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Metabolic activation of ftorafur [*R*,*S*-1-(tetrahydro-2-furanyl)-5-fluorouracil]: the microsomal oxidative pathway

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The pyrimidine antimetabolite ftorafur [*R*, *S*-1-(tetrahydro-2-furanyl)-5-fluorouracil, FT] is considered a prodrug of 5-fluorouracil (FUra), and it is slowly metabolized to FUra by several metabolic pathways [1]. FT and FUra have similar antitumor activities, but different tissue toxicities [2, 3]. Although FT is thought to act predominantly via FUra formation, plasma concentrations of FUra generated *in vivo* were shown to be negligible; this finding is consistent with the hypothesis that intracellularly formed FUra is further metabolized without subsequent redistribution via the systemic circulation [4, 5]. Therefore, the mechanism of metabolic activation of FT to FUra might be a determinant of its selective tissue toxicity towards the GI tract and the CNS. Several hydroxylated and one dehydrogenated metabolite of FT have been isolated, some of which may serve as metabolic intermediates in the formation of FUra [6, 7]. However, pharmacokinetic studies suggest that these metabolites account for a small fraction of the *in vivo* disposition of FT [5].

In a recent report, we identified two major *in vitro* pathways of FT activation to FUra that do not involve the known FT metabolites, one occurring in the 100,000 *g* microsomal pellet and one in the 100,000 *g* supernatant soluble enzymes of liver homogenates [1]. Involvement of the microsomal cytochrome P-450 enzyme system with the activation of FT was reported earlier [8], while the role of the supernatant activity was not noticed previously. γ-Butyrolactone (GBL) or its ring open congener, γ-hydroxybutyric acid (GHB), was isolated as a major metabolite in the supernatant soluble enzyme fractions, but not in the 100,000 *g* microsomal pellet incubation [1]. Figure 1 shows the potential activation pathways that can occur at either position C-2′ or C-5′ of FT. However, possible interconversion among the metabolic products of the tetrahydrofuran ring, e.g. of 4-hydroxybutanal and succinaldehyde to GBL/GHB, must be considered in establishing the mechanism of FT activation via these three pathways. We report here the identification of succinaldehyde as a metabolite of FT following *in vitro* incubations with microsomal hepatic enzymes and its subsequent conversion to GBL/GHB by soluble enzymes.

Materials and methods

Chemicals and reagents. All chemicals and reagents were of spectro-quality or analytical grade. FT was supplied by the Chemical and Drug Procurement Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. NADPH was purchased from the Sigma Chemical Co. (St. Louis, MO). SKF 525-A was received from the Smith, Kline & French Laboratories, Philadelphia, PA. Succinaldehyde was generated from 2,5-dimethoxytetrahydrofuran (Aldrich Chemical Co., Milwaukee, WI) under acidic conditions; 4-hydroxybutanal was generated from 2,3-dihydrofuran (Aldrich Chemical Co.) under acidic conditions [9].

Apparatus. Mass spectra were obtained on a Kratos MS double-focusing magnetic sector. The electron impact mass spectrometric (EIMS) analyses were carried out using a direct insertion probe, ionization energy of 70 eV, and an ion source temperature of 220°.

Liver preparations. Male Dutch rabbits were stunned and decapitated. The liver was excised and rinsed in ice-cold isotonic (1.15%) KCl. The liver tissue (12.5 g) was homogenized in 25 ml of ice-cold 0.01 M phosphate buffer (pH 7.4) using a Potter–Elvehjem Teflon pestle homogenizer. The homogenates were centrifuged at 10,000 *g* for 20 min at 0–4° to yield the 10,000 *g* supernatant fraction. The supernatant fractions were recentrifuged at 100,000 *g* for 1 hr at 0–4° to obtain the 100,000 *g* supernatant fraction. The sediment (microsomal pellet) was resuspended in buffer and used as the enzyme source immediately.

In vitro *incubations.* FT (5 mM) was incubated with the microsomal enzymes in the presence of 0.015 mM $MgSO_4$, 1 mM NADPH (final concentration of all preparations was standardized to 1 g wet weight of tissue per 4 ml incubation mixture) at 37° for 15 min in a metabolic shaker. At the

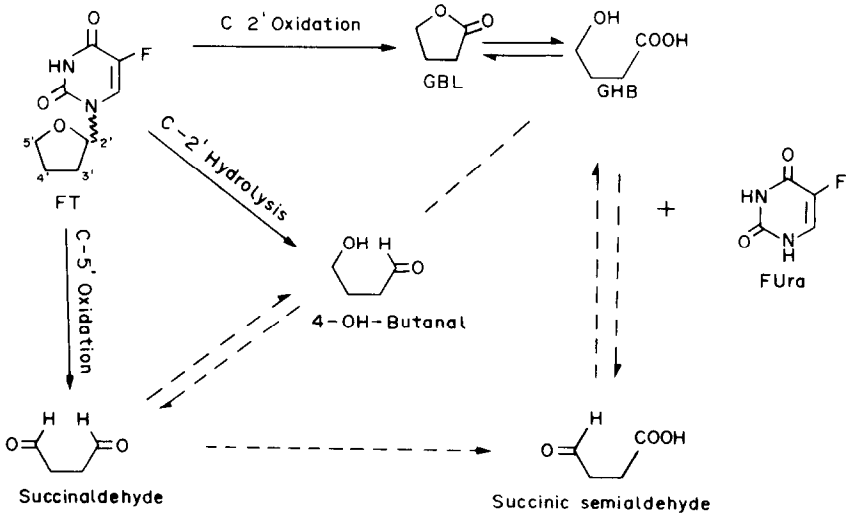

Fig. 1. Three possible activation pathways of FT that lead to FUra and the expected products of the tetrahydrofuran moiety. Potential interconversion among these products is also indicated.

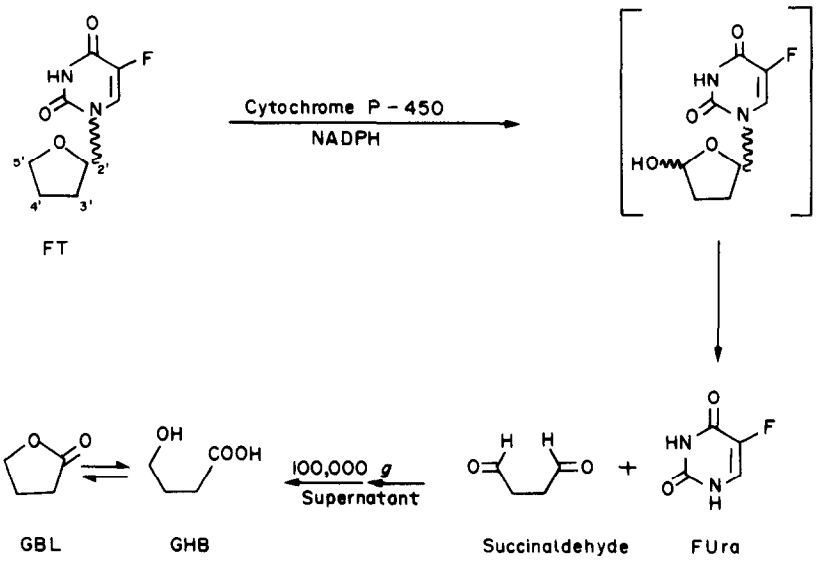

Fig. 2. Proposed activation pathway of FT to FUra by the microsomal enzymes and the major metabolic fate of succinaldehyde.

end of the incubation period, the incubates were extracted twice with 2 ml $CHCl_3$; the organic extracts were dried over anhydrous Na_2SO_4 and were reduced in volume to ~100 μl in a reaction vial under a stream of nitrogen. To derive aldehydic metabolic products, 100 μl of a pyridine solution (10%) of *p*-nitrobenzyloxyamine (PNBA) hydrochloride (Regis Chemical Co., Morton Grove, IL) was added, and the reaction mixture was heated at 70° for 30 min in the presence of a molecular sieve. The solvent was evaporated at 40° under a stream of nitrogen. The residue was dissolved in 2–3 ml of dichloromethane and washed with several portions of 0.1 N HCl and then water [10]. The dichloromethane layer was dried over anhydrous Na_2SO_4 and the derivatized products were identified using TLC (Silica gel 60 F-254, E. Merck, Darmstadt, West Germany) with $CHCl_3$:CH_3OH (9:1) as the developing system. The R_f value of the derivatized metabolic product was compared with that of authentic succinaldehyde carried through the same derivatization procedure and by mass spectral analysis. GBL/GHB was analyzed in the homogenates by the GC method published earlier [1].

Results and discussions

The formation of FUra from FT in the microsomal pellet is thought to be mediated by cytochrome P-450 requiring NADPH and O_2 [11–13]. The hypothetical product of FT oxidation at the C-2′ position, namely GBL/GHB, was not found in the incubates of the microsomal enzymes. Inability to detect GBL/GHB could have resulted from its rapid further metabolism. Therefore, GBL was incubated with 100,000 *g* microsomal pellet preparations in the presence of NADPH and O_2 at 37° for 1 hr; however, no loss of GBL occurred. Therefore, we have ruled out microsomal oxidation of FT at the C-2′ position. Furthermore, oxidation at the C-3′ and C-4′ positions of FT yield stable metabolic products [6] and, therefore, cannot account for the observed FUra formation by the microsomal enzymes. The remaining potential molecular site of microsomal oxidation is the C-5′ position of FT; the resulting 5′-hydroxyftorafur is expected to be chemically unstable [14] and to cleave spontaneously to FUra and succinaldehyde (Fig. 2). To detect succinaldehyde, a hydroxylamine derivatizing reagent was used in conjunction with TLC analysis. The metabolic derivative obtained from *in vitro* incubations of FT with the microsomal enzymes had the same R_f value (0.76) as authentic succinaldehyde derivatized under the same procedure. Mass spectrometric analysis confirmed the presence of the molecular ion, *m/z* 386, an authentic succinaldehyde dioxime derivative ($C_{18}H_{18}N_4O_6$, mol. wt 386) with an intensity of ~6% relative to the base peak (*m/z* 136). The other tetrahydrofuran ring products (e.g. GBL/GHB, succinic semialdehyde, and 4-hydroxybutanal) do not possess two aldehyde functions, and, therefore, cannot form the bis-oxime derivative of succinaldehyde.

Two possible pathways for the formation of succinaldehyde can be proposed. The first is C-2′ hydrolytic cleavage to give 4-hydroxybutanal (Fig. 1) with subsequent oxidation to succinaldehyde. However, no conversion of authentic 4-hydroxybutanal to succinaldehyde was observable in microsomal incubations, ruling out this pathway. The second is oxidation at the C-5′ position followed by spontaneous cleavage of 5′-hydroxyftorafur, the hemiacetal, to FUra and succinaldehyde (Fig. 2).

It has been suggested previously that hepatic microsomal cytochrome P-450 plays a role in FT activation to FUra [8, 10]. Higher rates of FT decomposition were observed with phenobarbital-induced liver microsomes harvested from mice [8]. We have shown here that SKF 525-A (500 μM) prevented the formation of succinaldehyde in microsomal incubations. In addition, incubation of FT in the absence of NADPH did not yield succinaldehyde. These results, together with the reported binding spectrum of FT with rat cytochrome P-450 [11], provide strong evidence that microsomal cytochrome P-450 is responsible for activation of FT to FUra and succinaldehyde according to the scheme shown in Fig. 2.

In a recent report [1], we have observed higher levels of GBL/GHB in 10,000 *g* supernatant incubations of FT than in 100,000 *g* supernatant (soluble enzymes) incubations. It is therefore possible that succinaldehyde generated from microsomal enzymes in the 10,000 *g* medium is further metabolized to GBL/GHB by soluble enzymes that are also present. To test this hypothesis, succinaldehyde was incubated with 10,000 *g* and 100,000 *g* supernatant fractions. Approximately 50–60% of the added succinaldehyde was converted to GBL/GHB within 1 hr. As depicted in Fig. 1, there are two possible mechanisms of GBL/GHB formation from succinaldehyde; it remains open which of these two is reponsible. These results demonstrate that GBL/GHB, which is detectable in plasma after FT administration to animals and patients, is generated via two separate pathways, i.e. the soluble enzymes pathway of yet

unknown mechanism and the microsomal pathway as shown in Fig. 2. Knowledge of the enzymatic mechanism of both pathways may lead to useful prodrugs with improved selectivity.

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Studies on a mode of resistance to m-AMSA

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Studies on patterns and modes of drug resistance in animal tumors have provided useful information regarding determinants of drug responsiveness. Several factors have been implicated in resistance to a group of natural and synthetic products including anthracyclines, actinomycin D, vinca alkaloids and alkylaminoanthraquinones. Mouse leukemia cell lines selected for resistance to any of these agents tend to be cross-resistant to the others [1–6]. This common mode of drug resistance appears to involve an operational barrier to drug accumulation which may be an energy-dependent active outward transport process [7–13]. The broad cross-resistance pattern could, therefore, reflect the specificity of an active exodus process [8, 10, 11].

Table 1. Drug responsiveness patterns *in vivo**

Drug	Dose (mg/kg)	P388	P388/AMSA
Aclacinomycin	12	88	88
Actinomycin D	0.36	96	116
Adriamycin	4.5	125	37
m-AMSA	12	112	12
Daunorubicin	3	62	27
Ellipticine	60	86	4
Vincristine	1.2	108	96

* Animals received 10^6 cells on day 0 and were treated with specified drug levels on days 1, 5 and 9. Results are described in terms of increased life-span of tumor-bearing animals relative to untreated controls.

In this study, we have examined a subline of the P388 murine leukemia selected [14, 15] for resistance to the synthetic aridine *N*-[4-(9-acridinylamino)-3-methoxyphenyl]-methanesulfonamide (m-AMSA), an antitumor agent [16] which binds to DNA [17] causing protein-associated DNA strand breaks [18]. The cross-resistance pattern in P388/AMSA was unusual, suggesting a new mode of drug resistance.

Derivation of the adriamycin-resistant P388/ADR cell line, together with procedures for measurement of drug-induced promotion of survival of tumor-bearing animals are described in Refs. 1 and 2. The P388/AMSA cell line was obtained by serial intraperitoneal passage of P388 cells in BALB/c × DBA/2 mice treated intraperitoneally with 4 mg/kg m-AMSA on days 4–10. After nine transplant generations, animals received drug on days 1–7; at twenty-four transplant generations, the m-AMSA resistant subline was obtained. Further characterization of this cell line is shown in Table 1. Full details are described in Ref. 15.

Drug resistance patterns were determined by *in vivo* studies involving treatment with anti-tumor agents on days 1, 5 and 9 after intraperitoneal transplant of 10^6 cells. Drug levels employed (Table 1) were non-toxic to control animals and represented the highest tolerated drug levels with this schedule. Drug-promoted response is reported in terms of percent increased life-span (%ILS). If the mean time to death after transplant for untreated animals is 7 days, a