Methotrexate: An Effective Agent for Treating Cancer and Building Careers. The Polyglutamate Era

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ABSTRACT

This paper chronicles developments in the laboratory of Dr. Bruce Chabner during the period 1978-1981. Initial work demonstrated that methotrexate is taken up by human breast cancer cells by a high-affinity, carrier-mediated, energy-dependent transport system similar to that described in murine leukemia cells. Conversion of methotrexate to a high molecular weight polyglutamate metabolite was also demonstrated to occur in human breast cancer cells. Polyglutamates became the predominant form of intracellular drug, both free in the cytosol and bound to dihydrofolate reductase, during a 24 h exposure to clinically achievable methotrexate concentrations. Intracellular retention of polyglutamates led to prolonged suppression of thymidine synthesis and loss of cell viability after removal of extracellular drug. This work identified methotrexate polyglutamates as biologically active enzyme inhibitors in human tumor cells and launched a series of investigations on the interaction of these derivatives with folate-requiring enzymes.

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INTRODUCTION

In July 1978, having completed a rigorous year of clinical training in the Medicine Branch of the National Cancer Institute, I joined the Clinical Pharmacology Branch to work in the laboratory of Dr. Bruce Chabner. I was looking forward to an intellectually challenging experience free from the emotional turmoil of caring for desperately ill patients with very advanced cancer. Although I had virtually no laboratory experience, I was determined to learn the tools of the trade and had chosen the area of clinical pharmacology as one that might have immediate relevance to improving the treatment options for cancer patients. Bruce immediately introduced me to Michael Cohen and Jim Drake, a visiting scientist and a technician, respectively, and suggested I begin to work with them on ongoing projects. Michael had become convinced that abnormalities in melatonin physiology were the root of breast cancer and was focused on characterizing the melatonin receptor in cultured human breast cancer cells. Jim was to introduce me to laboratory techniques but preferred to engage in a detailed analysis of each day’s Washington Post. Suffice it to say that by November 1978 I had determined that there was no future for me in melatonin research and had picked up relatively few lab techniques short of cell culture and newspaper folding!

Recognizing my frustration, Bruce next suggested that I work on characterizing methotrexate transport in cultured human osteosarcoma cell lines that had been established in Steve Rosenberg’s laboratory. This was a fine project since high-dose methotrexate was very much in vogue for treatment of osteosarcoma and little information was available on methotrexate transport in any human solid tumor. I soon learned, however, that human osteosarcoma cells grow extremely slowly in culture, making them impractical to study since it took months to prepare enough cells for an experiment. Demoralized by having nothing to show for my first six months in the lab, I was ready to quit and return to clinical medicine. Bruce, however, having learned that no knowledge is ever wasted, advised me to return to the human breast cancer cells that were being studied by Michael Cohen and to use these for my studies of methotrexate transport. Suddenly, things began to go my way and by February 1979 my experiments were moving rapidly ahead.

THE POLYGLUTAMATE ERA

Our initial work demonstrated that methotrexate was taken up by human breast cancer cells by a high affinity, carrier-mediated, energy-dependent transport system similar to that previously described for murine leukemia cells [1]. We
next asked whether there was evidence for methotrexate metabolism intracellularly, as conversion of naturally occurring folates to high molecular weight “storage forms” had recently been described in yeast. Using column chromatography on Sephadex G15, we found that human MCF-7 cells rapidly and quantitatively converted methotrexate to polyglutamate derivatives which became the predominant intracellular form of the drug following a 24-h exposure [2]. Moreover, examination of that portion of intracellular methotrexate bound specifically to dihydrofolate reductase revealed that polyglutamates became the predominant drug form bound to the enzyme during prolonged exposure, suggesting, for the first time, a role for these metabolites as biologically active enzyme inhibitors.

In July 1980, two significant additions to the laboratory arrived. Jacques Jolivet joined us from Montreal and new HPLC equipment was delivered. After spending a few months experiencing the trials and tribulations of new technology, Jacques and I set out to develop an HPLC assay for separation and quantification of methotrexate polyglutamates in cell extracts. HPLC technology allowed us to resolve multiple polyglutamate peaks that had not been detectable with Sephadex chromatography and demonstrated the capacity of human breast cancer cells to synthesize long chain polyglutamates [3]. Assay in hand, we set about to characterize more fully the synthesis, retention and biological activity of methotrexate polyglutamates in a variety of human breast cancer cell lines. These studies demonstrated considerable heterogeneity among cell lines in their capacity to form polyglutamates, and confirmed that polyglutamates were retained intracellularly for prolonged periods of time in excess of the dihydrofolate reductase binding capacity, leading to prolonged suppression of thymidylate synthesis and loss of cell viability after removal of extracellular methotrexate [4, 5].

CLINICAL PHARMACOLOGY OF LEUCOVORIN

After leaving the Chabner lab in 1981, my interests turned more to studies of the clinical pharmacology of anticancer agents, although I continued to have a special interest in folates and antifolates. When it became clear during the early 1980s that intracellular reduced folate pools were a critical determinant of cytotoxicity for 5-fluorouracil (5-FU), clinical investigators soon began to employ the combination of leucovorin (LV) with 5-FU in treatment of patients with advanced colorectal cancer and other malignancies. LV, a drug used for many years to prevent and ameliorate toxicity from methotrexate, was now being employed routinely to enhance the effectiveness of 5-FU. The only formulation of LV available for routine clinical use at that time was known to be a mixture of stereoisomers around the C6 carbon of the pteridine ring of LV. The individual isomers were known to differ significantly in their plasma clearance and route of elimination following i.v. administration, with the (6S) isomer being rapidly converted to 5-CH₂-tetrahydrofolate and the (6R) isomer being slowly eliminated by renal excretion. The high concentration ratio of (6R) to (6S) LV that accumulated in plasma following administration of a high i.v. dose raised concerns about the ability of (6R) LV to interfere with the biological activity of the (6S) isomer. Oral administration of LV had the potential to limit the accumulation of (6R) LV in plasma due to limited bioavailability of the (6R) isomer, leading to the hypothesis that it might be the preferred route of administration of high-dose LV if high concentrations of the (6R) isomer were indeed detrimental.

Approaching these questions at the cellular level required stereochromically pure preparations of the LV isomers, while studies of the clinical pharmacology of the LV isomers required more precise assay methods than the bioassays that had been used up to that time. My colleague Kyung Choi and I therefore set out to develop an HPLC method that could be used to prepare pure quantities of the LV isomers for in vitro work, and would have sufficient precision and sensitivity to permit additional studies of the clinical pharmacokinetics of the isomers following administration of clinically relevant doses. The chiral resolution was achieved isocratically using two bovine serum albumin-bonded silica columns connected in series and a sodium phosphate mobile phase. This separation method produced isomers of at least 99.9% purity. By performing reverse phase ion-pairing HPLC to separate LV from 5-methyl tetrahydrofolate, followed by chiral HPLC to separate the LV isomers, we were able to adequately quantitate the LV isomers in plasma [6].

We next undertook a study of the clinical pharmacokinetics of high-dose LV following i.v. and oral administration to normal volunteers. Five normal subjects received 1,000 mg racemic LV as a two-hour i.v. or in divided oral 100 mg doses over 24 h [7]. Following i.v. administration, peak plasma concentrations of (6R) LV, (6S) LV and 5-CH₂-THF were 148 ± 32, 59.1 ± 22 and 17.8 ± 17 micromolar, respectively. During oral administration of LV, virtually no (6S) LV appeared in the plasma. Steady-state concentrations of (6R) LV and 5-CH₂-THF were approximately 1.5 ± 0.23 and 2.8 ± 0.41 micromolar, respectively. Intravenous administration of LV resulted in an area under the curve (AUC) for (6R) LV that was more than four times that of the biologically active (6S) reduced folates, whereas oral administration produced an AUC for (6S) reduced folates that was approximately twice that of (6R) LV. After administration of high doses of LV i.v., conversion of (6S) LV to 5-CH₂-THF was saturable as indicated by the prolonged (6S) LV half-life, slow (6S) LV clearance and considerable amount of drug appearing unchanged in the urine. This study demonstrated that oral administration
of LV over 24 h produced an AUC of 5-CH$_3$-THF equivalent to that obtained after i.v. dosing in the presence of only small amounts of (6R) LV, and suggested that this might be the preferred route of administration of the drug if in vitro studies demonstrated that 5-CH$_3$-THF could effectively expand intracellular reduced folate pools and that high concentrations of (6R) LV did, indeed, impair the biological activity of the (6S) reduced folates.

**Modulation of 5-FU Catabolism**

The use of LV is one of a number of biochemical modulation strategies that have been attempted to improve the efficacy of 5-FU by enhancing its biochemical activation or binding to thymidylate synthase. An alternative modulation strategy is to impair 5-FU catabolism, thereby prolonging the half-life of the drug and eliminating 5-FU metabolites that could potentially interfere with the drug’s biological effects. 5-ethyluracil is a potent, mechanism-based inactivator of uracil reductase, the rate-limiting catabolic enzyme in the 5-FU degradation pathway. In preclinical model systems, 5-ethyluracil markedly alters the pharmacokinetic behavior of 5-FU resulting in longer half-life, delayed clearance, improved bioavailability, decreased inter-individual variability and linear pharmacokinetics. In tumor-bearing animals, the combination of 5-FU with 5-ethyluracil produces enhanced antitumor efficacy and an improvement in therapeutic index that may be due, at least in part, to the elimination of 5-FU metabolites that interfere with its antitumor effects.

Based on this preclinical rationale, we recently initiated a phase I clinical trial of 5-ethyluracil plus 5-FU in patients with advanced cancer. The study is being performed collaboratively with the University of Texas at San Antonio and the Burroughs-Wellcome Company, Research Triangle Park, NC. Eligible patients have microscopically confirmed advanced cancer refractory to standard therapy or for which no standard therapy is available, measurable or evaluable disease, Karnofsky performance status of at least 70% and adequate organ function. Patients initially receive 5-ethyluracil alone as a daily oral dose to provide us the opportunity to study the pharmacokinetics of the drug and its effects on uracil reductase activity in peripheral blood mononuclear cells. After a two-week washout, patients receive three consecutive daily doses of 5-ethyluracil with a single i.v. dose of 5-FU administered on day 2 to permit studies of 5-FU pharmacokinetics following administration of 5-ethyluracil. Two weeks later, patients receive seven daily oral doses of 5-ethyluracil with five daily i.v. doses of 5-FU. Fifteen patients have been treated to date. No toxicity attributable to 5-ethyluracil alone has been observed. At each dose level of 5-ethyluracil studied (0.74, 3.7, 18.5 mg/m$^2$), uracil reductase activity has been completely inhibited within one hour of drug administration. At the two higher doses, enzyme activity has remained at least 95% inhibited for at least 24 h following a single oral dose. As anticipated, plasma uracil concentrations become markedly elevated in a dose-dependent fashion. The plasma t$_{1/2}$ of 5-FU is markedly prolonged to approximately 4 h, and the plasma AUC of 5-FU following a dose of only 20 mg/m$^2$ is similar to that reported for a 24 h infusion of 1,000 mg/m$^2$. Biological activity, including tumor regression, has been observed at 5-FU doses as low as 20 mg/m$^2$ administered with 5-ethyluracil [8-10]. The study continues to enroll patients in an attempt to define the maximally tolerated dose of 5-FU that can be administered with 5-ethyluracil in the presence or absence of LV. Subsequent studies will determine the antitumor efficacy of this modulation strategy in patients with 5-FU responsive malignancies.

**Conclusions**

Although each of the trainees passing through the Chabner lab during the past 20 years worked on a different project, we all learned the same fundamental lessons. Bruce taught us all to be rigorous in developing and applying our methods, courageous in challenging dogma, critical in evaluating our results and modest in describing our accomplishments. He was genuinely interested in each of us, not as trainees, but as colleagues and, more importantly, as people. His approach to teaching was to learn with us and, in the process, to guide and inspire us.

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**References**


