate, in effect, the location and rate of release of PM and acrolein from AP. This point was perhaps more apparent in the context of sulfhydril compounds that were reported\textsuperscript{24-25} to “deactivate” and transport 4-HO-CP/AP by the reversible formation of 4-thiocyclophosphamide conjugates (7). Also, imino-CP had been reportedly identified\textsuperscript{26} and then criticized\textsuperscript{27} as a possible intermediate in these chemical transformations. Furthermore, we reasoned that if imino-CP was actually an intermediate, there could be bimolecular counterparts of imino-CP, namely, Schiff-base conjugates of AP. All of these reversible processes, as well as the irreversible fragmentation of AP (or 6\textsuperscript{23}) into acrolein and PM, and the alkylation chemistry of PM/PM- would be influenced by pH and metal ions. While data for all this complex chemistry was sorely lacking, information had been reported\textsuperscript{28} for the enzymatic “detoxification” of 4-HO-CP/AP that produces the urinary metabolites keto-CP and carboxy-CP.

With the aim of clarifying the foregoing chemistry issues and open questions, we used known synthetic methods to prepare \textit{cis}-4-hydroperoxycyclophosphamide (\textit{cis}-4-HO\textsubscript{2}-CP) as well as its 5,5-dideuterio (D = \textsuperscript{2}H) and 4-\textsuperscript{13}C isotopically labeled versions to allow unambiguously assignment of NMR signals to CP metabolites. This via initial reductive conversion of \textit{cis}-4-HO\textsubscript{2}-CP to \textit{cis}-4-HO-CP with sodium thiosulfate (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}) in 2,6-dimethylpyridine (lutidine) buffer at pH 7.4, 37 °C. Full details and the results obtained were published\textsuperscript{29} in 1984. Some of the salient findings and implications are briefly summarized as follows.

The stereospecific deoxygenation of synthetic \textit{cis}-4-HO\textsubscript{2}-CP with 4 equivalents of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} afforded, after ~20 min, a “pseudoequilibrium” distribution of \textit{cis}-4-HO-CP, AP, hydrated AP, 5 (Scheme 1), and \textit{trans}-4-HO-CP in the relative proportions of 57:4:9:30, respectively, which remained constant during their continual disappearance by irreversible reactions. NMR signals indicative of imino-CP and enol 6 (Scheme 1) were not detected (<0.5-1% of the synthetic metabolite mixture). A computerized least-squares fitting procedure was applied to the individual \textsuperscript{31}P-NMR derived time-courses for conversion of \textit{cis}-4-HO-CP, AP plus AP hydrate (“AP”), and \textit{trans}-4-HO-CP into acrolein and PM, the latter of which gave an expected array of thiosulfate S-alkylation products and other phosphorus-containing materials derived from secondary decomposition reactions. This kinetic analysis gave the individual forward and reverse rate constants for the apparent tautomerization processes, \textit{i.e.} \textit{cis}-4-HO-CP ↔ “AP” ↔ \textit{trans}-4-HO-CP, as well as the rate constant \(k_{AP}\) for the irreversible fragmentation of AP. Replacement of the HC(O)CH\textsubscript{2} moiety in AP with HC(O)CD\textsubscript{2} led to a primary kinetic isotope effect \(k_{H}/k_{D} = 5.6 ± 0.4\) for \(k_{AP}\), consistent with a primary effect for rate-determining removal of a proton that is adjacent to a carbonyl group. The apparent half-lives \(t_{1/2}\) for \textit{cis}-4-HO-CP, “AP”, and \textit{trans}-4-HO-CP, under the above reaction conditions, were each equal to ~38 min, which is considerably shorter than the then widely cited colorimetrically derived half-lives reported by earlier investigators.\textsuperscript{30}

Next, N-acetyl-L-cysteine (R*SH) was used to study its conversion of 4-HO-CP/AP to C4-SR* conjugates of the type previously reported by Hohorst et al.\textsuperscript{25} In brief, detailed analysis of the resultant complex \textsuperscript{31}P-NMR spectra supported the earlier conclusions by Hohorst et al.\textsuperscript{25} In the presence of the commonly used buffer tris(hydroxymethyl)aminomethane (Tris) at pH 7.4, 37 °C, AP gave rise to a \textsuperscript{31}P-NMR signal that was unambiguously identified as an aminal adduct, initiated by reaction of AP’s C(O)H moiety and the NH\textsubscript{2} group of Tris. Other investigators\textsuperscript{31} had mistakenly ascribed this \textsuperscript{31}P-NMR signal to 4-HO-CP. Final-
ly, $^{13}$C- and $^2$H-NMR studies of the decomposition of 5,5-dideuterio and 4-$^{13}$C isotopically labeled versions of cis-4-HO-CP revealed, via spectra of the isotopically labeled acrolein fragments $[H^{13}\text{C}(\text{O})\text{CH}=\text{CH}_2$ and HC(O)CD=CH$_2$], previously unrecognized chemical complexities. Namely, essentially all of this urotoxic metabolite was in rapid, reversible equilibrium with thermodynamically favored adducts at pH 7.4, 37 °C. This represented a caveat for metabolic and toxicological investigations of the “acrolein” metabolite produced by fragmentation of AP inasmuch as in vivo there may be essentially no free HC(O)CH=CH$_2$ per se.

As was mentioned above, there was no NMR evidence for detectable amounts of imino-CP, which Fenselau et al. had earlier proposed as a novel, chemically reactive metabolite of CP, as did Borsch et al. While the inability to detect imino-CP by NMR does not preclude its existence, it does suggest that, under the conditions of our reported studies, the concentration of imino-CP, if formed, is quite low. In order to optimize the conditions under which an imino oxazaphosphorine analog of imino-CP might form and be detected by NMR, I reasoned that possible addition and fragmentation pathways would have to be controlled. This led to the synthesis of 4-hydroxy-5,5-dimethylcyclophosphamide for NMR studies in a nonnucleophilic solvent, which we reported in 1987 (Boyd et al.). Like 4-HO-CP, this 5,5-dimethyl analog of CP can undergo ring-opening and dehydration reactions, thus affording the 5,5-dimethyl counterparts of AP and imino-CP, respectively; however, the absence of a C-5 proton blocks the formation of PM by an α,β-elimination mechanism. Characterization of the 5,5-dimethyl analog of AP would then provide NMR spectral benchmarks applicable to imino-CP. Using reduction of a corresponding cis-4-hydroperoxy precursor, as was mentioned above for CP, and anhydrous dimethyl sulfoxide (DMSO) solvent, it was possible to detect $^1$H, $^{13}$C, and $^{31}$P chemical shifts in NMR spectra that were unambiguously ascribed to 5,5-dimethyl imino-CP. In DMSO solution, concentrations of the dimethyl analogs of cis-/trans-4-HO-CP, AP, and imino-CP were found to be temperature-dependent with higher temperatures favoring aldehyde and imino analogs in a reversible manner, thus indicating that dimethyl cis-/trans-4-HO-CP, AP, and imino-CP were interconverting. Once the spectral characteristics of 5,5-dimethyl imino-CP were thus identified, they were used as benchmarks to locate the elusive imino-CP of the parent CP. Repeating the experiment with 4-hydroperoxy-CP led to the observation of authentic imino-CP. The addition of small amounts of water, a nucleophile, to DMSO solutions of imino-CP resulted in the immediate disappearance of its NMR signals. Thus, formation of any imino-CP in vivo is expected to lead to rapid conjugation reactions with biological nucleophilic species.

$^{31}$P-NMR SPECTROSCOPIC OBSERVATION OF THE INTRACELLULAR TRANSFORMATIONS OF CP METABOLITES

In the early 1980s, Jack S. Cohen and coworkers at the NIH pioneered metabolic studies of mammalian cells by $^{31}$P-NMR using a continuous perfusion technique wherein viable cells were embedded in a matrix of agarose gel in the form of fine threads which were continuously perfused in a standard NMR tube. While the small diameter of the thread allows rapid diffusion of metabolites and drugs into the cells. The changes in $^{31}$P-NMR signals, exemplified with ATP and Pi, levels, were followed as a function of time in response to perfusion with a glucose-containing medium, with isotonic saline and with a medium containing 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. These researchers suggested that “[t]his gel-thread perfusion method should enable routine NMR studies of cellular metabolism, and may have other potential biological applications.”

Around this time, I had already moved from CUA to the FDA Division of Biochemistry and Biophysics located on the NIH campus, and collaborated with my FDA colleague, William Egan, on the CP metabolite NMR investigations mentioned above. Since Egan had earlier worked with Cohen at NIH, he quite naturally became interested in applying Cohen's cell/gel-perfusion method to CP metabolites. The salient results of Egan's pursuit of such studies are briefly summarized as follows, and were reported in full detail in J Med Chem in 1986.

$^{31}$P-NMR spectroscopy was used to directly monitor, for the first time, the intracellular chemistry of the ultimate active metabolite of CP, namely, PM. These NMR studies utilized a human histiocytic lymphoma cell line (U937), embedded in agarose gel threads contained in a 10-mm NMR tube, as described by Cohen and coworkers. The cell/gel threads were perfused with medium containing a mixture of synthetically derived CP metabolites, namely, 4-HO-CP, AP, and PM (via reduction of cis-4-HO$_2$-CP in a separate solution). As can be seen from the time course of the $^{31}$P-NMR spectra (a-f) shown in Figure 5, the 4-HO-CP and/or AP metabolites readily crossed the cell membrane, and the increasing intracellular concentration of PM could, therefore, be...
attributed primarily to the intracellular fragmentation of AP. Signals suggestive of either carboxy-CP or keto-CP were not detected. In companion experiments, there was no measurable cellular uptake of PM, presumably due to its anionic character at physiological pH and the hydrophobic nature of cell membrane lipid bilayers.

Spectral data were used to calculate a rate constant for the intracellular disappearance of PM at 23 °C. The intracellular pH was determined to be 7.1 from the chemical shift of the internal inorganic phosphate signal. The intracellular disappearance of PM (see Figure 5 d-f) followed a first-order decay law. Least-squares fitting of the intracellular concentration of PM, as a function of time, to a simple first-order process provided a half-life (t_{1/2}) of 125 min at 23 °C. Considering variations in conditions, this intracellular half-life of 125 min compares favorably with t_{1/2} of 48 min reported by Voelcker et al.\textsuperscript{36} for PM in phosphate buffer at pH 7.0, 37 °C; allowing a factor of ~2 in rate for every 10 degrees in temperature, the adjusted half-lives are essentially the same. The value for the half-life in Tris buffer at pH 7.0, 37 °C determined by Engle et al.\textsuperscript{19} is somewhat short (14 min) relative to that found intracellularly, indicating that medium effects can be moderately significant.

In concluding this section on the first-ever demonstration of the use of NMR to study CP and its metabolites in living cells, it is worth noting that subsequent advances in NMR spectroscopy led to the design of surface coils to allow \textit{in vivo} detection of NMR signals of molecules that are present within the magnetic fields in tissue and/or blood vessels in proximity to the surface coil. The feasibility of this approach for CP was first reported in 2000 by Payne et al.\textsuperscript{37} at the CRC Clinical Magnetic Resonance Research Group in Surrey, UK, who detected \textsuperscript{31}P-NMR signals from CP in the livers of patients \textit{in vivo}. The signals were sufficiently large that they may be detected using simple pulse-and-acquire measurements employing a relatively small (8-cm diameter) \textsuperscript{31}P surface coil. The use of \textsuperscript{1}H-decoupling yielded substantial (~4-fold) improvement in sensitivity. In terms of nomenclature, following the development of NMR imaging using \textsuperscript{31}P or other non-radioactive nuclei, the preferred terminology was simplified from nuclear magnetic resonance (NMR) spectroscopy to magnetic resonance spectroscopy (MRS), thereby eliminating the “scary” (to non-scientists) word nuclear, and also differentiating MRS from signal-acquisition methods that are based on radioactive isotopes, such as positron emission tomography (PET).

**Figure 5.** \textsuperscript{31}P-NMR spectra (161 MHz) of U937 cells as a function of time and in the presence of added 4-HO-CP and after washing cells with fresh perfusate. Abbreviations: AP = aldophosphamide, PM = phosphoramide mustard, 4-OH-CP = 4-hydroxy cyclophosphamide, P\textsubscript{i} = inorganic phosphate, ATP = adenosine triphosphate, NAD\textsuperscript{+}/NADH = oxidized and reduced nicotine adenine dinucleotide, UDPG = uridine diphosphoglucose (galactose). An asterisk (*) denotes an unknown impurity (which was not observed in other runs). Reproduced with permission from J Med Chem 1986, 29, 1206-1210. Copyright 1986 American Chemical Society.

**CP TUMOR CELL TOXICITY VS. NORMAL CELL DETOXIFICATION, AND RESISTANCE**

The pharmacology of CP and its metabolites known in the 1970s was described by Connors et al.\textsuperscript{17} in their
publication of studies of the active intermediates formed in the microsomal metabolism of CP. Briefly, it was stated that CP (cf. Scheme 1) is first converted, presumably by mixed-function oxidases (e.g. cytochrome P450), into 4-HO-CP, which may then fragment by elimination of acrolein from 4-HO-CP's tautomeric form, AP, to yield the known cytotoxic agent PM. This purely chemical fragmentation of AP into acrolein and PM competes with the enzymic conversions of 4-HO-CP (by dehydrogenation) and AP (by oxidation) into the known in vivo metabolites of CP, keto-CP and carboxy-CP, respectively, each of which has low cytotoxicity. Keto-CP and carboxy-CP are the principal urinary metabolites of CP, but are non-toxic to Walker tumor cells in a reported bioassay system. In contrast, PM is markedly toxic and the dose needed to kill 75% of Walker tumor cells is low enough for it to be the toxic metabolite from “activated” CP. Acrolein, which has been detected following microsomal incubation of CP, had been suggested as the anti-tumor metabolite, but it is not as toxic as PM in this bioassay system. Connors et al. add that the highly selective antitumor action of CP could be explained if normal cells, but not tumor cells, could efficiently convert the primary metabolites, 4-HO-CP and AP, by further enzymatic oxidation and dehydrogenation into the non-toxic keto-CP and carboxy-CP metabolites, respectively. In other words, “[t]umor cells would be selectively killed if they did not efficiently perform the detoxification process.” A proportion of the primary metabolites entering the tumor cell would break down spontaneously into the highly toxic PM and acrolein. The selective effect of CP could thus be due to intracellular release of PM specifically in tumor cells while the whole animal toxicity could be due to breakdown of the primary metabolite in extracellular fluid. This proposal of cellular uptake of primary metabolites followed by intracellular release of PM were confirmed by the above-mentioned 31P-NMR data obtained with cell/gel perfusion (Figure 5).

Later, in 1994, 31P-NMR was used to monitor in real-time the reaction of 4-HO-CP/AP with glutathione (GSH) — a known agent for drug conjugations — to form a mixture of four detectable diastereomers of 4-GSH-CP conjugates. The 4-GSH-CP conjugates, which undergo the reverse reaction to reform 4-HO-CP/AP, were thus viewed as a “stabilized reservoir” of PM, which in turn can be intercepted (i.e. detoxified) by GSH through irreversible alkylation. This work was extended to show that levels of certain glutathione S-transferase isoenzymes in tumor cells can be related to development of resistance to CP.

Along similar lines, about the same time, it was shown by the Sladek lab that class 1 and class 3 aldehyde dehydrogenases (ALDH-1 and ALDH-3, respectively) catalyze the detoxification of CP by metabolism of AP. Thus, interindividual variation in the activity of either of these enzymes in, for example, breast cancers could contribute to the wide variation in clinical responses that are obtained when such regimens are used to treat these malignancies. Perhaps the most compelling data to support the critical importance of ALDH in the metabolism of CP and the role it plays in the serendipitous history of this drug was published in 1996 by Magni et al. Briefly, they tested whether ALDH-1 overexpression could directly induce CP resistance by cloning a full-length human ALDH-1 cDNA for retroviral vector transduction of CP-sensitive hematopoietic cell lines that were then tested for resistance to maphosphamide, a pre-activated analog of CP. Overexpression of the ALDH-1 gene resulted in a significant increase in CP resistance, thus indicating that ALDH-1-mediated conversion of AP to non-toxic carboxy-CP is sufficient for cellular resistance to CP.

Additional insights into the detailed pharmacology of CP and its metabolites were also obtained from stereochemical studies that are described in the next two sections.

SYNTHESIS OF THE ENANTIOMERS OF CP

As mentioned in the introduction, my initial interest in CP in 1971 was in large part stimulated by wanting to investigate whether CP's chirality at phosphorus would influence its metabolism and anticancer activity, about which there were no publications to my knowledge. By 1975, I had used optically pure (+)-(R)-PhCHMeNH2 to synthesize the two diastereomeric derivatives of CP having an N-C(Me)(Me)Ph (N-α-methylbenzyl) moiety attached to the ring nitrogen. Neither diastereomer underwent stereo-mutation at phosphorus when dissolved in human blood plasma at 37 °C for 14 h, or in H2O-DMSO at pH ~5.6 or ~8.4 at 50 °C for 15 h, which indicated that enantiomerically pure CP would not racemize during its transport to the liver mixed-function oxidase hepatic system which effects C-4 hydroxylation to 4-HO-CP. During the same time-frame, unbeknownst to me, Prof. Wojciech J. Stec and his students at the Polish Academy of Sciences Centre for Molecular and Macromolecular Studies in Lodz, Poland, had similar interests in CP stereochemistry, and likewise published in 1975 their synthesis of the same two diastereomeric N-α-methylbenzyl derivatives of CP. Importantly, however, their publication also reported that the separated diastereomers underwent hydrogenolysis over Pd/C in EtOH to yield the individual enantiomers of CP.
My lab soon thereafter carried out the same hydrolysis reaction to afford optically active (+)-CP that was proven by a novel NMR method\textsuperscript{44} to be enantiomerically pure. Through fortunate circumstances, my CP collaborator William Egan (see above) was acquainted with chemist Jean Karle, whose mother, Isabella Karle was a noted crystallographer and the wife of crystallographer Jerome Karle, who would later be awarded the 1985 Nobel Prize in Chemistry with Herbert A. Hauptman for their outstanding achievements in the development of direct methods for the determination of crystal structures. The Karles were keen on collaborating with Egan and my lab to apply the then well-known “Karle and Karle” procedure\textsuperscript{45} to determine the absolute configuration of phosphorus in (+)-CP without reference to a second asymmetric center of known chirality. After crystalline material was obtained, the crystal and molecular structure of enantiomerically homogeneous (+)-CP was determined by x-ray diffraction with the absolute configuration being established by the anomalous dispersion of the chlorine (Cl) and phosphorus (P) atoms (Figure 6).\textsuperscript{46} It was found that the dextrorotatory (+) enantiomer of CP ([α]D/20 = 2.3 ° (c 3.0, methanol)) has the R configuration at P (Rp). It was stated\textsuperscript{46} that “the presently reported R configuration for (+)-CP provides a convenient and reliable basis for the establishment of the absolute configuration at phosphorus in all of the known chiral metabolites of CP [cf. Scheme 1] which may be synthesized from CP using reactions that do not involve stereochemical changes at the asymmetric phosphorus center.” A footnote in this publication\textsuperscript{46} reported preliminary \textit{in vitro} kinetic measurements of liver microsomal “activation” of CP to give bis-(2-chloroethyl)amine (nor-HN2) wherein (-)-CP gave \( K_m = 0.57 \text{ mM} \) and \( V_{\text{max}} = 27.4 \text{ μmol of nor-HN2 equiv g}^{-1} \text{ h}^{-1} \) and (+)-CP gave \( K_m = 0.48 \text{ mM} \) and \( V_{\text{max}} = 22.2 \text{ μmol of nor-HN2 equiv g}^{-1} \text{ h}^{-1} \). These Michaelis–Menten kinetic parameters implied that there was relatively little enzymatic discrimination between the (-)-CP and (+)-CP enantiomers, under these \textit{in vitro} conditions. Additional findings later reported by others concerning the influence of CP stereochemistry on CP metabolism are given in the next section.

In the same year as our x-ray analysis of (+)-CP was published, Adamiak and Saenger at Abteilung Chemie, Max-Planck-Institut für experimentelle Medizin in Göttingen, Germany, in collaboration with the Stec lab in Lodz, Poland, reported\textsuperscript{47} the results of an x-ray-diffraction study of (-)-CP that established the S absolute configuration at phosphorus (\( S_p \)), consistent with our independent finding \( R_p \) for (+)-CP.

**EFFECTS OF STEREOCHEMISTRY ON CP METABOLISM IN HUMANS**

The above-mentioned independent syntheses of the (-)-CP and (+)-CP enantiomers by Stec’s laboratory and my group were quickly followed by a number of studies that compared the biological effects of the enantiomers with each other and with the racemate, the latter of which up to that time was the composition of CP administered to patients. The major question was whether evidence would be obtained indicating a substantially higher therapeutic index for either (+)-CP or (-)-CP compared to conventional racemic CP, (±)-CP.

The results obtained by my group were published\textsuperscript{48} in 1979 and were comprised of \textit{in vitro} metabolism studies and, through an NCI screening program, \textit{in vivo} animal experiments. Briefly, separate incubation kinetic measurements for the metabolic “activation” of (+)-CP, (-)-CP, and (±)-CP by identical phenobarbital (PB)-induced mouse liver microsomal mixed-function oxidase preparations gave, respectively, \( V_{\text{max}} = 13.8 ± 1.0, 20.0 ± 1.5 \) and \( 16.3 ± 1.1 \text{ μmol nor-HN2 equiv g}^{-1} \text{ h}^{-1} \) and \( K_m = 0.37 ± 0.02, 0.56 ± 0.04 \) and \( 0.45 ± 0.02 \text{ mM} \). The absolute magnitude of the apparent \( V_{\text{max}} \) kinetic parameter increased by ~50% in a subsequent comparative run between (+)- and (-)-CP using a second preparation of the hepatic microsomal oxidase; however, the relative behavior of CP enantiomers toward enzymatic “activation” was constant, within experimental error, and revealed that \( V_{\text{max}^-}/V_{\text{max}^+} = 1.34 ± 0.17 \) and \( K_m^-/K_m^+ = 1.35 ± 0.14 \). Removal and quantitative measurement, as a function of time, of free acrolein that is produced by incubation of CP with PB-induced microsomes repeatedly gave a roughly congruous family of “skewed bell-shaped” curves having maxima in the order (-)-CP > (+)-CP > (-)-CP; however, the differences between these acrolein time-course profiles were relatively small (~10–20%). Isolation of CP from separate (+)- and (-)-CP incubation mixtures, followed by determination of enan-

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**Figure 6. Stereodiagram of the absolute configuration of (+)-cyclophosphamide (CP).** The ellipsoids representing the thermal parameters are at a 50% probability level. Hydrogen atoms are represented by small spheres. Reproduced with permission from \textit{J Am Chem Soc} 1977, 99, 4803–4807. Copyright 1977 American Chemical Society.
tiomeric homogeneity by NMR methods, demonstrated that CP is not racemized during in vitro liver microsomal metabolism. Mouse screening data (test/control percentages) for (+)-, (-)-, and (±)-CP activity against mouse L-1210 lymphoid leukemia showed no significant differences in therapeutic value. Collectively, these various experimental results suggested to us that "there is an unusually low degree of biological stereoselectivity associated with the metabolism of CP enantiomers."48

Stec's laboratory carried out far more comparative experiments of this sort in vitro and in vivo in several animal species, namely, mouse, rabbit, and rat, in collaboration with investigators at the Institute of Cancer Research, Surrey, England, as summarized in the introductory section of a 1979 publication (Jarman et al.49) that extended these collaborative studies to humans. The protocol for this small pilot study49 involved 4 patients with carcinoma of the lung who had normal renal and hepatic function as measured by standard biochemical tests, and none had received CP previously. Doses (1 g i.v.) of (±)-CP, (+)-CP and (-)-CP were administered as a bolus, sequentially, with a 3-wk interval between doses. Blood samples were taken at 5, 10, and 30 min and at 1, 2, 4, 6, and 12 h. Urine was collected between 0 and 24 h after administration of the drug. The salient results of this first-in-humans pilot study were summarized by Jarman et al.49 as follows. The plasma levels of CP and the urinary output (24 h) of unchanged CP and of the two enzymatically produced metabolites, keto-CP and carboxy-CP (cf. Scheme 1), were determined using MS-stable isotope dilution. There was no significant difference between the three forms of CP with respect to plasma half-life (β phase) or in the urinary outputs of CP or carboxy-CP. The output of keto-CP after administration of (+)-CP was significantly greater than that produced from (±)-CP. CP recovered from the urine of patients given (±)-CP was either racemic or only slightly enriched in (-)-CP. The two enantiomers of CP were almost equally bound to plasma protein. Jarman et al.49 concluded that "[b]ased on these metabolic studies alone, there is little reason to predict that the enantiomers will differ from each other or from the racemate in their therapeutic effects in humans, but there are other factors, e.g., stereoselective uptake of the intermediary 4-hydroxylated metabolites [cis- and trans-4-HO-CP; cf. Scheme 1] by neoplastic cells, which could elicit such differences."

In concluding this section, it should be first mentioned that I was unable to find any subsequent publications on comparative therapeutic efficacy or toxicity of (+)-, (-)-, and (±)-CP in humans. The second point deals with the stereochemistry of ifosfamide (IFF), which is a chiral constitutional isomer of CP wherein one of CP’s CH₂CH₂Cl moieties is replaced with hydrogen and covalently bonded to the ring nitrogen. Racemic (±)-IFF received U.S. approval by the FDA in 1988 for use in combination with certain other approved antineoplastic agents for third-line chemotherapy of germ cell testicular cancer. Prior to this FDA approval of racemic IFF, Stec’s laboratory had published the syntheses of enantiomerically pure IFF and two of its chiral metabolites. 31P-NMR analysis of urine from patients treated with racemic IFF indicated “considerable stereoselectivity of in vivo formation of some chiral metabolites” of IFF.50 Much later, in 1999, an extensive amount of experimental and clinical data for IFF and CP was reviewed by Williams and Wainer,51 who stated that stereochemistry plays a minor role in the efficacy and toxicity of CP but is a major factor in neurotoxicity of IFF. Moreover, “[s]tudies have demonstrated that the use of a single IFF enantiomer, Rp-IFF, would retain the unique antitumor efficacy of this agent, while eliminating the major source of the observed IFF-associated neurotoxin, Sp-IFF."51 To my knowledge, racemic IFF is still used in the clinic, and in my opinion there is insufficient published information to speculate about the contrasting roles of stereochemistry on the biological activities of CP and IFF.

CONCLUSIONS

The history of the elucidation of the chemistry which underlies the anticancer activity of CP provides a remarkable example of serendipity, and the unanticipated nature and complexity of the biochemical processes underlying the selective toxicity of CP toward cancer cells. The original 1950s drug-design premise based on elevated levels of putative phosphamidase activity in cancer cells that could selectively release toxic nitrogen mustard was proven to be incorrect. Nevertheless, CP was found to be a clinically useful anticancer drug that indeed required enzymatic activation, but that was found to occur in the liver, and involved oxidative hydroxylation of the C4 position in CP mediated by the cytochrome P450 system. Serendipitously, the resultant metabolite, 4-HO-CP, happens to have a chemically labile hemiaminal moiety that allows, under physiological conditions, rapid equilibration between cis- and trans-4-HO-CP, through AP, its ring-opened aldehyde-bearing tautomer. The serendipity continues in that the chemical constitution of AP happens to allow, under physiological conditions, fragmentation into acrolein and PM at a rate which is slow enough to allow circulation and cellular uptake of 4-HO-CP/AP, as PM exists
under physiological conditions as its negatively charged conjugate base (PM-), which is therefore not readily taken up by cells. And, by the same token, anionic PM- does not readily efflux from cells after its generation therein from AP. It is fair to say, I think, that an expert medicinal chemist could not reliably design, \emph{a priori}, this overall multistep metabolic "Trojan horse" (Figure 4) scheme for the ultimate release of PM/PM- from CP. The serendipity continues with the unforeseen intervention of not one, but two additional enzymatic conversions to produce the metabolites keto-CP and carboxy-CP, which are non-toxic and, serendipitously, occurs more rapidly in normal cells compared to tumor cells, thus giving rise to the selective toxicity of CP toward cancers. This additional pharmacology is also something that an expert medicinal chemist could not reliably design, \emph{a priori}, in my opinion.

Immunosuppression is an aspect of CP chemotherapy that was not mentioned above, but is worth noting because it is yet another serendipitous aspect of CP. By the mid-1980s, and despite incomplete understanding of the exact mode of CP's immunosuppressive action, CP was being successfully used in certain nonmalignant diseases in which autoimmune phenomena are established or suspected in the pathogenesis of the disease.\textsuperscript{52} Much more is now know mechanistically, and CP remains an important treatment for life-threatening autoimmune diseases where disease-modifying anti-rheumatic drugs have been ineffective. For example, systemic lupus erythematosus with severe lupus nephritis may respond to pulsed CP, and CP is also used to treat severe rheumatoid arthritis and multiple sclerosis.

All of these non-cancer beneficial uses for CP derived from chance, albeit to prepared minds, as in the well-known adage. In this regard, readers may be interested in the essay titled \textit{On serendipity in science: discovery at the intersection of chance and wisdom} by Samantha Copeland.\textsuperscript{53}

From the perspective of analytical tools, NMR spectroscopy proved to be a powerful addition to MS methods by providing real-time kinetics and compelling structural information. Currently, there are \textasciitilde{}120 publications in PubMed that have CP and NMR/MRS in the title or abstract (and \textasciitilde{}1,800 anywhere in the article). Aside from the mentioned \textsuperscript{31}P-based \textit{in vivo} imaging of CP in humans,\textsuperscript{37} the treatment of human extremity sarcomas has been monitored by \textsuperscript{31}P-MRS, and profiling of urinary acetate and citrate by \textsuperscript{1}H-MRS following CP therapy.\textsuperscript{54}

From a personal perspective, my initial working hypothesis (and that of Prof. Wojciech J. Stec) that the stereochemistry of CP would markedly influence enzyme-mediated metabolic pathways turned out not to be the case. However, the saying "never say never" applies to science, and it is possible that future stereochemical studies may provide new and possibly surprising information.

After \textasciitilde{}60 years of investigating CP, which has generated \textasciitilde{}50,000 publications listed in PubMed that continue to show an upward trend (Figure 7), and \textasciitilde{}4,000 structural congeners of PM listed in \textit{Chemical Abstracts}, the amazingly serendipitous pharmacology of CP has been recently reported to have yet another surprise.

In 2019, Georg Voelcker, who has been investigating CP since the early 1970s, published a report\textsuperscript{56} stating that "[a]ttempts to improve this drug found by a lucky coincidence have failed until now. The efforts failed because they were based on wrong assumptions about the mechanism of action of CP." Voelcker provides new data to support his proposal that 3-hydroxypropanal (HPA), HOCH\textsubscript{2}CH\textsubscript{2}C(O)H, "the overlooked CP metabolite" derived from AP by a phosphodiesterase, which he identified in 2017, has known\textsuperscript{57} proapoptotic properties that contribute significantly to the anticancer activity of CP, in addition to the known\textsuperscript{58} cytotoxic apoptosis induced by PM alkylation of DNA. If this new mechanism of action for CP involving HPA withstands further experimental testing, it would represent yet more serendipity in the amazingly serendipitous story of CP chemotherapy.

In closing, and in view of the historical chart of CP publications in Figure 7, it can be said with a high degree of certainty that in 2034, on the 75\textsuperscript{th} anniversary of the approval of CP as a cancer drug, there will be far more publications than there are now. Based on extrapolation of a linear trend-line for the data in Figure 7 for 2019-2035, this future 15-year period would see \textasciitilde{}30,000

\textbf{Figure 7.} A chart of the number of annual publications in PubMed between 1959 and 2019 that have CP (or Cytoxan\textsuperscript{55}) in the title and/or abstract. Between 1959 and 2019 there have been \textasciitilde{}50,000 such publications, which show a continually increase trend, and over 1,800 in 2019.
additional CP-related publications. These future publications will likely include many investigations of the extent to which, and how, individual genomes influence CP activity and toxicity. This is already evident from a 2020 report noting that pharmacogenetic investigations have shown that CYP450 (which converts CP to 4-HO-CP), as well as aldehyde dehydrogenases (which converts 4-HO-CP to keto-CP), are associated with altered treatment response. Since individual genetics similarly applies to glutathione-S-transferase isoenzymes, it was suggested that “[a] shift from genetic-based studies to whole-genome-based investigations of CP-associated markers may contribute to personalizing CP therapies.” Indeed, El-Serafi et al.60 have clinically investigated and obtained data to support the potential importance of accounting for individual patient genotyping and levels of activating enzymes when personalizing treatment schedules in order to achieve optimal therapeutic drug plasma concentrations of CP.

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