Advances in Brief

Suicide Prodrugs Activated by Thymidylate Synthase: Rationale for Treatment and Noninvasive Imaging of Tumors with Deoxyuridine Analogues[1]

Jerry M. Collins,[2] Raymond W. Klecker, and Aspandiar G. Katki

Laboratory of Clinical Pharmacology, Food and Drug Administration, Rockville, Maryland 20850

Abstract

Most tumors are resistant to therapy by thymidylate synthase (TS) inhibitors due to their high levels of TS. Instead of inhibiting TS, we hypothesized that it was possible to use this enzyme to activate suicide prodrugs (deoxyuridine analogues) to more toxic species (thymidine analogues). Tumors with high levels of TS could be particularly sensitive to deoxyuridine analogues because they would be more efficient in producing the toxic methylated species. Furthermore, the accumulation of methylated species within tumors could be visualized externally if a tracer dose of the deoxyuridine analogue was tagged with an isotope, preferably a positron emitter, such as ^{18}F. Higher accumulation of isotope indicates higher activity of TS and lower sensitivity of the tumor to TS inhibitors, but perhaps more sensitivity to therapy with deoxyuridine analogues as suicide prodrugs. 2′-F-ara-deoxyuridine (FAU) was used as a prototype to demonstrate these concepts experimentally. FAU readily entered cells and was phosphorylated, methylated, and subsequently incorporated into cellular DNA. Among different cell lines, FAU produced varying degrees of growth inhibition. Greater DNA incorporation (*e.g.*, for CEM and U-937 cells) was reflected as increased toxicity. FAU produced less DNA incorporation in Raji or L1210 cells, and growth rate was minimally decreased. As the first demonstration that cells with high levels of TS activity can be more vulnerable to therapy than cells with low TS activity, this preliminary work suggests a new therapeutic approach for common human tumors that were previously resistant. Furthermore, it appears that the TS activity of tumors could be noninvasively imaged *in situ* by tracer doses of [^{18}F]FAU and that this phenotypic information could guide patient therapy.

Introduction

TS[3] is an essential enzyme for DNA synthesis. For decades, research and clinical studies have been directed toward inhibition of TS to shrink tumors. In some instances, this strategy has been modestly successful, *e.g.*, fluorouracil and floxuridine are used in the treatment of breast, colon, pancreas, stomach, ovarian, and head and neck carcinomas (1). Unfortunately, most tumors are inherently resistant to this strategy, and even those tumors that are initially sensitive develop resistance during the course of treatment (2). Recent applications of molecular probes for TS have demonstrated a consistent relationship between resistance and high expression of TS (3–7).

A new generation of drugs designed to inhibit TS is currently in the final stages of clinical testing (8). Despite the enormous resources that are being expended to improve the effectiveness of first-generation TS inhibitors, neither the existing drugs nor this new set of compounds are effective in tumors that have high levels of TS.

These observations suggest two currently unmet needs. (*a*) Because only a minority of tumors respond to TS inhibitors, a procedure for classifying sensitive and resistant disease would spare the majority of patients needless toxicity. It would be particularly helpful if a noninvasive measure of TS activity *in situ* could be developed. (*b*) A therapy that is specifically targeted toward tumors with high levels of TS could have widespread applicability.

Instead of inhibiting TS, we hypothesized that it was possible to use this enzyme to activate suicide prodrugs to more toxic species for therapeutic purposes. Among pyrimidine nucleosides, dUrd analogues are less toxic than their corresponding dThd analogues (9). Following entry into the cell and phosphorylation, an analogue of dUrd can serve as a suicide prodrug if TS methylates it to generate the corresponding dThd analogue. Thus, tumors that are resistant to TS inhibitors because of high levels of TS could be particularly sensitive to these dUrd analogues because they would be more efficient in producing the toxic dThd species.

Furthermore, if a tracer dose of the dUrd analogue is tagged with an isotope, preferably a positron emitter, accumulation of methylated species within tumors can be visualized externally. We hypothesize that sensitivity or resistance of the tumor, which is related to the activity of TS, can be determined by external imaging, *i.e.*, higher accumulation of isotope indicates higher activity of TS and lower sensitivity of the tumor to TS inhibitors

Received 2/5/99; revised 5/3/99; accepted 5/4/99.
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[1] Presented in part at the 89th Annual Meeting of the American Association for Cancer Research, March 28–April 1, 1998, New Orleans, LA.

[2] To whom requests for reprints should be addressed, at Food and Drug Administration, Room 314, 4 Research Court, Rockville, MD 20850. Phone: (301) 427-1065; Fax: (301) 427-1078; E-mail: collinsj@cder.fda.gov.

[3] The abbreviations used are: TS, thymidylate synthase; dUrd, 2′-deoxyuridine; dThd, thymidine; FAU, 2′-F-ara-deoxyuridine; FAUMP, FAU monophosphate; FMAUMP, 2′-F-ara-5-methyl-dUMP; FMAU, 2′-F-ara-5-methyl-deoxyuridine.

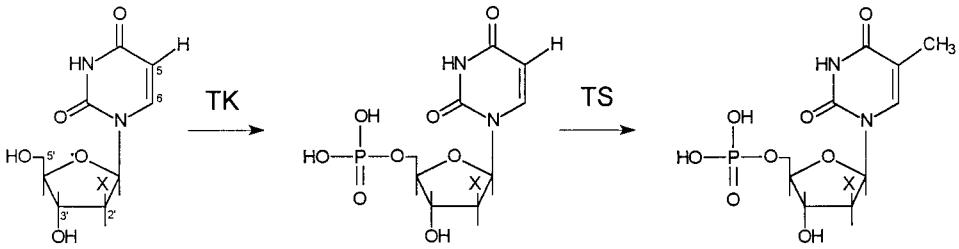

Fig. 1 Generalized structure for dUrd analogues and their intracellular activation pathways. For the endogenous nucleoside, dUrd, X = H. FAU has the substitution X = F. A phosphate group is attached to the sugar at the 5′-position by dThd kinase (*TK*) to form dUMP or its analogue, FAUMP. Subsequently, TS attaches a methyl group at the 5-position of the base to generate thymidylate, dTMP, or its analogue, FMAUMP.

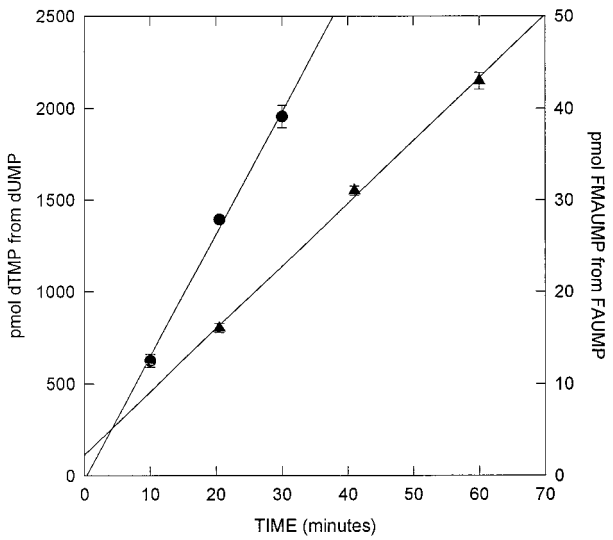

Fig. 2 FAUMP conversion to FMAUMP by TS in U-937 cell extracts, as demonstrated by the accumulation of tritiated water. The rate of conversion to FMAUMP was ~1% of the rate of dTMP formation from dUMP. Similar results were obtained for the other cell lines, with a range of 0.97–1.5%.

but perhaps more sensitivity to therapy with dUrd analogues as suicide prodrugs.

Here, we describe the concept and illustrate its experimental application using FAU as a prototype. FAU is readily transported into cells and converted by intracellular dThd kinase to its monophosphate, FAUMP. TS then methylates this species to form its dThd counterpart, FMAUMP. Cytotoxicity is produced via incorporation into cellular DNA, and external imaging can determine the extent of accumulation.

Fig. 1 shows the structure of dUrd analogues and the activation pathways. The base consists of uracil or various modifications, and the sugar is 2′-deoxyribose or a modification. dThd kinase adds a phosphate group at the 5′-position of the sugar to form dUMP or an analogue. The interaction with TS occurs at the 5-position of the base, where the hydrogen atom is replaced by a methyl group, which TS obtains from methylene tetrahydrofolate. The endogenous substrate for TS, dUMP, is transformed to dTMP. The original class of TS inhibitors, 5-fluorouracil (5-FUra) and 5-fluorodeoxyuridine (5-FdUrd), after intracellular conversion into 5-FdUMP, form a tight complex with TS and block the endogenous conversion of dUMP to dTMP.

Rather than attempting to block the 5-position as with 5-FUra and 5-FdUrd, our strategy was to preserve the hydrogen at the 5-position, encouraging the acceptance of the methyl donation. Thus, for those dUrd analogues that are less toxic than the corresponding dThd analogues, TS can increase cytotoxicity. Because the dThd nucleotide analogues accumulate within the cell, external imaging of tracer doses of radiolabeled dUrd analogues could monitor the activity of TS *in situ*.

In general, suitable dUrd prodrugs may consist of modifications of the base, sugar, or both. For our prototypical compound, FAU, a single atom substitution was made: the hydrogen atom at the 2′-position "above" the plane of the sugar (2′-F-arabino) was replaced by fluorine, *i.e.*, X = F in Fig. 1. For imaging, ^{18}F is the most suitable isotope for "tagging" of FAU.

We previously demonstrated (10) that FAU was phosphorylated intracellularly by U-937 and MOLT-4 cells to FAUMP; converted to its methylated form, FMAUMP; and incorporated into DNA. These prior observations suggested that FAU would be an appropriate prototype for testing our hypotheses regarding the cytotoxic and imaging potential of TS-activated prodrugs. To demonstrate the validity of the concept, we needed to: (*a*) determine that TS is the enzyme which catalyzed the methylation; (*b*) examine the net formation rates of methylated species in a variety of cells; (*c*) correlate the net formation rates of methylated species with TS activity; and (*d*) correlate accumulation of methylated species with cytotoxic effects.

Materials and Methods

Chemicals. FAU, FMAU, FAUMP, dUrd, and dUMP were obtained from Moravek Biochemicals (Brea, CA). The specific activities for [2-^{14}C]FAU, [^{3}H-CH$_3$]FMAU, [5-^{3}H]FAUMP, [6-^{3}H]dUrd, and [5-^{3}H]dUMP were 0.056, 0.33, 11, 11, and 17 Ci/mmol, respectively. All nucleosides and nucleotides used in this work were in the D-configuration. DNase I from bovine pancreas (type II), phosphodiesterase I from *Crotalus atrox* (type VI), formaldehyde, and tetrahydrofolate were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade.

Cells. The human-derived cell lines CEM, MOLT-4, Raji, U-937, and K-562 and the murine-derived L1210 were purchased from the American Type Culture Collection (Manassas, VA).

On the basis of preliminary screening, these lines were

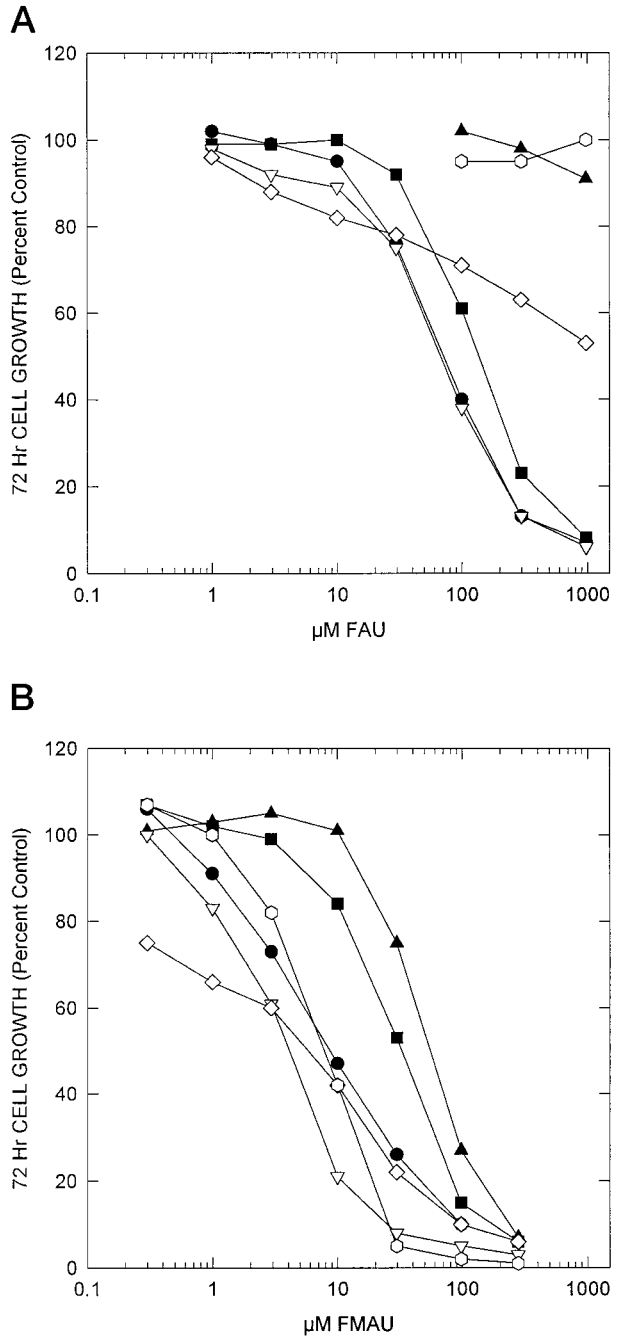

Fig. 3 Effect on cell growth of 72-h continuous exposure to FAU (*A*) or FMAU (*B*). ●, CEM; ■, MOLT-4; ▲, Raji; ▽, U937; ◇, K-562; ○, L1210.

chosen to assure a range of DNA incorporation values. Cells were grown and maintained as a suspension culture in RPMI 1640 containing L-glutamine and 10% (v/v) heat-inactivated FCS (Life Technologies, Inc., Rockville, MD). A solution of penicillin and streptomycin (Sigma) was added to achieve final concentrations of 100 units/ml and 100 μg/ml, respectively.

Methylation of FAUMP by TS in Cell Extracts. When TS adds a methyl group to the 5-position of dUMP to generate dTMP, the hydrogen atom at that location is released. When [5-^{3}H]dUMP is the substrate, TS activity in cell extracts can be assessed by monitoring the accumulation rate of tritiated water. In our adaptation of this procedure, [5-^{3}H]FAUMP was used as the substrate for methylation by TS, and the generation of FMAUMP was determined from the release of tritiated water. Cell extracts were prepared from each cell line by sonication of intact cells (11, 12). The methyl donor was provided by 5,10-methylene tetrahydrofolate, which was generated *in situ* by the addition of formaldehyde to tetrahydrofolate. At various times after the addition of substrate (20 μM either [5-^{3}H]dUMP or [5-^{3}H]FAUMP), the reaction was stopped by addition of HCl. Unreacted substrate was separated from tritiated water by adsorption onto activated charcoal. After centrifugation, an aliquot of the supernatant was counted for tritiated water.

Table 1 Intracellular nucleotides formed and incorporation into DNA from incubation of each cell line with 10 μM FAU for 24 h

Cell line	FAUMP (nmol/10^6 cells)	FMAUTP[a] (nmol/10^6 cells)	DNA (% incorporation)
CEM	0.96 ± 0.24	2.30 ± 0.28	0.81 ± 0.10
U-937	2.70 ± 0.69	1.99 ± 0.08	0.50 ± 0.02
MOLT-4	ND[a]	1.92 ± 0.55	0.19 ± 0.01
K-562	11.1 ± 0.9	ND	0.22 ± 0.002
Raji	0.95 ± 0.17	ND	0.09 ± 0.002
L1210	ND	ND	0.08 ± 0.005

[a] FMAUTP, FMAU triphosphate; ND, not detectable.

Growth Inhibition Studies. All cell lines, except for L1210, were suspended in fresh medium at 30,000 cells/ml. L1210 cells were suspended at 10,000 cells/ml. Cells (2 ml) were added to each of the wells of 24-well plates and incubated with either 0–1000 μM FAU or 0–300 μM FMAU. Incubations were conducted at 37°C in a humidified 5% CO_2 atmosphere for 72 h. Inhibition of cellular growth was assessed by cell counting (Elzone 180; Particle Data, Inc., Elmhurst, IL). Under these conditions, the control doubling times for CEM, MOLT-4, Raji, U-937, and K-562 cells were 21–22 h, whereas the doubling time for L1210 was 8–10 h.

Intracellular Nucleotide Formation and Incorporation into DNA. All cell lines except for L1210 were resuspended in fresh medium at 300,000 cells per ml with the appropriate amount of radioactive drug. L1210 cells were resuspended at 150,000 cells per ml. After 24 h at 37°C in a humidified 5% CO_2 atmosphere, cells were harvested for nucleotide measurement and DNA incorporation. Soluble nucleotides were determined for each cell line following exposure to 10 μM FAU. Incorporation of FAU into DNA (as FMAU) was determined over a range of FAU concentrations from 1 μM to 1 mM. As described previously (10, 12), DNase I and phosphodiesterase I were used to release the nucleosides from DNA. These nucleosides and soluble nucleotides were determined by previously reported high-performance liquid chromatography-based methods (10).

Drug incorporation into cellular DNA was determined using the equation: percentage incorporation = 100 × ([drug]/([dThd] + [drug]).

TS Activity in Intact Cells. Yalowich and Kalman (13) described various methods for determination of TS activity in

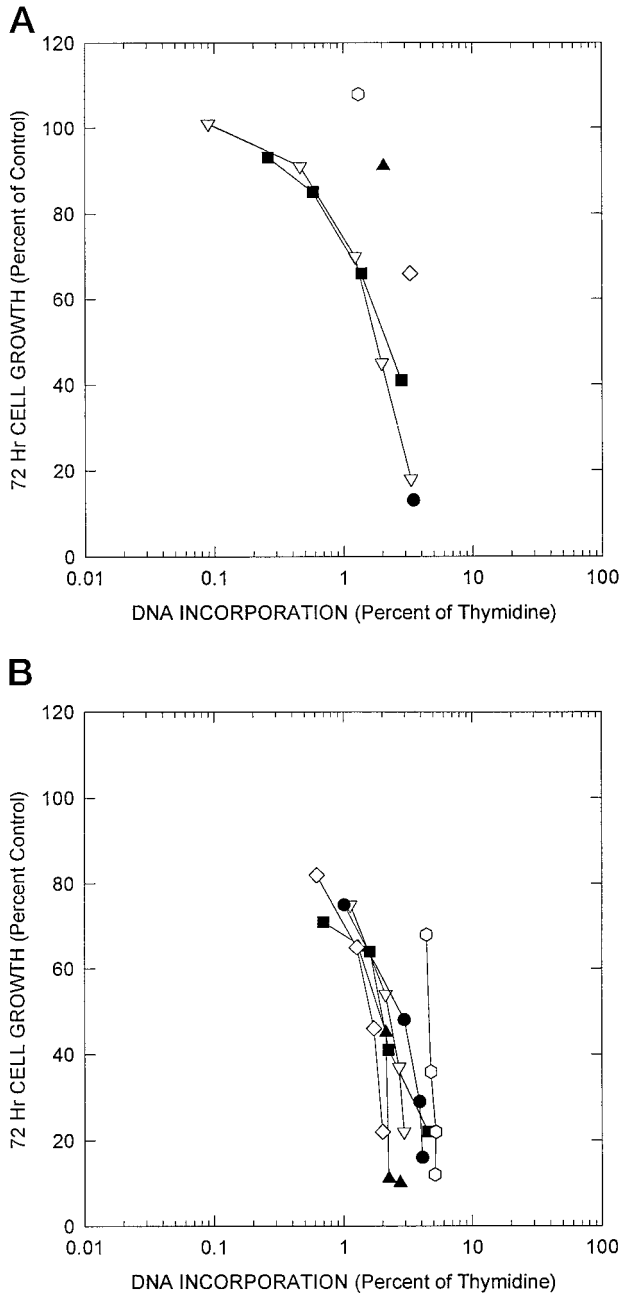

Fig. 4 Association of DNA incorporation with effect on cell growth. The FMAU in DNA was derived from exposure to FAU (*A*) or FMAU (*B*). ●, CEM; ■, MOLT-4; ▲, Raji; ▽, U937; ◇, K-562; ○, L1210.

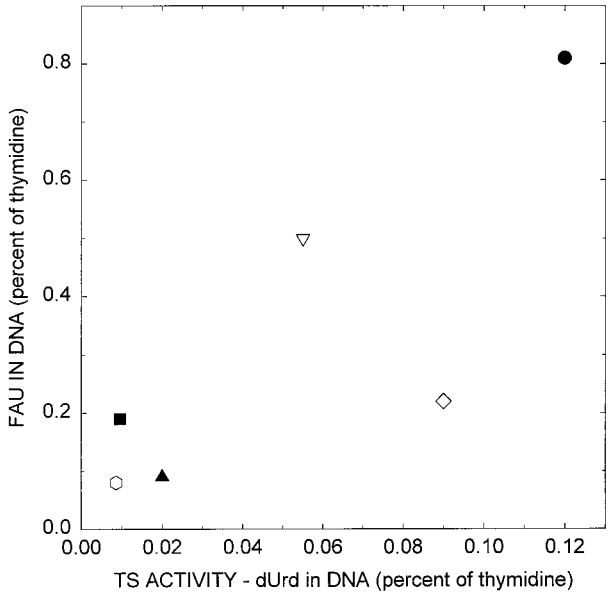

Fig. 5 Comparison of incorporation of FAU into DNA with TS activity in intact cells, as determined by [6-^{3}H]-dUrd incorporation into DNA. ●, CEM; ■, MOLT-4; ▲, Raji; ▽, U937; ◇, K-562; ○, L1210.

intact cells, including measurement of the incorporation of radiolabeled dUrd into DNA. We adapted their method, with the substitution of [6-^{3}H]dUrd for [2-^{14}C-dUrd]. Cells were resuspended in fresh medium at 300,000 cells/ml. [6-^{3}H]dUrd (50 nM) was added, and the cells were incubated for 3 h at 37°C. Cells were harvested, and DNA incorporation of dUrd (in the form of [6-^{3}H]dThd) was determined as described above for DNA incorporation of FAU.

Results

FAUMP was converted to FMAUMP by TS in cell extracts, as demonstrated by the accumulation of tritiated water. The rate of conversion to FMAUMP was ~1% of the rate of dTMP formation from dUMP (Fig. 2).

Continuous incubation of cells for 72 h with the dUrd analogue, FAU, produced varying degrees of growth inhibition (Fig. 3*A*). At 100 μM, CEM and U-937 cells were >50% inhibited and MOLT-4 and K-562 were somewhat less inhibited, but Raji and L1210 cells were completely uninhibited.

In contrast, the corresponding dThd analogue, FMAU, was more potent and consistently toxic. FMAU produced a concentration-dependent inhibition of growth for all cell lines (Fig. 3*B*). At 100 μM, all cell lines studied were almost completely inhibited (>80%). Most strikingly, L1210 cells were very sensitive to FMAU but were not inhibited by FAU, even at 1 mM.

CEM and U-937 were the most efficient cell lines at forming FMAU triphosphate from FAU, and subsequently, FMAU was incorporated to a higher extent into the DNA of these cell lines (Table 1). This greater DNA incorporation was reflected as the increased toxicity noted for CEM and U-937 in Fig. 3*A*. In contrast, FAU produced less incorporation of FMAU into the cellular DNA of the Raji and L1210 cell lines. This was reflected as a <10% decrease in growth rate, even at 1 mM. K-562 cells had a noticeably higher intracellular FAUMP pool. Only trace amounts of FMAUMP or FMAUDP were found.

When cell growth was plotted *versus* percentage incorporation of FMAU in DNA (Fig. 4), on the same scale used for extracellular concentration, the response curves were much steeper. Furthermore, the scatter among cell lines in toxicity referenced to percentage incorporation in DNA showed much less variation than when referenced to extracellular concentration. Full curves were obtained for FMAU in all six cell lines.

For FAU, due to the larger quantities of drug substance that were required, full curves were done in only two cell lines, and single points were obtained for the other four cell lines.

The data from Table 1 for incorporation of FAU into DNA (as FMAU) were plotted *versus* TS activity in intact cells (Fig. 5). TS activity was determined by incorporation of [6-^{3}H]dUrd into DNA, as [6-^{3}H]dThd. Generally, there was a clear association between the amount of FAU in DNA and TS levels, except for K-562 cells.

Discussion

Tumor cells with high levels of TS represent a common therapeutic challenge for which no specific treatment is currently available. Our therapeutic and imaging strategies are completely novel but also highly complementary to all prior approaches toward TS as an antitumor target. The growth of tumor cells with high TS can be preferentially inhibited with a dUrd analogue, and radiolabeled dUrd analogues can permit external imaging of TS activity. Using FAU as a prototype, we have successfully demonstrated these concepts. Its monophosphate, FAUMP, was converted by TS in cell extracts to the corresponding dThd form, FMAUMP. The incorporation of FAU into DNA increased at higher levels of TS. Incubation of FAU with tumor cell lines in culture inhibited their growth to a variable extent, depending upon the efficiency of activation via TS. This work is the first demonstration that cells with high levels of TS activity can be more vulnerable to therapy than cells with low TS activity. Furthermore, it appears that tumors with high TS activity could be noninvasively imaged by tracer doses of [^{18}F]FAU.

Wide variation among cell lines in growth inhibition and also relatively shallow slopes for the response *versus* extracellular concentration curves were observed. As a consequence, extracellular concentration of FAU was a weak predictor of cytotoxicity. In contrast, there was a steep response curve for growth inhibition *versus* incorporation of drug into DNA. Furthermore, there was similarity among cell lines in toxicity at similar values for percentage replacement of dThd in DNA by FMAU. Thus, for equal exposure to the prodrug, selective toxicity could be related to differences in the rate of conversion to dThd analogues by TS. However, although conversion by TS is a necessary condition for toxicity, it is not sufficient. Opportunistic utilization of elevated TS activity also relies upon other steps, especially kinases and polymerases, as well as competition with endogenous synthesis. Both growth inhibition and external imaging ultimately depend upon the net action of all of the pyrimidine pathways.

It is possible that FAU has autonomous biological effects separate from FMAU nucleotides. For example, compared with the other cell lines, K-562 cells have very high levels of intracellular FAUMP and a more shallow toxicity curve. However, there were indications that formation of FMAU by TS was adequate to explain the majority of observed effects. In our experimental design, comparison to the direct use of FMAU gave the opportunity to demonstrate that the toxic effects were dominated by FMAU nucleotides, especially similarity in DNA relationships. Nonetheless, under other experimental conditions, if there are differences among cells in transport, phosphorylation, or related pathways, then these factors can also influence response in addition to TS activity. This may be particularly important for folate-based TS inhibitors (14).

This work suggests a promising avenue for noninvasive biochemical diagnosis and attack of common human tumors that have previously been resistant to therapeutic approaches. Although FAU was used to demonstrate the principle, other synthetic modifications of dUrd could also serve as TS substrates. FAU was not very potent and may not necessarily be the optimal compound in its class. The rate of methylation by TS was rather low, only 1% compared with the endogenous substrate, dUMP. Despite this low rate, substantial amounts of FMAU were incorporated into DNA and toxicity was observed. In contrast, the isomeric form, L-FMAU, is phosphorylated by mammalian cells but is not a substrate for DNA polymerases (15).

Of course, further work is required to assess the practical value of the concept. Testing of other dUrd analogues, and use of a wider variety of tumor types will be helpful. A report that normal host tissues have low levels of TS (16) is encouraging, but demonstration that dUrd analogues are selective will require assessment in normal tissues. Preclinical toxicology studies for FAU are currently underway.

If studies were continued to the clinical stage, the ability to phenotype tumors for TS activity via external imaging would enable a variety of interesting therapeutic strategies. For tumors with high baseline TS activity, it would be attractive to spare patients needless toxicity from TS inhibitors. The availability of a drug, such as FAU, that would specifically target tumors with high TS would directly fill a large gap in current treatment strategies. In addition, FAU could also provide an alternative for patients whose tumors initially respond to treatment with fluorouracil (or another TS inhibitor), but whose eventual tumor progression is associated with increased levels of TS expression (2). Because high TS could generate collateral sensitivity to FAU, therapy could then be switched to FAU (or similar compound) to take advantage of higher activating capacity. External imaging could guide the development and implementation of these and other maneuvers.

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Clin Cancer Res 1999;5:1976-1981.