The Mechanism of Action

of

Methotrexate

by

Elizabeth Ann Jacobson

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"THE MECHANISM OF ACTION OF METHOTREXATE"

by

E. ANN JACOBSON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABBREVIATIONS USED ARE:

MTX, methotrexate; DHFR, dihydrofolate reductase enzyme;

^{3}H-UdR, deoxyuridine tritiated in position 6;

^{3}H-TdR, thymidine tritiated in the methyl group;

^{3}H-dUMP, deoxyuridine monophosphate tritiated in position 5;

FH_2, dihydrofolate; FH_4, tetrahydrofolate;

TS, thymidylate synthetase enzyme; AdR, deoxyadenosine

SUMMARY

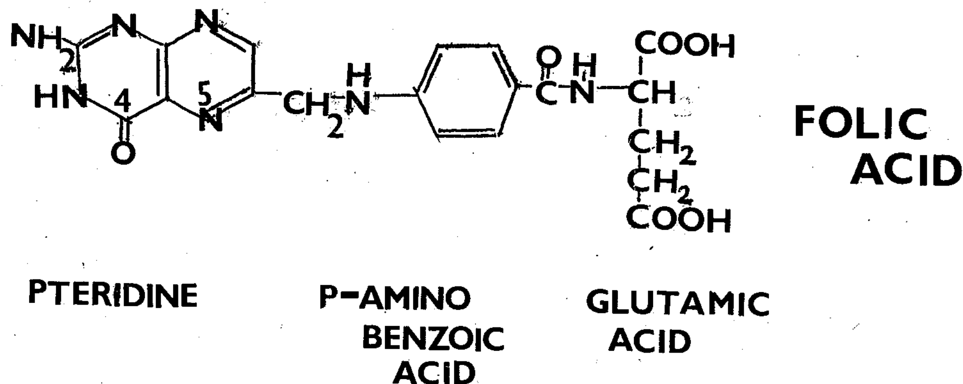
The 'calculated tritiated deoxyuridine rate' has previously been correlated with a methotrexate (MTX)-induced thymineless death as measured by cloning in L5178Y cells. This study extends the correlation to mid-log and resting cultures of CHO cells. The correlation could not be shown in early log CHO cultures due to the presence in fetal calf serum of dialyzible substances that protect against the cytocidal effects of MTX. Mid-log CHO cultures could also be protected against MTX by supplementing the medium with additional fetal calf serum at the time of drug treatment.

A MTX-induced drop in thymidylate synthetase activity was observed in extracts of both log and resting cultures of L5178Y cells. The addition of an exogenous purine source prevented the MTX-induced suppression of protein synthesis and prevented the drop in thymidylate synthetase activity. MTX may inhibit dihydrofolate reductase, rapidly deplete the L5178Y cells of reduced folates and thus produce a thymineless and purineless state. The purineless state, by inhibiting the synthesis of thymidylate synthetase may facilitate the deepening of the thymineless state.

An increased sensitivity to sonication of thymidylate synthetase activity from log cultures over resting cultures of L5178Y cells was detected. The recognition of this effect along with the discovery of protecting factors in fetal calf serum casts doubt upon many of the conclusions from previous studies of the mechanism of action of MTX where these variables may not have been adequately controlled.

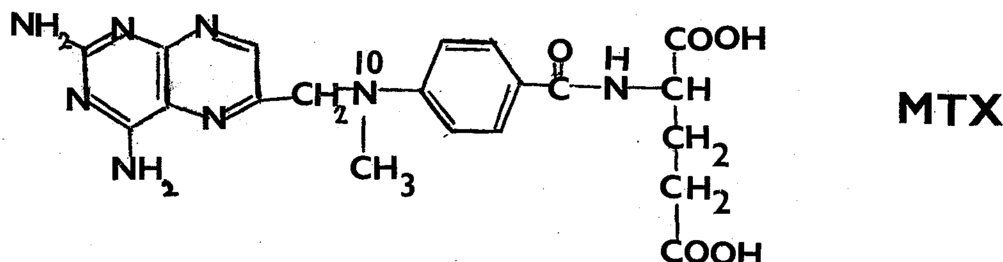
HISTORICAL REVIEW

Megaloblastic anemia has been treated with folic acid conjugates with great success since 1943 (9). Soon after the development of this therapy, folates were used in an unsuccessful attempt to treat the anemia of acute lymphoblastic leukemia. Since the leukemic process was in fact enhanced by the folate, producing an 'acceleration phenomenon', it occurred to Farber that an anti-folate might be of value in the treatment of patients with acute leukemia (30). Following his suggestion, Subbarow and colleagues developed a series of folic acid analogs in 1948. The first analog was aminopterin which was folic acid with an amino group substituted for the oxygen at position C_4 of the pteridine ring.



Ten out of 16 leukemic children treated with aminopterin by Farber exhibited rapid remission of signs and symptoms of their disease. However, eventually all relapsed and died despite continued treatment. This failure was not necessarily due to the drug but

rather the choice of dosage and scheduling. (60). Methotrexate (MTX), or amethopterin another analog, is aminopterin with an additional methyl group at position N_{10}.



Because aminopterin caused more gastrointestinal toxicity in animals with no increase in tumour cell kill, MTX is used more extensively in the clinic. MTX therapy has been found to cure some malignant diseases, and produce long survivals in others. However, in most solid tumours, it has been of limited usefulness.

One aspect of the action of MTX is interesting. If the malignancy is sensitive to MTX even _in vitro_, that malignancy is amenable to other forms of chemotherapy. For example, the degree of sensitivity to MTX _in vitro_, of cells from patients with acute myeloblastic leukemia (AML) appears to correlate with survival even though those patients are treated with drugs other than MTX (62). Thus, MTX appears to detect some vulnerability in the malignancy that makes it amenable to treatment. This susceptibility to MTX, if defined, might be exploited in the treatment of the malignancy. In order to understand how MTX might exert its antitumour activity, it is first necessary to review some basic concepts in chemotherapy of malignancy. One main theoretical scientific approach to the drug

treatment of malignancy was first suggested 15 years ago with the development of the cell kill hypothesis.

CELL KILL AND CANCER CHEMOTHERAPY

The concept of cell kill and its relationship to successful antitumour chemotherapy was first investigated by Skipper and Bruce (18,102,103). From their work evolved a more rational approach to drug therapy.

As defined by Skipper and Bruce, drug-induced cell kill was achieved when a cell's metabolic processes were perturbed to the point where it was unable to replicate itself even when no longer exposed to the drug. A cell might be intact physically and be capable of metabolism, e.g., limited protein synthesis, but still be 'dead', in the sense of being incapable of giving rise to clones or colonies.*

Skipper based his studies upon the ability of L1210 murine leukemia cells to proliferate in BDF_1 mice. Bruce and his colleagues developed a model to measure the differences in the effect of chemotherapeutic agents on normal marrow or spleen cells and malignant lymphoma cells in AKR mice.

The concepts developed with the use of these two model systems may be summarized as follows:

1. Injection of a single L1210 leukemic cell in a BDF_1 mouse would result in the death of the animal.

2. The larger the number of cells injected, the sooner the animal died.

*Clonable cells are assayed by transplanting small numbers of cells in drug-free medium on petri dishes, into soft agar, or suitable mice, and counting the number of colonies formed. The number of colonies is linearly related to the number of viable proliferating cells.

3. The time of death of the host could be predicted from a knowledge of the number of cells injected and their doubling time because these factors determined when the tumour cell population reached the critical size required to kill the animal.

4. Chemotherapy reduced the number of clonable cells remaining. Prolongation of survival was attributable to the percentage of cells killed.

5. Indefinite survival resulted when all the tumour cells were eradicated.

6. Skipper noted that for cells with equivalent growth rates, a given dose of drug killed a constant percentage of cells regardless of the tumour load. A single dose that reduced the leukemic cell population by 99.9999% (6 logs of kill) 'cured' none of a group of animals bearing 1×10^9 cells, 40% of those bearing 1×10^6 cells or all bearing 1×10^4 cells.

7. Three classes of dose-response curves were noted for the chemotherapeutic agents tested:

(A) For nitrogen mustard and gamma radiation, cell kill was exponential with respect to dose. The sensitivity of normal and lymphoma cells was identical. These agents killed cells in all portions of the cell cycle. Sensitivity did not depend upon the proliferative state of the cells.

(B) In the second class of chemotherapeutic drugs, were ^{3}H-thymidine (^{3}H-TdR),vinblastine, and MTX. The lymphoma cells were more sensitive than the normal cells. The survival decreased as a function of dose but eventually a dose was reached whereat further dose increments did not kill any more cells. It was suggested that

these drugs acted at one portion of the cell cycle and those cells not killed were in non-sensitive portions of the cell cycle. On the average, lymphoma cells complete a generation cycle about every 10 hours. If the agents kill during a particular portion of the cell cycle, i.e., DNA synthesis as for ^{3}H-TdR, exposure of the cells to the cytotoxic agent for 24 hours would result in substantial loss of the proliferative capacity. On the other hand, the normal hematopoietic colony-forming cells pass through the cycle less frequently; most are thought to be in a non-proliferative or G_o state, are not as susceptible to the same drug, and therefore fewer are killed per unit time of drug treatment.

(C) Treatment with agents of the third class, 5-fluorouracil, actinomycin D and cyclophosphamide, resulted in cell survival curves which decreased exponentially with dose. There were marked differences between the kill curves for malignant and normal cells. Since there was no nadir with increasing doses, it was hypothesized that the cells were sensitive to these agents throughout their generation cycle. It was suggested that the differences in sensitivity were a direct consequence of the differences in proliferative rate. Thus, Skipper and Bruce hypothesized that the prolongation of life span with chemotherapy was due to the cytocidal effects on the tumour cell population. The delay in host death was the time required for the tumour cells surviving chemotherapy to proliferate to a lethal number and was not thought to be consistent with a drug-induced lag phase or change in generation time (103).

With these cell models, it was possible to investigate the differences in the effect of chemotherapeutic agents upon the normal

and malignant cells and to devise regimens using combinations of drugs which allowed the killing of all tumour cells yet sparing the host from lethal toxicity.

It should be noted that this hypothesis does not take into consideration drug-induced or spontaneous mutations in tumour cells resulting in resistance to chemotherapy or the possibility of viral re-induction of new tumours after the original tumour has been eradicated. G_o was a theoretical concept that has not been verified. For example cells said to be in G_o could be a subpopulation of cells still in cycle but very resistant to drugs for biochemical reasons. A change in dose and scheduling of drug therapy might result in increased kill beyond the plateau reached by the schedules used in the experiments of Skipper and Bruce. Besides, not all tumour cells are equally exposed to the drugs, since not all agents can pass the blood-brain barrier (85). Furthermore the sensitivity of L5178Y lymphoblasts to nitrogen mustard, an agent of the first class has been shown to be a function of the proliferative state of the cells (38). Despite these reservations, the cell kill hypothesis has been useful framework within which to study chemotherapeutic agents. Based upon the assumption that the antitumour activity of MTX is related to its ability to kill cancer cells, the aim of the present research is to investigate how MTX kills cells. From this it is hoped one could predict which cells would be sensitive and which resistant to the drug.

MTX AS A CHEMOTHERAPEUTIC AGENT

MTX has been useful in managing acute leukemia in children, osteogenic sarcoma, squamous cell carcinoma of the head and neck, choriocarcinoma in the female, African Burkitt's lymphoma and breast cancer. It is of limited value in adult leukemias and most solid tumours.

Cell kill by MTX cannot be measured in human malignant cells directly sampled from humans, since they are not readily clonable with high efficiency. However, if one could derive biochemical correlates of cell kill from a clonable cell line, one might then predict the cytocidal effects of MTX on non-clonable cells (such as those from patients with acute leukemia) and, therefore, might predict survival of the patient after treatment.

"Predictive procedures are important for rapidly assessing probable drug responsiveness. In AML, unless remission is achieved rapidly, the patient may die before a second empirical therapy can be tried. It is important to avoid the use of an ineffective drug or drug combination, since the success of any chemotherapeutic program depends upon the rapidity with which the most appropriate drugs can be selected" (20). However, attempts at predicting the results of chemotherapy on the basis of _in vitro_ tests have met with limited success.

The _in vitro_ effect of 5-fluorouracil, an inhibitor of thymidylate synthetase, on ^{3}H-TdR incorporation in gastrointestinal tumour and normal tissue was almost identical and therefore could not be regarded as a valuable _in vitro_ test even though the drug is useful in the treatment of gastrointestinal cancer (118).

In patients with acute nonlymphoblastic leukemia, the highest percentage of marrow cells labelled with thymidine (TdR) was found in patients who later went into complete remission. The rate of incorporation of C^{14}-TdR after incubation with combinations of cytotoxic drugs was found to be depressed in cells of patients who later were found to be responders to that combination (121).

Some correlation was found between clinical response to the

drug and uptake by leukemic cells of MTX during short term in vitro culture. This correlation was found only if extracellular drug levels were kept sufficiently low (72). This work has not yet been confirmed by other researchers. When the effect of 1×10^{-6}M MTX on ^{3}H-TdR incorporation was studied after 4 hour treatment of suspensions of human acute leukemia blasts in vitro, there was in general, a correlation between sensitivity to MTX in vitro and duration of survival of the patients regardless of whether these patients were treated with MTX, or other agents (62). However, this test did not predict responsiveness of each individual patient with a high degree of accuracy. Thus, the search for a test predicting MTX responsiveness continues. In designing such a test, attention should be focused upon those pathways affected by the drug. These pathways relate to folate biochemistry.

FOLATE BIOCHEMISTRY

Folic acid is a nutritional factor comprised of residues of glutamic acid, p-aminobenzoic acid and pterin. A large portion of the folic acid in food is protein-bound. It exists in the polyglutamate form with linkage in the gamma position and deconjugation by intestinal conjugases is necessary to facilitate absorption. The precise mechanism of absorption is unknown but appears to be energy-requiring at low concentrations and occurs at the level of the small intestine. Vitamin B_{12} is necessary for the effective transport of folates into the liver and probably across other cell membranes (37).

Once in the cell, folic acid is reduced to the dihydro (FH_2) compound and subsequently to the tetrahydro form (FH_4) by the action of dihydrofolate reductase (5,6,7,8-tetrahydrofolate NAD(P) oxido-

reductase (E.C. 1.5.1.3) (DHFR) (9). The nitrogen atoms at positions 5 and 10 are the sites for the subsequent reactions in which FH_4 acts as a carrier of one carbon-containing groups at three levels of oxidation: methanol, formaldehyde, and formic acid as well as the formimino group.

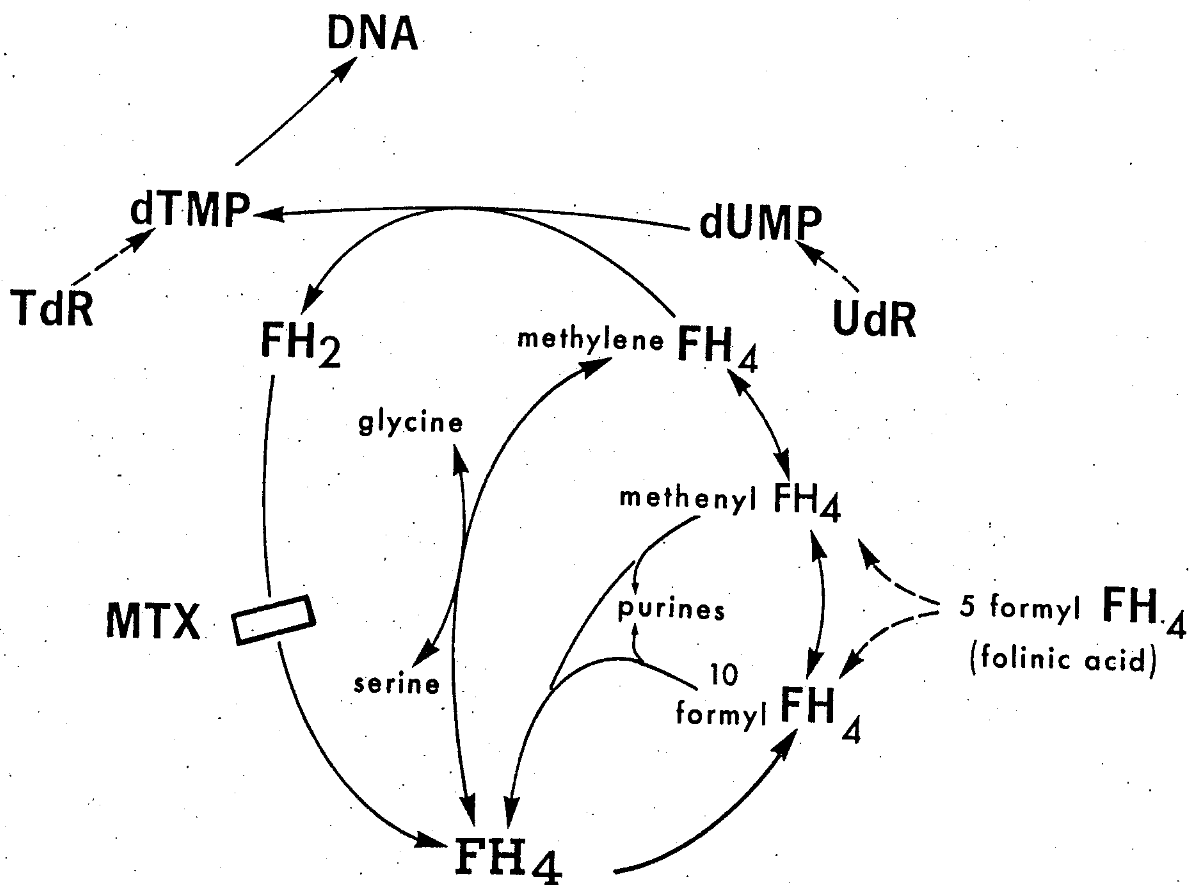
FH_4 is converted to N^5, N^{10} methylene FH_4 with the production of glycine by serine hydroxymethyltransferase. The methyl group can then be transferred to deoxyuridine monophosphate (dUMP) by thymidylate synthetase (TS), to form thymidylic acid (dTMP). In this reaction, FH_2 is formed and must be reduced to FH_4 by DHFR. Thymidylate can also be formed from thymidine by thymidine kinase (TK), a scavenger reaction.

In purine biosynthesis, carbons 2 and 8 come from FH_4 cofactors. Glycinamide ribonucleotide transformylase catalyses the transfer of a carbon atom from N^5, N^{10} methenyl FH_4 for the C_8 of the purine. C_2 is inserted by a transformylase acting on N^{10} formyl FH_4. N^{10} formyl FH_4 results from the hydrolysis by cyclohydrolase ofN^5, N^{10} methenyl FH_4. This compound is formed by the oxidation of N^5, N^{10} methylene FH_4 by methylene FH_4 dehydrogenase. Histidine is involved in folic acid metabolism since it utilizes a portion of an existing purine nucleus in bacteria.

Methylene FH_4 reductase reduces N^5, N^{10} methylene FH_4 to give N^5 methyl FH_4, the main transport and storage form of reduced folates. This methyl group is transferred intact for the de novo synthesis of methionine, and is also used in the synthesis of choline, sarcosine, and dimethyl glycine.

Reduced folates can also arise from the action of N^5 formyl FH_4 isomerase on folinic acid, which is present in small amounts in

the diet, to give N^5, N^{10} methylene FH_4 or N^{10} formyl FH_4.



Folate coenzymes function in the synthesis of RNA directly through the synthesis of purine bases and indirectly through the contribution to DNA synthesis. Protein synthesis is influenced by the interconversion of serine from glycine as well as by the contribution of folate coenzymes to RNA synthesis. In prokaryotic systems, folate coenzymes are necessary in the formylation of methionine esterified to transfer RNA (tRNA). N formyl methionyl

tRNA provides the N-terminal in the growing polypeptide chain, but an absolute requirement for the formyl group has not been shown in mammalian cells even though eukaryotic mitochondrial protein synthesis is initiated with N formyl methionyl tRNA. Formylation of labelled methionine continues in the presence of MTX indicating that the formyl group may not be contributed by an FH_4 derivative (111).

CHEMISTRY, PHARMACOLOGY AND TOXICITY OF MTX

MTX, a weak organic acid, is readily absorbed from the alimentary tract but may be given parenterally. At low doses the drug does not pass the blood-brain barrier in sufficient amount to give clinically significant concentrations in the cerebro-spinal fluid (85). It can be injected intrathecally as prophylaxis or to treat already established leukemic involvement of the central nervous system (49,83). Plasma levels of the drug are dose-related with a mean plasma half-life of 2 hours. The antifolate distributes itself to total body water within 1 hour following an intravenous or small oral dose. 50 to 90% of absorbed MTX is excreted unchanged in the urine within 48 hours (51).

The tissues that retain the largest amounts of MTX are the organs that contain the highest content of DHFR and of folate coenzymes (68, 120). These are the kidney, liver, gut and marrow.

When rats were given intraperitoneal injections of MTX and then sacrificed, the intracellular localization of the drug in the liver was determined by homogenizing the tissue and fractionating by differential centrifugation. The bulk of the drug was lost with a half-life of 120 to 360 hours and enzyme activity partially reappeared when the supernatant was dialyzed against sucrose (0.25 M).

The rest was displaced when the samples were dialyzed against sucrose containing folate (2 x 10^{-3}M). This resulted in complete reactivation of DHFR. Thus a significant portion of MTX is tightly bound to DHFR (114).

The rapidly exchangeable intracellular MTX not bound to DHFR is likely present in the cytoplasm in an osmotically active state. A small amount of the exchangeable fraction is loosely adsorbed at or near the cell surface (7).

In the rat liver, MTX appears to follow the biosynthetic routes of folic acid in being raised to the poly-gamma-glutamyl derivatives (3). The rate of disappearance of the polyglutamate forms of MTX from the liver and kidney was 8 times faster than that of unconjugated MTX (115). In _S. faecium_, increasing numbers of glutamyl residues led to decreasing inhibition of bacterial growth possibly due to reduced penetration of the drug or reduced inhibition of DHFR (84).

After administration of MTX to humans, some persists in the liver bound to DHFR and is released only slowly over a period of months. This most likely is due to slow dissociation from the enzyme which it stabilizes against degradation. The slow release could also be due to metabolism to a form with less affinity for DHFR or to the eventual degradation of the enzyme. However, at the usual doses used, the drug is metabolized to an insignificant degree, initially all appearing in the urine in unchanged form.

Toxic effects can be seen 3 to 4 days after administration of a large single dose to a normal human. The major lesion occurs in the proliferating tissues, i.e., intestinal tract, bone marrow,

and hair follicles. Ulceration of the oral mucosa is the first to occur. Bone marrow toxicity is manifested by pancytopenia and may be fatal. The loss of hair occurs frequently but surprisingly the hair regrows despite continued administration of the drug. With high doses, histological changes occur in the convoluted tubules with impaired renal clearance. Extensive desquamation of epidermal cells can occur in severe toxic reactions (9).

MTX TRANSPORT

The rate of transport of MTX is a critical determinant of its cytotoxicity. The higher the free intracellular MTX level acheived and the longer it is sustained, the longer the interval over which the DHFR will be inactivated.

Influx of MTX into cells follows Michaelis-Menten kinetics, has marked oxygen, pH and temperature dependence and is abolished by p-chloromercurobenzoate, a sulfhydryl reactive reagent which may inhibit active transport. An electrochemical potential gradient for free intracellular MTX can be demonstrated at low levels of extracellular MTX. Folinic acid, (N^5 formyl FH_4) when added to the extracellular medium competitively inhibits the influx and promotes the efflux of MTX, indicative of counter-transport. In addition, when the cells are preloaded with folates and MTX added to the extracellular medium at a steady state, there is enhancement of the efflux of folates. This suggests that the naturally occurring folates not only inhibit the interaction between MTX and a carrier but at least in part utilize the same carrier. Thus, MTX might exert a cytotoxic effect by first inhibiting the cellular uptake of N^5methyl FH_4 the main reduced folate extracted by cells from the extracellular compartment, and