Metabolic Activation of R,S-1-(Tetrahydro-2-furanyl)-5-fluorouracil (Ftorafur) to 5-Fluorouracil by Soluble Enzymes

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ABSTRACT

There are two major R,S-1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) activation pathways to 5-fluorouracil, one that is mediated by microsomal cytochrome P-450 oxidation at C-5' of the tetrahydrofuran moiety and one that is mediated by soluble enzymes. This report demonstrates that the soluble enzyme pathway proceeds via enzymatic cleavage (possibly hydrolytic) of the N-1—C-2' bond to yield 5-fluorouracil and 4-hydroxybutanal, which is immediately further metabolized to γ-butyrolactone or γ-hydroxybutyric acid. The soluble activation pathway was present in liver, small intestine, and brain. Because of the limited distribution of cytochrome P-450 in body tissues and because of the lack of redistribution of 5-fluorouracil via the systemic circulation after ftorafur administration, we propose that the soluble enzyme pathway is at least in part responsible for organ toxicity and possibly antitumor effect. Distinction of the microsomal (C-5') and the soluble enzyme (C-2') activation pathways can be exploited in the design of more selective prodrug analogues.

INTRODUCTION

The pyrimidine antimetabolite ftorafur has shown activity against several adenocarcinomas with less myelotoxicity but more central nervous system toxicity than those of 5-fluorouracil (29). Ftorafur is considered a prodrug of 5-fluorouracil, and it is slowly metabolized to 5-fluorouracil by several metabolic pathways (3, 4, 10).

Recently, we identified 2 major in vitro pathways of ftorafur activation to 5-fluorouracil that do not involve the known ftorafur metabolites (that retain the tetrahydrofuran moiety), one occurring in the 100,000 g supernatant fraction (soluble enzymes) of liver homogenate and one in the 100,000 g microsomal pellet (3, 10). The microsomal pathway involving cytochrome P-450 produces succinaldehyde and 5-fluorouracil via oxidation at the C-5' position of ftorafur (10). In contrast, GBL or its ring open homolog GHB was isolated as a major metabolite in the supernatant soluble enzyme fractions. Chart 1 shows the potential activation pathways that can occur at either position C-2' or C-5' of ftorafur with GBL or GHB as the possible common end product.

Precise knowledge of the activation mechanisms of ftorafur may lead to the development of more selective 5-fluorouracil prodrugs. In this report, we provide evidence that the soluble enzyme pathway of ftorafur activation proceeds via cleavage of the N-1—C-2' bond of ftorafur to yield 4-hydroxybutanal as the intermediate which is further metabolized to GBL or GHB.

MATERIALS AND METHODS

All chemicals and reagents were of spectroquality or analytical grade. Ftorafur was supplied by the Chemical and Drug Procurement Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. NADPH and NADH were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Alcohol dehydrogenase from horse liver (specific activity, about 5.7 units/mg) was purchased from Boehringer Mannheim, Indianapolis, Ind. Because of their chemical instabilities, 4-hydroxybutanals and succinaldehyde had to be synthesized immediately preceding their use. 4-Hydroxybutanals was generated from the precursor 2,3-dihydrofuran (Aldrich Chemical Co., Milwaukee, Wis.) under acidic conditions (21). Succinaldehyde was generated from 2,5-dimethoxytetrahydrofuran (Aldrich Chemical Co.) under acidic conditions (17).

Tissue Homogenate Preparations and In Vitro Incubations. Male Dutch rabbits were stunned and decapitated. The liver, brain, and small intestine were excised and rinsed in ice-cold isotonic (1.15%) KCl. Each organ tissue was homogenized in ice-cold 0.01 M potassium phosphate buffer (pH 7.4) containing 15% KCl (1:2, w/v) using a Potter-Elvehjem Teflon-pestle homogenizer. The homogenates were centrifuged at 10,000 g for 20 min at 0–4° to yield the 100,000 g supernatant fraction. The supernatant fractions were recentrifuged at 100,000 g for 1 hr at 0–4°. The 100,000 g supernatant fraction may be used immediately or frozen at −20° until use.

Several experiments were carried out with dialyzed 100,000 g liver homogenates. The dialysis tubes containing the homogenates were incubated in 200 volumes of 100 mM Tris buffer, pH 7.4, for 5 hr at 4°. A high concentration of Tris buffer was chosen to accelerate solute exchange; moreover, the buffer was replaced every hr.

All in vitro incubations were carried out at pH 7.4 at 37° for 1 hr. Final concentration of all preparations was standardized to 1 g tissue (wet weight) per 2 ml incubation mixture. Chemical reagents and cofactors were added at the concentrations indicated. The concentrations of NADPH and NADH were 1 mM each. When incubations were performed for more than 1 hr, additional amounts of NADPH and NADH were added every hr (yielding concentration increments of 1 mM each) in order to prevent cofactor depletion. Higher concentrations of NADPH and NADH failed to increase the rate of product formation. The rate of GBL or GHB formation from ftorafur was linear over at least 4 hr.

GC Assay for GBL or GHB, 4-Hydroxybutanal, and 4-Butanediol. GBL or GHB, 4-hydroxybutanal, and 1,4-butanediol were measured by the previously published GC assay of GBL or GHB (3) with the following modifications. The column, injector, and detector temperatures were 145, 190, and 210°, respectively. GC retention times for GBL, 4-hydroxybutanal, and 1,4-butanediol were 3.2, 1.5, and 9.5 min, respectively. There was no detectable conversion of 4-hydroxybutanal and succinaldehyde to GBL or GHB under the assay conditions. Sensitivity for GBL and GHB formation from ftorafur was linear over the range of 0.5 to 200 μM/ml. There is no measurable decomposition of ftorafur to GBL at concentrations up to 200 μg/ml during the assay procedures.

High-Pressure Liquid Chromatographic Assay of 5-Fluorouracil. 5-Fluorouracil was measured in the in vitro incubates with a modified high-
pressure liquid chromatographic assay (30) using preparativeparative separation of 5-fluorouracil from the endogenous interferences present in the tissue homogenates. To 1 ml of the in vivo incubation of flurafur (2 mw) with the soluble enzyme of the 100,000 g supernatant, 0.1 ml of 0.5 M NaH₂PO₄ and 5 μg of 5-

 bromouracil:50 μl methanol as internal standard were added. Samples were then extracted with 2 × 10 ml ethyl acetate, and the ethyl acetate layers were evaporated to dryness under nitrogen gas at 60°. The residue was dissolved in a small volume (100 μl) of methanol and subjected to column chromatography (14 × 0.9 cm, inside diameter; silica gel, 100 to 200 mesh; Bio-Rad, Richmond, Calif.), eluting with 10 ml acetonitrile:chloroform:water (40:4:1) (20). The appropriate eluate fraction was evaporated to dryness under nitrogen gas. The residue was dissolved in 100 μl of methanol, and 25 to 50 μl were injected into the liquid chromatograph under the following conditions: C₁₈-HydrophilicBondapak (30 cm x 4 mm, inside diameter; Waters Associates, Milford, Mass.); mobile phase, 2 mM sodium acetate buffer (pH 4.2):acetonitrile (59:1, v/v); flow rate, 2.0 ml/min; detector, UV, 280 nm. The retention times of 5-

fluorouracil, 5-bromouracil, and flurafur were 2.5, 5.5, and 23 min, respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approximately 50%. 5-Fluorouracil concentrations were respectively. Overall analytical recovery of 5-fluorouracil from the homogenate was approxima...
Ftorafur Activation by Soluble Enzymes

Charts. Conversion of 4-hydroxybutanal to GBL or GHB by the 100,000 g supernatant of rabbit liver homogenates under different incubation conditions. The incubation time was 1 hr. Hydrazine was tested in the absence of reducing cofactors, since they are not required but indeed inhibit the conversion of 4-hydroxybutanal to GBL or GHB. Reagent concentrations were 1 mM NADPH, 2 mM NADH, 2 mM hydrazine, and horse liver alcohol dehydrogenase (ADH) (4 mg/ml).

Metabolic Conversion of Ftorafur to GBL or GHB and 5-Fluorouracil. Chart 4 shows the conversion of ftorafur to GBL or GHB (percentage of control) under different 100,000 g liver homogenate incubation conditions. Potential inhibitors selected for this experiment were those active as inhibitors of either 4-hydroxybutanal or succinaldehyde conversion to GBL or GHB. Ftorafur is converted to GBL or GHB in highest yield without cofactor addition. Hydrazine did not reduce GBL or GHB yield, while addition of NADPH and, more strongly, NADH (± horse liver alcohol dehydrogenase) inhibited GBL:GHB formation from ftorafur to a similar extent as that from 4-hydroxybutanal. Incubation of ftorafur with phosphate buffer (pH 7.4) at 37° for 1 hr resulted in no detectable 4-hydroxybutanal or GBL or GHB. In addition, preheating of the liver homogenates (100° for 30 min) eliminated the formation of GBL:GHB from ftorafur, which suggests that the reaction is enzymatic.

In order to address the question of whether or not thymidine or uridine phosphorylase is responsible for metabolic activation of ftorafur to 5-fluorouracil, we carried out in vitro incubations with 10 mM thymidine and with 2'-deoxy-5-trifluoromethyluridine at a concentration (1.6 mM) that was shown to inhibit nucleoside phosphorylase (7, 14). Under these conditions, ftorafur conversion to GBL or GHB was affected only to a small extent (Table 1). Furthermore, the enzyme preparation was dialyzed as described ("Materials and Methods") in order to remove phosphate which is required for the phosphorylation reaction. Subsequent incubation of ftorafur with the dialyzed enzyme preparation with or without phosphate buffer (10 mM) yielded similar amounts of GBL or GHB. However, dialysis resulted in partial loss (~70%) of enzymatic activity, which prevents any firm conclusions that may have been derived from this experiment.

Activation of Ftorafur by Different Tissue Homogenates. In vitro experiments were carried out using various organ tissue homogenates. Table 2 represents the results of in vitro incubations of ftorafur with rabbit liver, brain, and small intestine 100,000 g supernatant enzyme fractions. The yield of 5-fluorouracil from ftorafur is lower than that of GBL or GHB. Therefore, the rate of disappearance of 5-fluorouracil (77 μM) was measured in 100,000 g liver supernatants (without the addition of any cofactor). Within the first 20 min of incubation, 5-fluorouracil was rapidly metabolized by more than 80%, which was possibly a result of the presence of residual endogenous cofactor (e.g., NADPH, ribose 1-phosphate, 5-phosphoribosyl 1-pyrophosphate). After this initial rapid phase, 5-fluorouracil metabolism was much slower (t½ ~ 150 min). Although exact quantitative calculations of the expected 5-fluorouracil yield from ftorafur were not readily possible under these conditions, the data are roughly compatible with the hypothesis that 5-fluorouracil and GBL or GHB were generated in stoichiometric amounts. Liver homogenates showed highest levels of ftorafur activation, followed by small intestines and brain. Although 5-fluorouracil concentrations were rather small in the brain homogenates, they are clearly above the nonenzymatic background (<0.08% conversion). In order to estimate the Michaelis-Menten constant (Km) for the overall reaction of ftorafur to GBL or GHB, we carried out...
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**DISCUSSION**

The formation of GBL or GHB and 5-fluorouracil from ftorafur in the soluble enzyme preparation of 100,000 g supernatant fractions may be mediated by either of the 3 pathways described in Chart 1. These include oxidation at C-2’ and C-5’ positions which leads to chemically labile intermediates that spontaneously cleave to 5-fluorouracil and to GBL or GHB (C-2’ oxidation) or to succinaldehyde (C-5’ oxidation). Another potential pathway of ftorafur activation involves C-2’ hydrolytic cleavage which produces 5-fluorouracil and 4-hydroxybutanal. Furthermore, succinaldehyde and 4-hydroxybutanal can undergo enzymatic conversion to GBL or GHB. In fact, GBL or GHB was isolated as a major metabolite of ftorafur in the soluble enzyme fraction of 100,000 g supernatants (5).

Since GBL or GHB is a major product of several possible metabolic pathways of ftorafur activation, it is necessary to first study the metabolic activation of the postulated intermediates, 4-hydroxybutanal and succinaldehyde. The specific inhibition of their conversion to GBL or GHB can then be applied to test GBL or GHB formation pathways from ftorafur.

The formation of GBL or GHB from succinaldehyde in the soluble enzyme preparation could be achieved by two mechanisms: (a) reversible reduction to 4-hydroxybutanal followed by oxidation; or (b) oxidation to succinic semialdehyde followed by reversible reduction to GBL or GHB. It has been previously reported that succinic semialdehyde (an intermediate metabolite in γ-aminobutyric acid metabolism) is reversibly metabolized to GHB by soluble enzyme fractions of brain and other tissue homogenates (11). The conversion of succinaldehyde to GBL or GHB requires NADPH as a cofactor, and this conversion was strongly inhibited by hydrazine (an aldehyde-trapping reagent which can undergo bifunctional condensation with succinaldehyde to form a heterocyclic ring structure) (Chart 2). Kaufman and Nelson (18) had previously used hydrazine to trap succinic semialdehyde produced from oxidation of GHB in vitro. In contrast, hydrazine had very little effect on the conversion of both 4-hydroxybutanal and ftorafur to GBL or GHB (Charts 3 and 4). This result rules out succinaldehyde as an intermediate of the conversion of ftorafur to GBL or GHB and thus rules out C-5’ oxidation by the soluble enzymes as the major activation pathway.

There are also several pathways of 4-hydroxybutanal metabolism by the soluble enzymes of 100,000 g supernatants (Chart 1). 4-Hydroxybutanal could be directly oxidized to GBL or GHB, reversibly oxidized to succinaldehyde or reversibly reduced to 1,4-butanediol. Each of these reactions could involve a different enzymatic mechanism, requiring different cofactors. The metabolic conversion of 1,4-butanediol to GBL or GHB as the major product has been demonstrated previously (12, 24). We have found that the conversion of 4-hydroxybutanal to GBL or GHB in 100,000 g homogenates is extremely rapid. Therefore, it was not possible to directly demonstrate the presence of 4-hydroxybutanal in ftorafur incubations. Conversion of 4-hydroxybutanal to GBL or GHB did not require any external cofactor but it was slightly reduced by NADPH and strongly inhibited (80%) by NADH plus added horse liver alcohol dehydrogenase. Similarly, NADPH and, more strongly, NADH (± horse liver alcohol dehydrogenase) inhibited GBL or GHB formation from ftorafur to a similar extent as that from 4-hydroxybutanal. These results provide strong evidence in support of the hypothesis that 4-hydroxybutanal is an essential intermediate in the conversion of ftorafur to GBL or GHB. Moreover, the lack of any cofactor requirements argues against an oxidation of ftorafur at C-2’ to give 5-fluorouracil and GBL or GHB directly.

It has been previously reported that horse liver thymidine phosphorylase and rat liver uridine phosphorylase were inactive against ftorafur (5, 30). Recently, Kono et al. (19) suggested that the cleavage of ftorafur to 5-fluorouracil was catalyzed by a thymidine phosphorylase activity, which was enhanced in human tumor tissues. Moreover, ftorafur activation was suppressed in the presence of a 7-fold excess amount of thymidine (19). Lack of a rigorous quantitative analysis of the data makes it difficult to assess the significance of their results. In contrast, in our experiments, addition of thymidine had little effect on the yield of GBL or GHB, and the presence of the nucleoside phosphorylase inhibitor (2’-deoxy-5-trifluoromethyluridine) did not inhibit the metabolic activation of ftorafur to GBL or GHB. These results argue against the hypothesis that activation occurs via phosphorylosis of the N-1—C-2’ bond of ftorafur, yielding 5-fluorouracil and 2-hydroxytetrahydrofuran-2-phosphate, which is then converted to 4-hydroxybutanal by phosphatases. Rather, we propose that a hydrolytic enzyme mechanism is responsible for activation of ftorafur in the 100,000 g supernatants.

Although ftorafur is thought to act predominantly via 5-fluoro-
uracil formation, plasma concentrations of 5-fluorouracil generated in vivo were shown to be negligible (5); this finding is consistent with the hypothesis that intracellularly formed 5-fluorouracil is further metabolized without subsequent redistribution via the systemic circulation (2, 5). Therefore, the mechanism of metabolic activation of fluorafur to 5-fluorouracil might be a determinant of its selective tissue toxicity towards the gastrointestinal tract and the central nervous system as well as of its antitumor effects. We demonstrate here that these tissues are capable of enzymatically activating fluorafur to 5-fluorouracil by soluble enzyme catalysis. Although the extent of in vitro activation is rather small, it must be considered that fluorafur is present in rather large concentrations after therapeutic doses. Moreover, because of the long elimination half-life (6 to 17 hr) of fluorafur (5, 6, 16), tissue exposure to 5-fluorouracil as a result of the soluble fluorafur activation pathway could be sufficient to account for the observed organ toxicities of the drug. On the other hand, we cannot rule out the possibility that metabolites other than 5-fluorouracil contribute to organ toxicity (1, 13). For example, GHB occurs physiologically in brain and cerebrospinal fluids (9, 23, 25, 28) and has been used as an anesthetic adjuvant (15). Its effects on the central nervous system lead to behavioral depression and an induction of an epileptic-like stupor (lethargy) (6, 27). However, the plasma concentrations of GBL or GHB required for such effects are far above those generated from fluorafur.

In conclusion, this report demonstrates that fluorafur is activated to 5-fluorouracil by the soluble enzymes of target tissues via cleavage of the N-1-C-2' bond (Chart 5). The intermediate product, 4-hydroxybutanal, is rapidly converted to GBL or GHB which has been observed previously as a major fluorafur metabolite in circulating plasma. The other major metabolic activation pathway, i.e., fluorafur oxidation at C-5' by a cytochrome P-450-mediated mechanism (10), leads to 5-fluorouracil and succinaldehyde (Chart 5), the latter being also converted, at least partially, to GBL or GHB. Since cytochrome P-450 is mainly localized in the liver, with lower levels in the gastrointestinal tract and much lower levels in the brain, and since 5-fluorouracil is not redistributed via the circulation (2, 5), it is possible that the soluble enzyme pathway is responsible for part of the organ toxicity as well as for the antitumor effects of fluorafur. Other previously reported activation pathways are unlikely to contribute significantly to the fluorafur effects. Further development of prodrugs related to fluorafur should therefore be directed towards separating the microsomal (C-5') and soluble enzyme (C-2') activation pathways of the tetrahydrofuran ring moiety. Moreover, assay of the soluble activation pathway of fluorafur may serve as a predictor of the antitumor efficacy of the drug.

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Chart 5. The 2 major activation pathways of fluorafur (FT) to 5-fluorouracil (Fura). Note that the 2 pathways attack at different sites of the fluorafur moiety. While only a fraction of the generated succinaldehyde (SA) is converted to GBL or GHB, 4-hydroxybutanal (4-HB) quantitatively yields GBL or GHB.
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