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Short Review

The Fluoropyrimidines: Biochemical Mechanisms and Design of Clinical Trials

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Summary. *Our understanding of the biochemical events of fluoropyrimidine-induced cytotoxicity remains incomplete. However, we have a good perception of the activation and degradation pathways of these agents. Additionally, from studies performed in vitro we are gaining a new appreciation of the interactions between methotrexate and the fluoropyrimidines. These studies suggest that the common clinical practice of simultaneously administering methotrexate and 5-fluorouracil may be disadvantageous. Several simple scheduling modifications of combination therapies with these two drugs could lead to improved clinical efficacy and deserve further investigation.*

Introduction

Despite almost 25 years of intensive basic and clinical research, our understanding of the fluoropyrimidines remains fragmentary. Many thousands of patients with a wide variety of malignant diseases have received these agents and currently three are used in common clinical oncologic practice – 5-fluorouracil (FU), 5-fluoro-2′-deoxyuridine (FUdR), and ftorafur (FT).

It is appropriate to review the current preclinical and clinical information on these agents in order to employ the currently available drugs in the most efficacious fashion, and to rationally design and screen new fluoropyrimidines.

Mechanism of Action of the Fluoropyrimidines

The fluoropyrimidines produce their cytotoxic effects by two separate biochemical mechanisms: the inhibition or alteration of RNA maturation and function by fluorouridine 5′-triphosphate (FUTP) incorporation into RNA, and the inhibition of DNA formation through blockade of thymidylate synthetase by 5-fluoro-2′deoxyuridine 5′-monophosphate (FdUMP) (Fig. 1). A detailed analysis of these mechanisms can be found in several recent review articles (Miller, 1971; Maley, 1977; Heidelberger, 1975; Myers et al., 1976). The inhibition of thymidylate synthetase is generally accepted as the major mode of cell toxicity of FU. However, interference with RNA function by FU may be equally important under certain conditions as indicated by the fact that both thymidine and uridine are required to completely overcome toxic FU effects (Maley, 1977; Doolittle et al., 1973). Unfortunately, the contribution of RNA effects to clinical drug efficacy remains unknown. Effective thymidine rescue treatments against the cell killing effects of FU in vitro and in vivo provide evidence that thymidylate inhibition indeed probably represents the primary mechanism of action.

Depletion of the thymidylate pool by FdUMP triggers a chain of biochemical events resulting in DNA breakage and cell death, also known as "thymine-less death" (Cohen, 1971). There are three phases of the biochemical mechanism of cell kill by FU: 1. FU metabolism to the active nucleotide FdUMP; 2. effects on the production of thymidylate (dTMP); and 3. subsequent cellular events leading to DNA breakage and "thymine-less death."

1. Metabolic Activation of Fluoropyrimidines. The activation of FU to FdUMP can proceed via several pathways as shown in Figure 1. In general the metabolism of FU closely parallels that of its endogenous congener uracil except for 5-methylation by thymidylate synthetase. The pyrimidine-specific enzymes which participate in FU activation are listed in Table 1. The major route of activation appears to occur via 5-fluorouridine-5′-monophosphate (FUMP) and ribotide reductase, since

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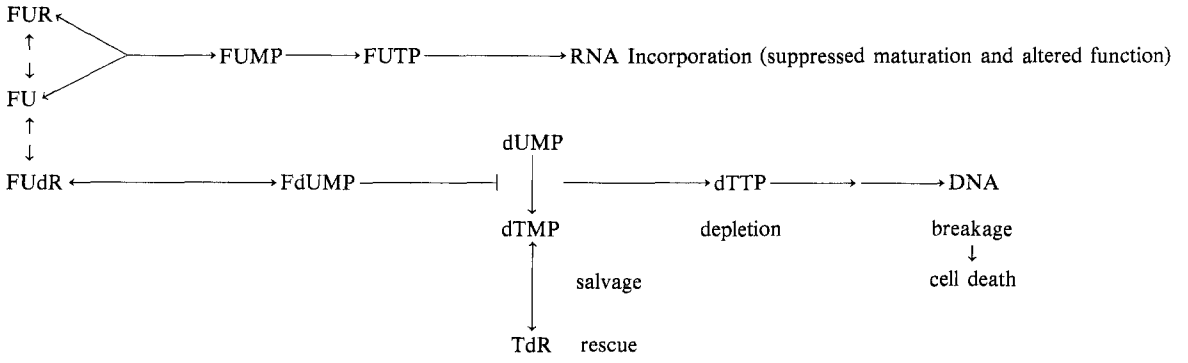


Fig. 1. Schematic representation of the biochemical mechanism of action of fluoropyrimidines

Table 1. Pyrimidine specific enzymes activating FU and its analogs FUdR and FUR

Uridine phosphorylase (FU ⇄ FUR)
Uridine kinase (FUR → FUMP)
Uracil phosphoribosyl transferase (FU → FUMP)
Deoxyuridine – thymidine phosphorylase (FU ⇄ FUdR)
Thymidine kinase (FUdR → FdUMP)
UDP reductase (FUDP → FdUDP)
Various phosphatases and kinases

FUdR is rapidly converted to FU by phosphorolysis in vivo (Chadwick and Rogers, 1972). It should be noted that thymidine kinase not only activates FUdR directly to FdUMP but is also capable of bypassing the de novo thymidylate inhibition by salvaging preformed thymidine to dTMP. This mechanism could account for the paradoxical finding that high levels of thymidine kinase may actually protect the cell against FUdR toxicity (Heidelberger, 1975). The fluoropyrimidine analogs FU, 5-fluorouridine (FUR), and FUdR, although metabolically interconvertible, show quite different quantitative activities. In particular FUR is rather more toxic to the host, a finding which so far has precluded its wider clinical use. However, some tumors containing low uridine phosphorylase levels may respond better to FUR than to FU (Kessel et al., 1971; Burchenal et al., 1975). The recently introduced FU derivative FT represents a chemical depot form of FU, which is slowly released in vivo (Lu et al., 1975). Thus, FT and slow intravenous infusions of FU have low bone marrow toxicity; however, the high incidence of CNS toxicity of FT may preclude its general clinical use.

2. Inhibition of dTMP Formation. The dominant factor of the inhibition of de novo dTMP flux is the extent of FdUMP formation in the target cell. However, several additional factors modulate thymidylate synthetase activity. This key enzyme in the de novo synthesis of dTMP catalyzes the reductive methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) using 5,10-methylene tetrahydrofolic acid (CH_2-THF) (Maley, 1977; Heidelberger, 1975; Pogolotti and Santi, 1977). Regeneration of the CH_2-THF cofactor requires a one carbon donor, such as serine, and dihydrofolate reductase, which is inhibited by methotrexate (MTX). Binding of FdUMP to thymidylate synthetase is initiated by attack of a nucleophile (most likely a cysteine residue) at the C-6 position of FdUMP, resulting in a covalent enzyme-substrate complex (Heidelberger, 1975; Pogolotti and Santi, 1977). Stability of this complex is dramatically increased by addition of CH_2-THF, which then produces essentially irreversible enzyme inhibition by FdUMP (Myers et al., 1975). All of these factors, therefore, participate in the control of de novo thymidylate formation. Our understanding of these biochemical mechanisms is further complicated by the fact that a second independent salvage pathway exists for dTMP formation based on thymidine kinase activity, which in turn correlates with cell proliferation rate (Ohashi and Teguchi, 1976). Exogenously administered thymidine itself may at high doses be toxic or at lower doses inhibit the toxicity of concomitant application of fluoropyrimidines (Morse et al., 1965; Tattersall et al., 1975).

3. Potential Mechanisms of "Thymine-less Death". The cellular events subsequent to thymidylate depletion and prior to DNA breakage are less well studied. One of the logical direct consequences is a reduction of thymidine 5′-triphosphate (dTTP) levels, which has been observed in several cell lines (Tattersall and Harrap, 1973; Kimmer and Kraml, 1977) and has been correlated with inhibition of DNA formation. However, in one report no changes of dTTP pools following FUdR were observed in the presence of significant inhibition of DNA synthesis (Baumunk and Friedman, 1977). These authors postulate that a compartmental dTTP pool exists that is not accessible for DNA synthesis. Further studies will be

needed to clarify the role dTTP pools and turnover rates play in the biochemical activity of FU (particularly in those tumor cells with very large dTTP pools).

Following a decrease in active dTTP levels, further changes of the mononucleotide metabolism are likely to occur, since dTTP functions as a feedback inhibitor of a number of other pathways. These include ribonucleotide reductases, thymidine kinase and other enzymes directly involved with DNA metabolism (Tattersall and Harrap, 1973). Thus, FU administration causes a reduction of dTTP levels, but an increase of 2′-deoxycytidine 5′-triphosphate (dCTP), 2′-deoxyadenosine 5′-triphosphate (dATP), and 2′-deoxyguanosine 5′-triphosphate (dGTP) levels (Tattersall and Harrap, 1973). These changes lead to an imbalance of deoxyriboside triphosphates, which potentially causes DNA breakage and cell death. As such, the mechanism underlying the "thymine-less death" may not be unique. Excessive amounts of thymidine or deoxyguanosine (Tattersall et al., 1975) and blockade of dCTP utilization by cytosine arabinoside may cause dNTP imbalances leading to similar DNA breakage. It is interesting to note that cytosine arabinoside and FU in combination display antagonistic effects, which might result from the correction of a critical imbalance of dNTP utilization after doses of either agent alone (Momparler, 1973).

Additionally, "thymine-less death" appears to be dependent upon a continuous supply of nutrients other than thymidine, a situation which has been called "unbalanced growth" (Cohen, 1971). A critical factor in accentuating the toxic effects of FU is the availability of folate cofactors, which promote protein and purine productions (without directly affecting thymidylate formation in the presence of FU). Possibly the cell has to proceed through the cell cycle in order to be maximally sensitive to thymine starvation and this might explain the need for general nutrients. However, increases in FU toxicity by folinic acid have also been associated with the increased binding affinity of FdUMP to thymidylate synthetase in the presence of CH_2-THF (Santi et al., 1977).

We do not as yet know what precise cellular mechanisms are responsible for the "thymine-less death". This information could be of tremendous value in the design of clinical trials with fluoropyrimidines, particularly in combination chemotherapy. Our current knowledge, however, may allow us to design several clinical trials which reflect the known biochemical determinants of FU activity.

Determinants of FU Activity

Relatively minor biochemical differences may convey resistance of tumor cells to 5-fluoropyrimidines, whether they preexist or are acquired through the genetic selection pressure of chemotherapy. No absolute cell specificity exists, and therapeutic efficacy is determined by the relative degree of host and tumor cell resistance rather than an all-or-none phenomenon. Thus, determinants of FU activity in cell cultures lack reference to the host cell toxicity, and one has to be careful in interpreting mechanisms of resistance in vitro as therapeutic indicators. However, predictive tests of FU efficacy would be of great importance, since the response rate of some adenocarcinomas is still low and unnecessary exposure to toxic agents such as FU should be avoided.

Biochemical factors of cell resistance have been recently reviewed (Myers et al., 1976). Many previous studies have focused on the nature and abundance of pyrimidine enzymes that convert FU to its active form, FdUMP. Most of the enzymes listed in Table 1 have been implicated in determining cell sensitivity and resistance, most notably uracil phosphoribosyl transferase, uridine phosphorylase, and uridine kinase (Heidelberger, 1975; Reyes and Hall, 1969). Most studies were performed in vitro and indicate that the extent of FdUMP formation is the single major determinant of FU activity.

Inhibition of thymidylate flux by FdUMP, however, is also determined by the physical-chemical nature of the enzyme thymidylate synthetase and its formation rate (Heidelberger et al., 1960; Maley, 1977) as well as by the extent of dUMP pool size expansion following FU administration (Myers, et al., 1975). A gradual increase of dUMP levels protects newly formed thymidylate synthetase against irreversible inhibition by FdUMP and correlates with recovery of ^{3}H-2′-deoxyuridine incorporation into DNA.

Incorporation of ^{3}H-2′-deoxyuridine into DNA appears to be the parameter most useful in monitoring FU therapy, since it reflects all of the known biochemical determinants of FU activity (Myers et al., 1976). This biochemical test might prove useful in conjunction with surgical tumor excision, which allows ready access to tumor and host tissue.

Determinants of cell resistance in the cellular events after thymidylate depletion are largely speculative. Yet, they might be equally important in the clinical success of FU treatment, and further investigations are needed.

Biochemical Basis of a Combination Regimen: FU, MTX, and Folinic Acid

The biochemical and clinical interactions between FU, MTX, and folate cofactors have been investigated in several studies, since FU-MTX combinations are currently in clinical use. Surprisingly, most of the cell culture and animal tumor studies indicate antagonism rath-

er than therapeutic synergism between these two agents. Three hypotheses have been proposed to account for this finding. 1. MTX increases the dUMP pool size, since it blocks reduction of dihydrofolate (DHF) to THF, which is a required cofactor in the methylation of dUMP to give dTMP (Tattersall, Jackson, et al., 1973). High levels of dUMP protect thymidylate synthetase against FdUMP inhibition and thereby reduce FU toxicity. 2. MTX causes depletion of CH_2-THF which is required for tight binding of FdUMP to thymidylate synthetase. In the absence of sufficient amounts of CH_2-THF, FU toxicity might be reduced, while coadministration of folinic acid enhances FU toxicity in support of this hypothesis (Santi et al., 1977). However, in the absence of sufficient CH_2-THF pools, thymidylate flux is already reduced and complete inhibition should require less rather than more FU. 3. According to the imbalanced growth hypothesis of the "thymine-less death" (Cohen, 1971) one would expect antagonism between FU and MTX (since MTX depletes the folate pool) and synergism between FU and folinic acid (which replenishes the folate pool). The folate cofactors are essential in the continued growth of the cell, which causes increased cell death in the absence of thymine.

None of these hypotheses accounts for the synergistic results between MTX and FU observed by Bertino et al. (1977) in a mouse sarcoma 180 tumor model. We have to realize that some tumor systems can respond in dramatically different ways which are still entirely unknown. Yet reasonable predictions can be made based on the data obtained from previous results of FU-MTX combinations and should be applied to new clinical trials with these agents to avoid antagonism and maximize synergism by proper dosage regimens.

Specific thymidine rescue rather than folinic acid rescue has attracted several clinical investigators. It must be recognized, however, that clinical trials employing FU and thymidine rescue are empirical, since the nature of the "thymine-less death" remains unknown.

Clinical Consequences

The above preclinical data have obvious practical importance in the clinical management of patients with malignant disease. Firstly, the use of fluoropyrimidines in combination chemotherapy programs must be reexamined. Usually drug schedules are based on physician or patient convenience, rather than biochemical or kinetic rationale. Moreover, the necessary information about the interaction of the fluoropyrimidines and other antitumor agents is largely lacking. However, we do have at least some understanding of the interactions between FU and MTX. Based on the previously noted laboratory data, the clinical use of these two drugs together could result in additive, synergistic, or even antagonistic effects. Since both drugs are frequently used simultaneously, this inquiry is not mere academic curiosity.

The combination of cytoxan (CTX), FU, and MTX for patients with disseminated breast cancer is an appropriate example for this type of study. A commonly used program of these 3 drugs (CMF) is:

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CTX	×	×	×	×	×	×	×	×	×	×	×	×	×	×
FU	×							×						
MTX	×							×						

Repeated q 28 days

Approximately 60% of patients evidence an objective tumor regression lasting a median of 9 months (Carbone et al., 1977). Nevertheless, a significant proportion of treated patients fail to benefit. Even in those who do benefit, responses lasting a year or more are unusual. Several in vitro studies suggest that simultaneously employed FU and MTX are antagonistic. Therefore, modifications of the "standard" CMF program (CTX, MTX, FU) should be designed which reflect the biochemical rationale of MTX-FU interactions. Reasonable programs might be:

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CTX	×	×	×	×	×	×	×	×	×	×	×	×	×	×
FU	×							×						
MTX		×							×					
or:														
CTX	×	×	×	×	×	×	×	×	×	×	×	×	×	×
FU	×							×						
MTX		×		×					×		×			

Repeated q 28 days

These simple modifications avoid simultaneous antagonistic FU and MTX administration. FU administration before MTX should eliminate antagonistic effects on FU activity, while the effects on MTX activity remain unpredictable.

Recognizing the requirement for reduced folate cofactors in order for FU to efficiently kill target cells, we envision a further modification of CMF:

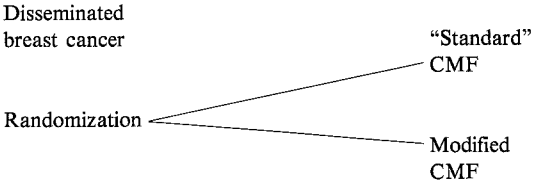
Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Folinic acid	×							×							
CTX		×	×	×	×	×	×	×	×	×	×	×	×	×	×
FU			×						×						
MTX				×						×					

Pretreatment with an agent such as folinic acid may cause an initial growth-promoting effect on the tumor; however, the expected enhancement of FU efficacy might outweigh this problem. The exogenously supplied folinic acid (in modest dosage) would help prevent subclinically detectable folate deficiency in this patient population. These doses of folinic acid are not likely to interfere with MTX administered 3 (or more) days later.

Further and more aggressive modification using high dosage MTX and leukovorin rescue could be explored. For example (Program see below).

Bertino and coworkers (Bertino et al., 1977) have suggested that high dosage MTX may enhance FU efficacy and this in vitro data deserves careful clinical attention. The programs suggested above should be regarded as preliminary estimates. The schedule of administration of these drugs is a particularly critical factor since duration of tissue exposure, rate of cell recovery, and response to rescue treatment are a function of the biochemical and cell kinetics of target and host tissues. Labeling of DNA by ^{3}H-deoxyuridine may represent an appropriate in vitro test to monitor tissue response for further improvements of the initial dosage regimen.

In order to test these hypotheses a large scale prospective trial would be required. For example:



Unless dramatically improved response rates or durations of responses were observed these modified approaches would seem unwarranted, since a more complicated schedule would be used. Therefore, the number of patients studied need not be prohibitively large.

Secondly, another promising area for clinical investigation involves the use of specific biochemical rescue techniques for the fluorinated pyrimidines. Based on the successful use of leukovorin to ameliorate otherwise fatal MTX toxicity, thymidine might improve the therapeutic index of the fluoropyrimidines. Kroll and associates (1976) have reported a program of FUdR (0.5 mg/kg/d) followed by 5-iodo-2′-deoxyuridine (IUdR) (40 mg/kg/d) for 3 days with thymidine rescue (32 mg/kg/d q 2 h × 5 doses for 3 days). They describe significantly lessened myelotoxicity in patients receiving thymidine compared to those not receiving it. Additionally, phase I data on very high dose FU (3–16 g/m^2/d infusion × 7–14 days) has been presented by Spiers et al. (1977). The dose-limiting factors are bone marrow and gastrointestinal toxicities. If rescue programs can be identified, this aggressive approach would deserve further study, especially in gastrointestinal malignancies.

Thirdly, the biochemical examination of human normal and tumor tissues could meaningfully assist in the clinical prediction of response and toxicity to fluoropyrimidine therapy. Currently, we are recognizing that several mechanisms are responsible for clinical resistance to FU therapy. Assessment of the biochemical milieu of drug activation (uridine kinase, thymidine kinase, phosphoribosyl transferase, etc.) and inactivation (uracil dehydrogenase) might prove useful. Quantitative and qualitative examination of thymidylate synthetase kinetics and DNA formation rate by measuring ^{3}H-2′-deoxyuridine incorporation into DNA appears to represent the most promising approach to monitor FU therapy. Since so few patients benefit from these drugs as they are currently employed, it is mandatory to understand tumor resistance to therapy.

Much information is being amassed concerning the pharmacology and biochemistry of the fluoropyrimidines. It is not only necessary that these studies continue, but also that this data is applied in clinical trials. New

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
High dose MTX	×																		
Leukovorin rescue		×	×																
FU				×								×							
CTX				×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×

clinical approaches are needed now. After the Battle of Waterloo the Duke of Wellington is reported to have said: "They came at us in the same old way, and we turned them back in the same old way". Unless oncologists thoughtfully employ basic information in the design of clinical studies, we will continue the pedestrian approaches which have offered too little to too few patients.

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