# From Plasma Kinetics to Cellular Pharmacology

*H.M. Pinedo*
Academisch Ziekenhuis, Vrije Universiteit, Department of Medical Oncology, Amsterdam, The Netherlands

**Key Words.** Methotrexate • CFU-C

**Abstract. This paper primarily summarizes the work done under the guidance of *Dr. Bruce Chabner* in 1975 and 1976. During these years I studied the role of drug concentration, duration of exposure, and endogenous metabolites in determining methotrexate (MTX) cytotoxicity to the bone marrow stem cell (CFU-C). We found that the rate of loss of CFU-C during continuous intravenous infusion of methotrexate was related to the duration of exposure until the nadir in cell count was reached at 24 hours. Depletion of nucleated cells was mitigated, probably as a result of recruitment of previously uncommitted precursor cells to CFU-C, even while the MTX infusion was continued up to 48 hours. In vitro it was shown that methotrexate and leucovorin were transported competitively in the CFU-C, which was in clear contrast with rescue agents such as thymidine and nucleoside analogs. After my training my work has continued in close contact with *Dr. Chabner*, while the scope of both our interests broadened to include the MDR field. My own interest focused on functional studies in MDR.** *Stem Cells 1996;14:16-17*

In December 1974 I arrived at the medical branch of the NCI. Unfortunately, my host had taken a job elsewhere, so I felt I was left in the lurch in a huge institute in a foreign country. It was clear to me that I had to put together my own program. At that time I had never heard of *Bruce Chabner*. I sat down for a full week in the library studying bone marrow physiology and stem cell culture. At that same time I was intrigued by the introduction of high-dose methotrexate (MTX) into the clinic as a new treatment for osteosarcoma. My second topic to study was the preclinical pharmacology of this antimetabolite which seemed to be making furor. So another week was spent in the library to get acquainted with the pharmacology of MTX. The days thereafter were spent putting the two subjects together and preparing a workplan, which I presented to *Joan Bull* on the 3rd floor.

As I needed mice and a spectrophotometer, I intruded on the 6th floor and introduced myself to *Dan Zaharko*. I was offered space in the animal room and a bench next to a spectrophotometer. On a day in February, 1975, while measuring mouse plasma samples of MTX, I bumped into *Bruce Chabner*, who kept observing me working in the Pharmacology Branch. He asked for my research plan and was truly interested in my ideas. Soon thereafter we discussed my plan in detail. It seemed that the potential clinical impact of my work was constantly in his focus. In short, my project would study the effect of MTX concentration and its duration of exposure on the bone marrow stem cell, both in vitro and in vivo. This was the beginning of a very intense collaboration with *Bruce*.

The difficult early days proved to be the start of a most exciting year, in fact the most exciting one in my research career. This was a year without any administrative headaches, without responsibilities for running any department. In order to study the effect of constant exposure of MTX on bone marrow CFU-C, I used a glass tube device developed by *Dan Zaharko*. I am still very grateful to him. Indeed, he also had a very critical look at the data I generated. The infusion device permitted s.c. infusion of MTX into mice at a constant rate. Physical characteristics of devices ranging in size were studied. Plateau plasma concentrations of MTX ($1 \times 10^{-8}$ M to $1 \times 10^{-5}$ M) achieved from infusion rates varying over three orders of magnitude (<1 to >100 µg/hour) were studied and evaluated in terms of toxicity and pharmacokinetic behavior of this drug.

The effects of exposure of bone marrow to specific MTX concentrations were studied by constant infusion of the drug into mice. The residual marrow nucleated cell count was determined in mice at specific intervals. In vitro culture of CFU-C was also performed in these mice. Duration of exposure varied from 12 h to 72 h.

Correspondence: Dr. H.M. Pinedo, Academisch Ziekenhuis, Vrije Universiteit, Department of Medical Oncology, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.

Received January 4, 1996; accepted for publication January 4, 1996. 

Plateau plasma MTX concentrations were studied in the range from $10^{-8}$ to $10^{-5}$ M. Unexpectedly, the total number of nucleated cells per femur fell to a plateau of 30% of control for all drug concentrations studied. The nadir was reached earliest with the highest drug concentrations. The percentage of CFU-C per fixed number of nucleated cells plated increased at 48-hour infusions, compared to the percentage at 24 h. This increase was seen at all plasma concentrations studied. The total number of CFU-C at plasma MTX concentrations above $10^{-6}$ M decreased in the first 24 h, but then the number significantly increased between 24 h and 48 h. In contrast, no change was observed in CFU-C per femur between 24 h and 48 h during constant infusion at plasma concentrations below $10^{-6}$ M. Constant exposure to $10^{-5}$ M MTX produced a rapid decrease in total nucleated cells to 35% at 12 h, which remained at approximately this level throughout 48 h of drug infusion. A decrease in the number of both CFU-C and CFU-S per femur was observed, which paralleled the drop in nucleated cells during the first 24 h. However, in contrast to the increase in CFU-C during the next 24 h, an additional drop in the number of CFU-S was observed at this high concentration. These data indicated a self-limited cell kill of nucleated bone marrow cells, and suggested recruitment of CFU-C from the CFU-S pool between 24 h and 48 h of infusion despite continued MTX infusion.

Thereafter, the cytotoxic effect of MTX for mouse bone marrow cells was studied by in vitro culture of granulocyte precursor cells (CFU-C). The formation of colonies was inhibited to 50% of control by $10^{-8}$ M MTX. Further increases in MTX concentration rapidly abolished colony formation by CFU-C. The potential of leucovorin and nucleosides to rescue the CFU-C from MTX toxicity was studied. Toxicity of $10^{-7}$ M MTX was completely reversed by equimolar concentrations of leucovorin, but with higher MTX concentrations, relatively more leucovorin was required. While MTX was rescued by leucovorin, rescue of the toxic effect of $10^{-4}$ M MTX by $10^{-3}$ M leucovorin was not observed. In contrast to the rescue by leucovorin, toxicity of all MTX concentrations up to $10^{-4}$ M was completely prevented by $10^{-5}$ M thymidine with $10^{-5}$ M adenosine, inosine, or hypoxanthine. Single nucleosides of thymidine with guanosine were ineffective, as were lower concentrations ($\leq 10^{-6}$ M) of the effective combinations. Thus, while leucovorin reversed the MTX toxicity to CFU-C competitively, rescue by nucleosides was noncompetitive.

Since my return to the Netherlands in 1976 my scientific work continued to focus on cellular pharmacology. In the beginning, I performed several in vitro experiments on L1210 cells. Grossly, the findings were pretty much in agreement with the in vitro observations for the CFU-C's. In those days *Al Leyva*, who was recommended by *Dr. Chabner*, joined my group in Utrecht. In the eighties, after moving with my group to Amsterdam, the MTX work made way for 5 fluorouracil and PALA. The type of studies did not change, however. Studies now focused on the target enzymes. Cellular resistance in the MDR field gradually came into focus, a field into which both *Bruce* and I moved. Because of the potential presence of a number of cellular proteins keeping the drugs from reaching their targets, my group switched to functional assays. We first applied acute myeloid leukemia as a model, but more recently we are attempting to dissociate solid tumors to perform similar functional studies. However, the tumor stroma is making such an approach extremely difficult. In the clinic we both embarked on MDR reverting trials. Our trials with reverting agents in solid tumors have been quite disappointing. At this year's AACR, *Bruce* will be organizing a symposium on controversies in this field, which without any doubt will be of great interest. Part of the discussion will be the lack of predictiveness of preclinical models of drug resistance for the clinic.

It may ultimately appear that we have been focusing too much on Pgp as a resistance marker, while reality is closer to Pgp being a marker for aggressiveness. Pgp might appear to be an ATP-dependent pump for endogenous components. If true, one would expect its affinity for such compounds to be higher, and with a higher concentration of the biological products at the pump than of any reverter administered. This would leave a very narrow window for reverters and for clinical trials to be successful. The situation may prove slightly different for acute leukemia and myeloma where the extracellular concentration of the reverter may be sufficient to compete with endogenous produces.

*Bruce Chabner* and *Dan Longo* joined me as editors of the successful Cancer Chemotherapy Annual. Moreover, I have greatly appreciated *Bruce's* co-chairmanship of the NCI/EORTC New Drug Symposia during the past 10 years. In conclusion, more than 20 years have passed since *Bruce Chabner* triggered my interest in the field of pharmacology of antineoplastic agents. Both our research interests were initially dominated by the antimetabolites. Thereafter, there was a broadening of the scope with the introduction of the MDR research. As with his other disciples, I have maintained close contact with him despite our physical distance, exchanging data on a routine basis.