THE EXPANDING ROLE OF FOLATES AND FLUOROPYRIMIDINES IN CANCER CHEMOTHERAPY

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Edited by

Youcef Rustum and
John J. McGuire

Roswell Park Memorial Institute
Buffalo, New York

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

International Symposium on the Expanding Role of Folates and Fluoropyrimidines in Cancer Chemotherapy (1988: Buffalo, N.Y.)

The expanding role of folates and fluoropyrimidines in cancer chemotherapy / edited by Youcef Rustum and John J. McGuire.

p. cm. — (Advances in experimental medicine and biology; v. 244)

"Proceedings of an International Symposium on the Expanding Role of Folates and Fluoropyrimidines in Cancer Chemotherapy, held April 28–29, 1988, in Buffalo, New York" — T.p. verso.

Includes bibliographies and index.

ISBN 0-306-43100-9

1. Fluoropyrimidines — Therapeutic use — Testing — Congresses. 2. Folinic acid — Therapeutic use — Testing — Congresses. 3. Fluorouracil — Therapeutic use — Testing — Congresses. 4. Cancer — Chemotherapy — Congresses. I. Rustum, Youcef M. II. McGuire, John J. III. Title. IV. Series.

[DNLM: 1. Fluorouracil — therapeutic use — congresses. 2. Folic Acid — therapeutic use — congresses. 3. Neoplasms — drug therapy — congresses. QZ 267 16175e 1988]

RC271.F55I59 1988

616.99'4061 — dc19

DNLM/DLC

for Library of Congress

88-38105

CIP

Proceedings of an International Symposium on the Expanding Role of Folates and Fluoropyrimidines in Cancer Chemotherapy, held April 28–29, 1988, in Buffalo, New York

A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

Printed in the United States of America

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PREFACE

Although 5-fluorouracil (FUra) is the drug of choice for the treatment of patients with advanced colorectal cancer, this agent has limited effectiveness with a reported response rate of 10-20% and a duration of response of only 6-9 months. The large percentage of treatment failures with this agent has spurred a continuing effort to delineate the mechanism(s) of resistance to FUra and to evaluate approaches that would selectively modulate the therapeutic efficacy of this agent.

The therapeutic efficacy of FUra has been attributed to its selective incorporation into RNA and to its inhibition of thymidylate synthase, leading to potent inhibition of DNA synthesis. Studies of cell lines in vitro and model systems in vivo have demonstrated that although mechanisms of sensitivity and resistance to FUra are multifactorial, in the presence of citrovorum factor (CF, 5-formyltetrahydrofolate) the site of action of FUra becomes predominantly the pronounced and prolonged inhibition of thymidylate synthase. This action is the result of stabilization of the covalent ternary complex between FdUMP, an active metabolite of FUra, 5,10-methylenetetrahydrofolates, and thymidylate synthase. This effect of CF is thus an example of the concept of metabolic modulation.

CF is commercially available as a racemic mixture of diastereoisomers (6R and 6S). The 6R isomer is considered to be biologically inactive; the 6S isomer is the biologically active form that is metabolized intracellularly to form the various folate cofactor pools including 5,10-methylenetetrahydrofolates. Although the extent of metabolism of folates in normal and tumor tissues has not been clearly delineated, it is possible that in some tumor tissues the formation of folylpolyglutamates is a function of both the dose and schedule of CF administration. Thus, it appears that for optimal modulation of FUra activity several factors must be considered simultaneously. These include the dose and schedule of administration of CF, the intracellular concentrations of the various folylpolyglutamate forms, the level of thymidylate synthase, and the degree and duration of inhibition of thymidylate synthase. The latter is also influenced by the absolute and relative intracellular concentrations of FdUMP and the competing metabolite, dUMP.

This symposium had four goals:

1) To discuss the biochemical, pharmacological and molecular determinants of response to FUra in combination with CF (FUra/CF).

2) To identify conditions for optimal modulation of FUra activity.

3) To update and review the response rate and duration of response of patients treated with this combination.

4) To define the future direction for this combination in patients with advanced malignancies.

On day one of this symposium, studies related to the first two goals were discussed. Identification and evaluation of determinants of response to FUra in combination with CF were emphasized. The role of the 6R and 6S diastereomers of CF, and the effects of the schedule and route of administration of CF in FUra modulation were points of focus.

On the second day of this symposium, a review and update of the clinical results with FUra/CF in patients with various malignancies were discussed. Since various doses and schedules of FUra and CF have been employed clinically, it was hoped that knowledge would be gained as to the optimal conditions for FUra modulation. The question of whether CF is selectively modulating the therapeutic efficacy of FUra was addressed by a number of the participants. It was clear that the clinical pattern of host tissue toxicity of FUra had been altered by CF, with mucositis and gastrointestinal toxicities predominating.

The results of these various clinical trials, based on strong rationales derived from in vitro and in vivo laboratory studies, reinforces the need for further laboratory investigations aimed at optimization of conditions and parameters responsible for selective modulation of FUra by CF. It is clearly evident from the results of clinical trials conducted to date that a better understanding of the role of dose, schedule and route of administration of CF in the selective modulation of fluoropyrimidines is required.

On behalf of the organizing committee (Drs. Rustum, Creaven, McGuire, Mihich and Mittelman), we would like to take this opportunity to thank the speakers, discussants and attendees for their valuable contributions to this symposium. The success of this symposium should be credited to the tireless efforts of Ms. Gayle Bersani and Ms. Geri Wagner to whom we are indebted. We would like also to thank Ms. Cheryl Melancon and Ms. Mae Brown for their help in typing manuscripts, transcribing the discussions, and preparing correspondence.

Major support of this symposium was generously provided by Burroughs Wellcome, the Lederle Division of American Cyanamid, Kyowa Hakko Kogyo, Co., and the Food and Drug Administration. Without their generous financial support this 2 day symposium would not have been possible. Additional support was provided by Hoffman-LaRoche, Bristol Myers, Upjohn, and Marine Midland Bank.

This symposium was held to honor Maire T. Hakala, Ph.D. for her outstanding contributions to the field of cancer research. Recently, Maire retired from active scientific duties following 31 years of productive research at Roswell Park Memorial Institute. It is her research on mechanisms of action of fluoropyrimidines alone and in combination with CF that provided the scientific basis for the development of clinical trials at Roswell Park Memorial Institute and elsewhere. We all shall miss her and wish her the best in all future endeavors.

Youcef M. Rustum
Co-Editor

John J. McGuire
Co-Editor

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OVERVIEW: RATIONAL BASIS FOR DEVELOPMENT OF FLUOROPYRIMIDINE/5-FORMYLTETRAHYDROFOLATE COMBINATION CHEMOTHERAPY*

F.M. Huennekens, Y.D. Montejano and K.S. Vitols

Division of Biochemistry
Department of Basic and Clinical Research
Research Institute of Scripps Clinic
La Jolla, California 92037

SUMMARY

Fluorodeoxyuridylate (FdUMP) and thymidylate synthase (TS) are one of the better understood systems of drug-target interaction in cancer chemotherapy. Isolation and characterization of TS (initially from *Lactobacillus casei* and later from a variety of other sources), cloning and sequencing of the gene, determination of the 3-D structure of the enzyme by X-ray diffraction, and elucidation of the structure of both the catalytic intermediate and the enzyme-inhibitor complex have revealed critical parameters of the target at the molecular level. Potentiation of FdUMP binding by 5,10-methylenetetrahydrofolate (CH_2-FH_4), discovered at the enzymatic level, has been exploited to increase the clinical effectiveness of fluoropyrimidines. CH_2-FH_4 can be generated from folate, 5-methyltetrahydrofolate, or 5-formyltetrahydrofolate (citrovorum factor, CF); the latter is the compound of choice for therapeutic regimens. Transformation of CF to CH_2-FH_4 can occur via two pathways: (a) CF —> 5,10-methenyltetrahydrofolate —> CH_2-FH_4; or (b) CF —> tetrahydrofolate —> CH_2-FH_4. The relative importance of these pathways in various cells is not yet clear. The role of CH_2-FH_4 in FdUMP toxicity, and its central position in folate coenzyme-dependent C_1 metabolism, emphasize the need for development of methods to quantitate intracellular levels of this compound.

EMPIRICAL AND RATIONAL APPROACHES TO DRUG DEVELOPMENT: SEPARATE ROADS TO A COMMON OBJECTIVE

Cancer chemotherapy continues to improve, although still too slowly, by optimizing the regimens of existing drugs and by the development of new drugs. The latter is accomplished either by an empirical approach in which large numbers of compounds (naturally-occurring or synthetic) are screened for anti-tumor activity or by a rational approach in which smaller numbers of specific compounds are synthesized with some *a priori* target or strategy in mind. Each approach has its merits; each has

* This is publication 5359-BCR from the Research Institute of Scripps Clinic. Experimental work was supported by Grant CA-39836 (Outstanding Investigator Award) from the National Cancer Institute and Grant CH-31 from the American Cancer Society.

produced useful drugs. Acivicin, for example, was encountered in a survey of fermentation broths, and maytansine was found in the extract of an exotic plant. Tiazofurin and N-(phosphonacetyl)-aspartate (PALA), alternatively, were synthesized for specific purposes, viz., to inhibit IMP dehydrogenase and aspartate transcarbamoylase.

Despite these and other clear accomplishments, there still lurks the belief that "rational" is a complimentary term, while "empirical" is one of opprobrium. More balanced views, fortunately, have placed this matter in proper perspective. Hitchings (1), for example, has commented that "One has heard a great deal about the need for, and the possibility of, a rational chemotherapy. In the minds of some, chemotherapy would be rational only when new agents could be produced on demand, fully formed, like the heroes who arose from the dragon's teeth of Jason. Perhaps this rational chemotherapy will arrive in one glorious stroke of genius. It seems more likely, however, that it will arrive by small increments of progress and for this reason unheralded". And, similarly, Friedkin (2) advised that "Insight alone, although seemingly superior to the empirical approach, is not enough. Our knowledge is too fragmentary. We may be in the position to make a good guess about potential efficacy but we cannot predict toxicity. After we are all through inhibiting our favorite enzymes, we simply cannot be certain that our treated patient will still have a clear mind, good circulation, a steady heart, clear skin, toes that don't tingle, kidney and liver in good shape, and unblemished chromosomes".

Acceptance of fluoropyrimidines as important agents in the chemotherapeutic arsenal has resulted from the work of a number of investigators, travelling on both empirical and rational roads. The use of 5-formyltetrahydrofolate (folinate; citrovorum factor) to potentiate the cytotoxicity of fluoropyrimidines stands as an excellent example of a rational approach based upon an understanding of the target enzyme (thymidylate synthase) and of the pathways for interconversion of folate coenzymes. These subjects are reviewed briefly in the following sections.

FLUOROPYRIMIDINES IN CANCER CHEMOTHERAPY

The chemical synthesis of 5-fluorouracil (FUra)[1] by Heidelberger and colleagues in 1957, and the subsequent demonstration of its cytotoxicity toward several animal tumors (3), stimulated research in many laboratories. The close structural relationship of FUra to uracil, and reversal of its cytotoxicity by thymine, suggested that the drug interfered in some manner with the conversion of uracil to thymine. Subsequent work proceeded in two general directions: (a) identification of the fluoropyrimidine species responsible for the cytotoxicity; and (b) delineation of the target enzyme.

Fluorouracil is metabolized by two different pathways (Fig. 1). Reaction with deoxyribose-1-P yields the deoxynucleoside (FdUrd), which becomes phosphorylated to 5-fluorodeoxyuridylate (FdUMP). Alternatively,

1. Abbreviations: FUra, 5-fluorouracil; FdUrd, fluorodeoxyuridine; FUrd, fluorouridine; FdUMP, fluorodeoxyuridylate; TS, thymidylate synthase; F, folate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; 5-CHO-FH_4 (also CF), 10-CHO-FH_4, 5,10-CH-FH_4 (or CH-FH_4), 5,10-CH_2-FH_4 (or CH_2-FH_4) and 5-CH_3-FH_4, 5-formyl-, 10-formyl-, 5,10-methenyl-, 5,10-methylene-, and 5-methyl-tetrahydrofolate.

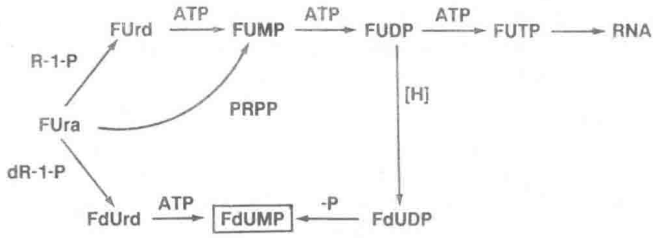

Fig. 1. Metabolism of 5-fluorouracil. For abbreviations, see footnote 1.

reaction of the base with PRPP produces 5-fluorouridine (FUrd) which, after conversion to the nucleoside triphosphate, can be incorporated into RNA. Although interference with RNA function is important, the primary basis for cytotoxicity of FUra is believed to be the inhibition of thymidylate synthase by FdUMP. Discovery of this target/drug combination was made independently by the laboratories of Cohen (4) and Heidelberger (5).

THYMIDYLATE SYNTHASE

Recognition of TS as the enzyme responsible for the conversion of dUMP to dTMP began with the work of Friedkin and Kornberg (6), who demonstrated that, in extracts of *Escherichia coli*, the reaction required serine (as the C_1 source) and FH_4. Further studies by Humphreys and Greenberg (7), using extracts of rat thymus, implicated 5,10-methylene-tetrahydrofolate (CH_2-FH_4) as the co-substrate with dUMP, and the lack of requirement for any additional oxido-reduction components led these investigators to conclude that the tetrahydrofolate moiety supplied the reducing power for the methylene —> methyl conversion. The overall stoichiometry for dTMP synthesis was thus established:

$$CH_2\text{-}FH_4 + dUMP \xrightarrow{TS} FH_2 + dTMP \qquad (1)$$

It was soon recognized, however, that the *continuous* synthesis of dTMP required regeneration of CH_2-FH_4 via a cyclic process (Fig. 2) involving TS, dihydrofolate reductase and serine hydroxymethyltransferase. Since TS

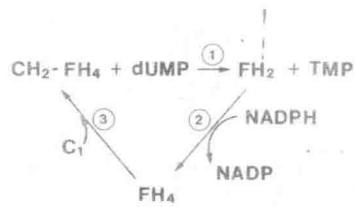

Fig. 2. Thymidylate synthesis cycle. 1, thymidylate synthase; 2, dihydrofolate reductase; 3, serine hydroxymethyltransferase.

was the target for FUra (as FdUMP) and dihydrofolate reductase had been identified previously as the target for Methotrexate (8), this cycle became the focus of attention for investigators in both the antifolate and fluoropyrimidine fields.

Initial studies with TS were conducted with partially purified preparations from different sources (reviewed in (9)). The enzyme was then purified to homogeneity and crystallized from Methotrexate-resistant strains of *L. casei* (10,11). Although it was not known at the time, the elevated levels of TS (up to several hundred-fold), and also of dihydrofolate reductase, were the result of drug-induced gene amplification in these mutants. For a number of years the *L. casei* enzyme served as the prototype TS. Key properties common to various thymidylate synthases (e.g., dimeric structure consisting of two 35 kDa peptides, and the presence of one critical -SH group among the total of four in the dimer) were first encountered in the bacterial enzyme.

In subsequent studies, TS was isolated from other sources (reviewed in (12)) and amino acid and gene sequences are being determined (reviewed in (13,14)). The three-dimensional structure of the *L. casei* enzyme has been determined recently by X-ray diffraction (15). The kinetics and mechanism of the enzyme-catalyzed reaction (eq. 1) and its inhibition by FdUMP have been investigated extensively (reviewed in (12)). Some salient findings are: (a) Polyglutamates of CH_2-FH_4 show significantly lower K_m values than the monoglutamate (16). (b) The dimeric enzyme displays a curious half-of-active-sites behavior with respect to the binding of dUMP and FdUMP. Depending upon the conditions used (e.g., presence or absence of CH_2-FH_4), one or two molecules of these nucleotides are bound to the complex of identical subunits (reviewed in (12)). (c) In both the enzyme-catalyzed reaction (eq. 1) and the aborted reaction in the presence of -FdUMP, the first step involves the critical -SH group (see above), identified as Cys-198 in the *L. casei* enzyme (17), reacting with the 5,6-double bond in dUMP to form an enzyme-substrate complex. This activates C-5, allowing it to react with the 5-iminium cation form of CH_2-FH_4. The resulting intermediate (whose structure was first proposed by Friedkin (18)), has the -CH_2 group bridged between N-5 of tetrahydrofolate and C-5 of the pyrimidine. Ejection of the proton from C-5, and attack by a hydride ion emanating from C-6 of tetrahydrofolate, cleaves the linkage to N-5 to yield dTMP and dihydrofolate. A similar sequence (reviewed in (12) and (19)) occurs when FdUMP is present at the pyrimidine binding site, but the inability to release fluorine from C-5 produces a stable enzyme-substrate-inhibitor complex (Fig. 3).

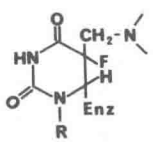

Fig. 3. Complex of TS with substrate (CH_2-FH_4) and inhibitor (FdUMP). -CH_2-N< denotes N-5 of tetrahydrofolate and the bridging methylene group (linked covalently to C-5 of the pyrimidine). Enz denotes enzyme whose -SH group is linked covalently to C-6 of the pyrimidine.

POTENTIATION OF THE BINDING OF 5-FLUORODEOXYURIDYLATE TO THYMIDYLATE SYNTHASE

Studies on the interaction of TS with FdUMP demonstrated that drug binding is enhanced in the presence of CH_2-FH_4 (reviewed in (12)). Although in enzyme catalysis, binding of one substrate often induces conformational changes in the protein that enhance binding of the second substrate, the situation is more complicated with TS. Both the normal and inhibited reaction proceed via an ordered sequence in which the pyrimidine nucleotide interacts first (20), and subsequent binding of CH_2-FH_4 produces a ternary complex in which the substrates are fused covalently and one of them (dUMP or FdUMP) is also linked covalently to the enzyme (see above).

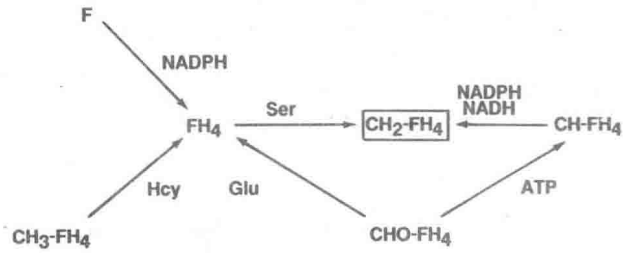

Fig. 4. Pathways for generation of 5,10-methylenetetrahydrofolate.

From these considerations, it seemed possible that the cytotoxicity of fluoropyrimidines could be maximized if high intracellular levels of CH_2-FH_4 were attained. The latter compound, however, is ordinarily present at relatively low concentrations in cells (see below), and co-administration of CH_2-FH_4, even in cell culture experiments, is not feasible because of its lability. Inspection of the network of reactions linking various C_1-FH_4 adducts (Fig. 4) suggests that the desired build-up of the 5,10-methylene derivative can be achieved by several routes: (a) from F, via the NADPH-dependent reduction to FH_4, followed by addition of the CH_2 group from serine; (b) from CH_3-FH_4, via the B_{12}-dependent methionine synthetase, to yield FH_4 (and hence, CH_2-FH_4); and (c) from CHO-FH_4, via two possible pathways (conversion to FH_4 or CH-FH_4). Processing of CH-FH_4 would then involve reduction by the NADPH- or NADH-dependent CH_2-FH_4 dehydrogenase. 5-Formyltetrahydrofolate is the agent of choice, because it is more stable than the methyl derivative and more readily transported into cells than folate. Translating theory into practice, several groups (21-23) reported that incubation of tumor cells with high levels of CHO-FH_4 increased their sensitivity to FUra. Representative results, from the work of Dr. Maire Hakala and her colleagues illustrating the effectiveness of the CHO-FH_4/FUra combination against S-180 and Hep-2 cells, are summarized in Table I. It is thus fitting that the present Symposium, which includes the extension of this therapeutic regimen to cancer patients, honors Maire for her fundamental contributions in this field.

Table I. Potentiation of FUra Toxicity by CF*

Cells	Drug	IC_{50}
		μM
Hep-2	FUra	430
	FUra + CF (300 μM)	170
S-180	FUra	7.2
	FUra + CF (300 μM)	1.7

* Data have been calculated from Chart 2 in reference (23).

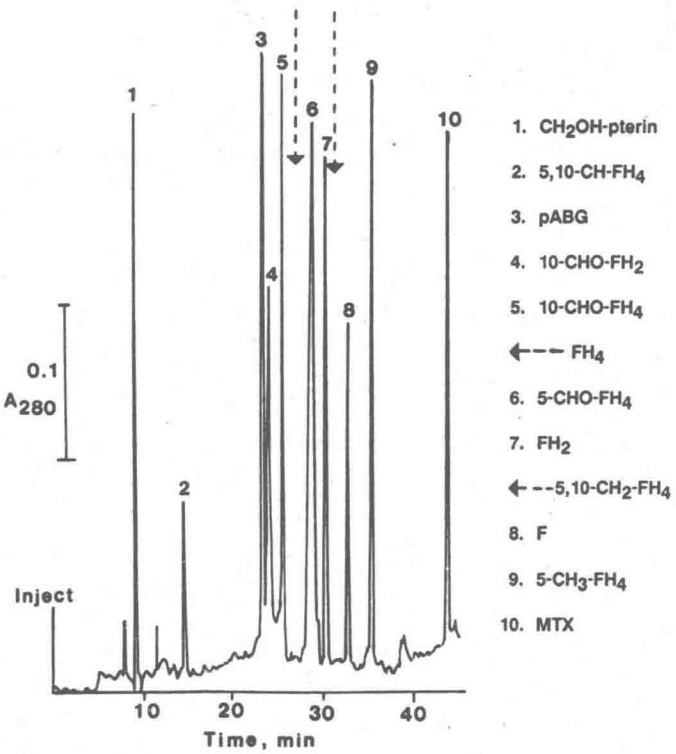

Fig. 5. HPLC of folate compounds. Experimental details as in (28) except that the gradient was changed to: 100% A for 4 min; 40% B for 1 min; linear gradient to 100% B for 60 min; 100% A for 20 min (the latter at 1.5 ml/min). Elution position of CH_2-FH_4 and FH_4 are indicated by arrows.