Evidence from Rat Hepatocytes of an Unrecognized Pathway of 5-Fluorouracil Metabolism with the Formation of a Glucuronide Derivative

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ABSTRACT

Isolated rat hepatocytes in suspension were exposed to [3H]-5-fluorouracil for intervals over 2 h, following which the cells were removed from the media and sonicated, and the cytoplasm was sampled. High-performance liquid chromatography was used to separate 5-fluorouracil (FUra) from its known anabolites and catabolites, with subsequent quantitation of these metabolites by measurement of radioactivity. As the extracellular concentration of FUra was increased above 30 μM, the intracellular levels of FUra increased, with detection of a new peak of radioactivity distinct from any of the known anabolites or catabolites. This new metabolite, "G," increased in concentration as the extracellular concentration of FUra was raised above 1 mM. Inhibition of FUra catabolism by 2 mM thymine resulted in a further increase in intracellular FUra (approaching the extracellular concentration) and was accompanied by a further increase in the intracellular concentration of "G," demonstrating that "G" was not formed via the catabolic pathway. The increase in intracellular FUra and "G" was not accompanied by an increase in intracellular anabolites, suggesting that "G" was formed via a novel metabolic pathway. "G" was retained within the hepatocytes, although it was not bound to intracellular macromolecules.

"G" was converted to FUra in the presence of β-D-glucuronidase; this reaction was inhibited with the addition of saccharo-1,4-β-lactone, a specific inhibitor of the β-D-glucuronidase. This data, together with evidence from hepatocyte homogenates in which formation of "G" was shown to be dependent on the concentration of uridine-5'-diphosphoglucuronic acid, demonstrates that "G" is a glucuronide of FUra. The formation of "G" suggests that FUra is metabolized via a previously unrecognized metabolic pathway.

INTRODUCTION

Since the synthesis of FUra25 years ago, most studies of fluoropyrimidine metabolism have concentrated on anabolism, focusing on the conversion of FUra or its nucleosides to ribosyl or deoxyribosyl nucleotides and the subsequent effects on DNA or RNA synthesis or function (2, 12-15, 19, 22, 23, 28). In contrast, until recently, there have been few studies (3, 4, 21) that have examined FUra catabolism, despite the fact that, in humans, approximately 90% of administered FUra is metabolized via the pyrimidine catabolic pathway (2), with the major site of FUra catabolism believed to be in the liver (7).

The development of a highly specific HPLC methodology that can rapidly resolve all of the known anabolites and catabolites of FUra has permitted a reexamination of fluoropyrimidine metabolism (26). We have recently demonstrated the usefulness of the isolated rat hepatocyte model for studying fluoropyrimidine metabolism because of its excellent agreement with the metabolic fate of FUra in humans (6). In a previously reported study (26), hepatocytes in suspension were exposed to 30 μM FUra [a concentration corresponding to that found after systemic administration of FUra (2, 6)]. Intracellular and extracellular FUra and its metabolites were quantitated, demonstrating a transport-limited, rapid, and complete conversion of FUra to FUH2 with FUH2 being the major intracellular catabolite, with subsequent conversion to FUPA and then to FBAL, but with no evidence for FUra anabolism (26).

In order to better understand the metabolism of FUra at the hepatic site with concentrations more representative of FUra levels achieved following various clinical regimens, including i.v. bolus injection (2), studies were undertaken with FUra concentrations higher than 30 μM. In the present study, we demonstrate that, as extracellular FUra is increased, the catabolic enzyme dihydrooracil dehydrogenase (EC 1.3.1.2) becomes saturated, resulting in increased intracellular levels of FUra. However, this is not accompanied by evidence of increased anabolism. Most notable is the appearance of a new metabolite distinct from any of the known anabolites or catabolites of FUra that appears to be a glucuronide of the FUra base formed via a previously unrecognized metabolic pathway.

MATERIALS AND METHODS

Chemicals

[6-3H]FUra (20 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (City of Industry, CA) and purified by the HPLC technique described below. [Carboxyl-14C]Inulin (2.5 Ci/g) was purchased from Amersham-Searle Corp. (Irvine, CA). FUra and authentic standards of FUH2, FUPA, and FBAL were kindly supplied by Hoffmann-La Roche Laboratories (Nutley, NJ and Basel, Switzerland). β-D-Glucuronidase (from bovine liver, type B-3), saccharo-1,4-β-lactone, and thymine were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade.
Preparation of Freshly Isolated Rat Hepatocytes and Incubation Conditions

Hepatocytes in suspension were prepared from male Sprague-Dawley rats (200 to 300 g) by a modification of the method of Berry and Friend (1), which increases cell yield and viability (10). Cell membrane integrity was determined by trypan blue exclusion; only preparations in which 90% of the cells excluded the dye were used. Hepatocytes in suspension (5 x 10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer containing 0.25% gelatin and 10 mM glucose. The pH was maintained at 7.4 by passing warmed and humidified 95% O_2/5% CO_2 over the cell suspension.

Experiments were initiated with the addition of sufficient [3H]FUra (specific activity, 3 to 15 mCi/mM) to achieve final concentrations between 30 μM and 1 mM. Times of incubations in the text represent the length of time between introduction of radiolabel into the cell suspension and starting of the microculture. Portions of the cell suspension (0.5 ml) were layered onto 400 μl of inert silicon oil of density 1.04 (5) in 1.5-ml plastic microfuge tubes. The tubes were immediately centrifuged at 15,000 x g for 15 s to sediment all of the cells. The pellet was then frozen quickly in a dry ice/acetone bath. The tip of the tubes contained radiolabel representing the total intracellular fraction (unchanged FUra and its metabolites). The oil layer contained no radioactivity, and the remaining upper fraction contained the extracellular medium.

Analysis of Intracellular and Extracellular FUra and its Metabolites by HPLC

Portions of the buffer (25 to 50 μl), after separation from cells, were analyzed by HPLC without further processing. The frozen cell pellet (see above) was aspirated into the tip of a Pasteur pipet, extruded into a plastic tube immersed in ice, and subjected to sonic oscillation (3 to 4000 Hz) in 1 ml of 2 mM potassium phosphate buffer (pH 7.4) for 30 s to disrupt the cells and release the intracellular 3H. The sonicate was centrifuged at 25,000 x g at 0°C for 15 min to sediment the debris. Portions of the supernatant (100 to 200 μl) were analyzed by HPLC within 12 h following exposure of the hepatocytes to FUra. Over this interval, FUH_2 and the other catabolites are stable in the presence of cellular protein at pH 7.4 and -20°C (26).

Analyses were performed with a Hewlett-Packard Model 1084 B liquid chromatograph equipped with automatic injector, variable wavelength spectrophotometer, and chromographic terminal (Hewlett-Packard 79850 ALC). The extracts from cells or medium were examined by reversed-phase ion pair HPLC using 2 Brownlee RP 18 columns (25 x 0.46 cm) connected in tandem and packed with 5 μm of Hypersil ODs and 5 μm of Spherisorb ODs, respectively. The mobile phase was 0.005 M of tetrabutylammonium hydrogen sulfate and 0.0015 M potassium phosphate buffer (pH 8) with a flow rate of 1 ml/min. The parent drug and its metabolites were detected and quantitated by subsequent measurement of radioactivity in the postcolumn eluent with comparison of their retention times with standards. The retention times of the unlabeled drug and its metabolites were: FBAL, 6.7 min; FUH_2, 7.8 min; FUPA, 11 min; and FUra, 17 min. FUra anabolites were also eluted with this method, with the nucleosides fluouridine and fluorodeoxyuridine having retention times of 31 and 39 min, respectively. The nucleotides which were formed included: FBAL, 6.7 min; FUH_2, 7.8 min; FUPA, 11 min; and FUra, 17 min. The nucleotides which were formed included: FBAL, 6.7 min; FUH_2, 7.8 min; FUPA, 11 min; and FUra, 17 min.

Isolation and Preliminary Identification of a Novel FUra Metabolite

Initial studies in which hepatocytes in suspension were exposed to extracellular FUra concentrations >30 μM demonstrated a peak of radioactivity (referred to here as "G") on the HPLC radiochromatogram of the intracellular fraction which did not correspond to a retention time of any known catabolite or anabolite derivative. The following methods were used for isolation and preliminary identification of "G".

Isolation. To obtain a sufficient quantity of pure "G" to enable further identification of this unknown, hepatocytes in suspension (10^6 cells) were incubated for 2 h at 37°C with 2 mM [3H]FUra (specific activity, 1.5 mCi/mM). After discarding the extracellular fluid, intracellular 3H was extracted from the cell pellet as described above. Portions of 8 to 10 ml of the resulting supernatant were successively passed through an Amicon filter (stirred ultrafiltration cells Model 8010, equipped with PM 10 filters) and lyophilized. The dry residue was dissolved in 2 to 3 ml of water, and 200-μl portions were chromatographed as described above. Under these conditions, "G" eluted at 28 min. This peak was collected and lyophilized, and the content was pooled. The entire purification procedure was repeated with additional cells and [3H]FUra until approximately 3 x 10^6 dpm of "G" were isolated.

Analysis of Binding to Cellular Macromolecules. After sonication of the cell pellet and centrifugation of the cellular debris, 100-μl portions of the supernatant were layered onto a minicolumn of Sephadex G-25 equilibrated with 5 mM sodium phosphate buffer at pH 7, and the minicolumn was centrifuged at 1000 x g for 3 min. This procedure, described in detail previously (8), permits separation of protein-ligand complexes, which pass through the minicolumn, from free ligand, which is retained completely within the column.

Incubation with β-o-Glucuronidase. "G" (approximately 25,000 dpm) was incubated at 37°C for 1 h with 10,000 units of β-o-glucuronidase in the presence of 0.2 mM sodium acetate, pH 4.5, in a capped minivalve with a final volume of 1.1 ml. Following incubation, 0.9 ml of the sample was transferred to a 15-ml glass centrifuge tube containing 0.1 g of ammonium sulfate and vortexed for 30 s. After the precipitate settled into the top of the tube, a 1-butanol extract was obtained, with recovery of approximately 80% of the radioactivity related to "G" and FUra. After centrifugation, the organic phase was evaporated to dryness at 50°C under N_2. The residue was suspended on 0.25 ml of water, and a 0.2-ml portion was chromatographed on HPLC. A subsequent assay was carried out simultaneously under the same conditions, in which 200 mM of saccharo-1,4-β-lactone, a specific inhibitor of β-o-glucuronidase (16, 17), was included in the incubation medium. Control incubations were performed with boiled enzyme.

Formation of "G" in Cell Homogenates. Hepatocytes were suspended in 3 volumes of 0.25 M sucrose:50 mM Tris-HCl buffer (pH 7.4) and subjected to sonic oscillation for 60 sec at 0°C. The cellular debris was separated by centrifugation at 100,000 x g for 20 min, with this supernatant used as the enzyme source for the subsequent assay. Reaction mixtures contained a final protein concentration of 3 to 6 μg/ml as determined by the method of Lowry (18). The assay mixture (total volume 0.5 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl_2, 6 mM saccharo-1,4-β-lactone, and 1.5 mM [3H]FUra (specific activity, 1.5 mCi/mM) and concentrations of UDPGA ranging from 0 to 5 mM. After 30 min at 37°C, the reaction was stopped by immersing the mixture into dry ice-acetone. Samples were then centrifuged at 15,000 x g in an Eppendorf model 5312 microcentrifuge for 2 min, and 50 μl of the resulting supernatant (approximately 150,000 cpm) was analyzed by the HPLC technique described above. Total radioactivity applied to the column was recovered (98.5 ± 3.2% (SD)).
RESULTS

Analysis of Intracellular \(^3\)H as Extracellular \([^{3}H]\)FUra is Increased

Chart 1 illustrates the HPLC analysis of intracellular \(^3\)H following exposure of hepatocytes to 30, 100, or 300 \(\mu M\) of \([^{3}H]\)FUra for 15 min. FUH\(_2\) was the major intracellular constituent, and it did not increase appreciably as extracellular FUra was increased. FUPA increased significantly (about 2.5-fold), but this increase was not proportional to the increase of the extracellular FUra concentrations (10-fold). Furthermore, the FBAL levels did not change at all. As reported previously (26), unmetabolized FUra was not detected at an extracellular concentration of 30 \(\mu M\), consistent with transport of FUra being rate-limiting to reduction to FUH\(_2\) under these conditions. When extracellular FUra was increased to 100 \(\mu M\), intracellular FUra was detected and further increased when extracellular FUra was increased to 300 \(\mu M\). Of note, however, was that, at the latter concentration, an additional radioactive chromatographic peak (to be referred to as “G”) appeared which did not coelute with any of the known anabolites or catabolites of FUra, all of which can be detected with this HPLC technique (26). It should be emphasized that, although FUra was observed intracellularly at the higher extracellular FUra concentration, no nucleoside or nucleotide metabolites of FUra were detected.

Time Course of Appearance of FUra and its Metabolites in Hepatocytes

Chart 2 shows the time course of appearance of FUra and its metabolites, FUH\(_2\), FUPA, FBAL, and “G,” in the intracellular water after incubation of a hepatocyte suspension with 300 \(\mu M\) FUra. Intracellular FUra levels of 161 ± 46 \(\mu M\) were achieved within 5 min (approximately 50% of the extracellular level), and they subsequently declined to 26.5 ± 5 \(\mu M\) by 2 h. By 1 h, FUH\(_2\), the predominant catabolite, reached a steady-state level of 1434 ± 278 \(\mu M\), which was maintained for the remaining 1 h of the experiment. Within 30 min, FUPA accumulated to a maximum level of 165 ± 9 \(\mu M\) and subsequently declined, while FBAL reached a steady-state level of 53.5 ± 6 \(\mu M\) by 30 min. “G” was detected within 1 min, reached a maximum intracellular level of 187 ± 42 \(\mu M\) within 1 h, and thereafter represented about 10% of the total intracellular \(^3\)H.

Relationship between the Extracellular FUra Level and the Intracellular Levels of “G”

The intracellular level of “G” after a 1-h exposure of hepatocytes with increasing extracellular FUra (0.1 to 2 \(\mu M\)) is illustrated in Chart 3. Intracellular formation of “G” increased until the extracellular level of FUra reached a value of 1 \(\mu M\). Thereafter, the level of “G” appeared...
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 FUra and 2 mM thymine. Hence, "G" represents a metabolite of FUra that is not a product related to the catabolic pathway described previously (26).

Membrane Permeability of "G". Despite the accumulation of high intracellular levels (Charts 2 to 4), "G" was not detected in the extracellular compartment. To determine whether the absence of extracellular "G" reflects low cell membrane permeability or hydrolysis in the incubation medium, cells were exposed to 300 μM FUra for 50 min, washed at 0°C, and resuspended in a large volume of FUra-free medium. The intracellular 3H was then analyzed. Chart 5 shows that total cell 3H initially declined rapidly, with 3.9 ± 0.9 nmol/mg protein of the intracellular 3H exiting the cell within 15 min. However, a slowly exchanging fraction was detected which did not decrease further, even after resuspension of cells into fresh FUra-free buffer. HPLC analysis of intracellular 3H before and after efflux demonstrated that the rapidly exchanging component was largely FUH2, with a lesser amount identified as FUPA, FBAL, and unmetabolized FUra. There was no loss of "G" from these cells. Further, as illustrated in the inset to Chart 5, HPLC analysis of intracellular 3H 50 min after resuspension into FUra-free buffer (when only slowly exchanging 3H was present in cells) indicates that peak "G" represents more than 95% of this nonexchangeable fraction. This is consistent with a low level of membrane permeability for "G".

To evaluate the extent to which this slowly exchanging fraction to approach a limiting value, suggesting saturation in the synthesis of this derivative. At 0.6 mM initial extracellular FUra, intracellular FUra equilibrated across the cell membrane with the extracellular FUra.

Further Characterization of "G"

Effects of Inhibition of the Catabolic Pathway by Thymine. In order to further characterize the pathway of "G" formation, hepatocytes were exposed simultaneously to 300 μM FUra and 2 mM thymine, an inhibitor of the catabolic enzyme, dihydrouracil dehydrogenase (29). Under these conditions, FUra catabolism was inhibited by more than 99%, FUH2, FUPA, and FBAL were not detected in the intracellular compartment over 2 h, and the intracellular concentration of FUra was increased markedly as compared to control cells achieving equilibrium with extracellular FUra. Likewise, "G" increased over 2-fold under these conditions to levels in excess of FUra to account for as much as 60% of the intracellular 3H. This is illustrated in Chart 4, which compares the intracellular profiles obtained 2 h following incubation of the hepatocyte suspension with either 300 μM FUra alone or 300 μM FUra and 2 mM thymine. Hence, "G" represents a metabolite of FUra that is not a product related to the catabolic pathway described previously (26).

Membrane Permeability of "G". Despite the accumulation of high intracellular levels (Charts 2 to 4), "G" was not detected in the extracellular compartment. To determine whether the absence of extracellular "G" reflects low cell membrane permeability or hydrolysis in the incubation medium, cells were exposed to 300 μM FUra for 50 min, washed at 0°C, and resuspended in a large volume of FUra-free medium. The intracellular 3H was then analyzed. Chart 5 shows that total cell 3H initially declined rapidly, with 3.9 ± 0.9 nmol/mg protein of the intracellular 3H exiting the cell within 15 min. However, a slowly exchanging fraction was detected which did not decrease further, even after resuspension of cells into fresh FUra-free buffer. HPLC analysis of intracellular 3H before and after efflux demonstrated that the rapidly exchanging component was largely FUH2, with a lesser amount identified as FUPA, FBAL, and unmetabolized FUra. There was no loss of "G" from these cells. Further, as illustrated in the inset to Chart 5, HPLC analysis of intracellular 3H 50 min after resuspension into FUra-free buffer (when only slowly exchanging 3H was present in cells) indicates that peak "G" represents more than 95% of this nonexchangeable fraction. This is consistent with a low level of membrane permeability for "G".

To evaluate the extent to which this slowly exchanging fraction...
is bound to cellular constituents, binding of intracellular $^3$H to cell macromolecules after FUH$_2$, FUPA, and FBAL had exited the cells was assessed by the Bio-Gel P2 minicolumn filtration technique as described by Fry et al. (8). With this technique, macromolecular-bound ligands are excluded from the column, while free ligands are retained. It was observed that 97 to 98% of $^3$H applied to the column was free within the intracellular water, based on its retention in the Bio-Gel column.

Evidence that "G" is a Glucuronide of FUra. Chart 6B demonstrates that, in the presence of $\beta$-d-glucuronidase, the chromatographic peak "G" was completely converted to a peak coeluting with authentic FUra standard. In the presence of boiled enzyme (Chart 6A) or saccharo-1,4-$\beta$-lactone, a specific inhibitor of $\beta$-d-glucuronidase (Chart 6C), no significant radioactivity was found in the FUra region on the radiochromatogram, indicating that conversion of "G" to FUra was totally suppressed.

Further evidence that "G" is a glucuronide of FUra was suggested by the net effect of exogenous UDPGA levels (a major factor regulating glucuronidation) (25) on "G" formation in the 100,000 x g supernatant fraction of the cell homogenate. Chart 7 demonstrates the net increase in glucuronidation of FUra observed after 30 min of incubation of the supernatant fraction with 1.5 mM of FUra at increasing UDPGA concentrations (0.01 to 5 mM).

DISCUSSION

In a previous report, a highly specific liquid chromatographic method for the detection of FUra, its catabolites, and anabolics was applied to investigate FUra metabolism in freshly isolated rat hepatocytes (26). This model was chosen for study because (a) it maintains many of the biochemical and physiological properties of the hepatocyte; (b) it permits the quantitation of transcellular and intracellular events mediated by hepatocytes distinct from the other cellular elements in the liver lobule; and (c) it obviates experimental difficulties presented by variabilities of blood flow, extracellular space, and vectorial flows of test compounds and their metabolites from the sinusoid to bile canaliculus.

The initial report evaluated FUra-hepatocyte interactions at low extracellular FUra concentrations (26). Membrane transport was found to be rate-limiting to the enzymatic conversion of FUra to FUH$_2$ in these cells. FUH$_2$ was the major intracellular catabolite; there was minimal conversion to FUPA and FBAL, with no formation of FUra nucleosides or nucleotides.

In the present study, unmetabolized FUra was detected in cells only when the extracellular FUra concentration was increased to 100 $\mu$M; intracellular FUra continued to increase as the extracellular level was increased further to 300 $\mu$M. The detection of FUra within cells appears to reflect saturation of the dihydrouracil dehydrogenase, as cellular accumulation of FUra is increased with increasing extracellular concentrations of the drug. This is consistent with the reported $K_m$ for this enzyme by Shiotani and Weber in the presence of uracil as substrate (24) and with the observation that intracellular FUH$_2$ levels are similar 15 min after incubation of the hepatocyte suspension with either 30, 100, or 300 $\mu$M FUra (Chart 1). However, even at this high extracellular FUra concentration (300 $\mu$M), intracellular FUra remains below the extracellular concentration due to rapid intracellular consumption. Hence, it is only when cells are exposed
to higher extracellular FUra levels (up to 2 mM) or are in the presence of sufficient thymine to abolish formation of FUH₂ by inhibiting dihydrouracil dehydrogenase that intracellular FUra achieves equilibrium with extracellular drug; this is consistent with a non-energy-dependent equilibrating process.

Of particular interest was the unexpected finding of a new metabolite of FUra that is detected only when substantial concentrations of free FUra accumulate within cells. This metabolite “G” was resolved from the known catabolites of FUra and did not coelute with any known anabolites. A number of characteristics of “G” formation and disposition in freshly isolated hepatocytes were established: (a) “G” formation proceeds even after FUH₂ synthesis is blocked by thymine. Indeed, in the presence of thymine, when the intracellular FUra was increased, “G” levels were increased to values in excess of the FUra concentration (Chart 4) while, at a relatively low FUra concentration, “G” was not detected at all (Chart 1). Hence, the rate of catabolism of FUra by dihydrouracil dehydrogenase must be so much greater than the rate of formation of “G” that negligible “G” appears until the dihydrouracil dehydrogenase saturates and sufficient FUra substrate accumulates within the cell to permit this to occur; (b) as extracellular FUra is increased to 300 μM, FUH₂ reaches maximum intracellular levels, indicating saturation of a rate-limiting step in FUH₂ formation (Chart 1). However, as extracellular FUra is increased to levels in excess of 1 mM, “G” formation continues until the capacity of this metabolic process becomes saturated. This further links “G” production directly to the cellular FUra level by a route distinct from the reductive pathway; (c) “G” exits from the cell very slowly, once formed within the intracellular compartment, to achieve and sustain very high transmembrane gradients. Indeed, only negligible levels of extracellular “G” could be detected, even at the highest intracellular levels of “G” accumulated in the presence of thymine. This poor permeation of “G” may reflect a large hydrophilic carbohydrate moiety on this compound and/or the existence of “G” as an ionized form in the intracellular compartment, under these conditions; (d) “G” appears to be present in cells free within the intracellular water, as tight binding of “G” could not be detected by gel filtration.

Although sufficient “G” could not be isolated to allow detailed chemical characterization, a variety of studies were done to characterize “G.” (a) “G” was completely hydrolized by β-d-glucuronidase to the FUra base. This reaction was completely suppressed in the presence of saccharo-1,4,β-lactone, a specific inhibitor of β-d-glucuronidase (16, 17); (b) “G” formation in supernatant fractions was enhanced several-fold following addition of exogenous UDPGGA, a cofactor which has been shown to increase the rate of glucuronidation in hepatic cells in vitro (20, 25, 27). Hence, these data are consistent with “G” being a glucuronide of FUra.

This is the first report of this metabolite in mammalian cells. Further studies will examine the formation of “G” in vivo and the extent to which “G” may serve as a depot for slow release of FUra after administration of this drug. The presence of this metabolite in hepatocytes exposed to FUra at concentrations comparable to those found in the hepatic vasculature following a conventional bolus dose of FUra (2) suggests the need for future studies to evaluate the biological significance of this metabolite.

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