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In Vitro Biological Evaluation of the R and S Isomers of 1-(Tetrahydrofuran-2-yl)-5-fluorouracil[1]

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SUMMARY

The S isomer of Ftorafur was synthesized and the ability of the latter to inhibit growth of cultured human fibroblasts was determined relative to both the R isomer and the racemic mixture (Ftorafur) that is presently used clinically. No significant difference in the cytotoxic effects or the relative abilities to prevent an increase in cell numbers was observed with the three forms. Inhibition of DNA synthesis in murine L1210 leukemia cells by either isomer was observed only after prolonged (18-hr) exposure. The data suggest that Ftorafur is a repository form of 5-fluorouracil and that activity is manifested equally by both isomers.

INTRODUCTION

Clinical evaluation (1, 3, 12) of the antitumor activity of Ftorafur [1-(tetrahydrofuran-2-yl)-5-fluorouracil; *1a,b*][3] indicates that the agent is less toxic and more effective than is FU[4] in the treatment of cancer of the breast (7) and gastrointestinal tract (9). It has been suggested on the basis of pharmacological studies that *1a,b* is a repository form of FU (10, 11).

Ftorafur has been synthesized by the condensation of both the chloromercuri- (4) and bistrimethylsilyl (5) derivatives of FU with 2-chlorotetrahydrofuran. More recently, Earl and Townsend (2) described an alternative synthetic route to *1a,b* via direct fluorination of 1-(tetrahydrofuran-2-yl)uracil. However, the absence of stereochemical control in these condensation reactions results in each case in a racemic mixture (*1a,b*).

Recent work in our laboratory led to a practical conversion of 2′-deoxynucleoside uronic acids to both pyrimidine and purine 2,3-dihydrofuryl derivatives via a single-step decarboxylative elimination reaction (13). Catalytic reduction of the dihydrofuryl intermediates (*e.g.*, *4a*) affords purine and pyrimidine tetrahydrofuran derivatives with preservation of the original anomeric (R) configuration. Thus, the 2-reaction sequence (see below) provided us with Ftorafur of the R configuration (*1a*).

It was of interest to compare the biological activity of the individual R (*1a*) and S (*1b*) isomers and the corresponding olefinic precursors (*4a* and *b*) with racemic Ftorafur and with FU to determine whether greater activity is associated with a particular configuration or whether both isomers are repository forms of FU. Accordingly, we prepared *1b* by an extension of our original synthetic approach to the R form (*1a*).

Cultured human fibroblasts served as the biological material for determining the ability of these compounds to kill cells or prevent cell multiplication. The extent of inhibition of DNA synthesis by these agents was measured with murine L1210 leukemia cells.

MATERIALS AND METHODS

Chemical

R,S-Ftorafur (*1a,b*) was generously donated by Dr. L. B. Townsend, Department of Chemistry, University of Utah. 3′,5′-Di-*O*-(*p*-toluoyl)-5-fluoro-α-2′-deoxyuridine (6) and FUdR were kindly provided by Dr. A. L. Nussbaum, Hoffman LaRoche, Nutley, N. J. FU was obtained from Nutritional Biochemicals Co. Cleveland, Ohio. All fluoro derivatives tested were chromatographically homogeneous (<0.1% contamination) by TLC.

Melting points were determined in a Thomas-Hoover Unimelt apparatus and are uncorrected. Evaporations were carried out in a Büchi rotary evaporator in a vacuum at 25° except for removal of DMF, which was carried out at a bath temperature of 50°. TLC was performed on 6- x 2-cm, precoated, Silica Gel F-254 aluminum foils (Merck, Darmstadt, Germany) in chloroform:methanol (9:1). Preparative TLC was performed on 2-mm thick 40- x 20-cm loose layers of silica gel (70 to 325 mesh ASTM; Merck) containing 1% of fluorescent indicator (Leuchtpigment ZS Super, Riedel-De Haën, Hanover, Germany) in the same system. Compounds were eluted from the silica gel with chloroform:methanol (1:1). Paper electrophoresis was conducted in a

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[3] Ftorafur is the Russian trade name assigned to the product. Direct translation of this name has produced the English equivalent, "Fluorofur." The Japanese literature frequently refers to this same compound as FT-207.

[4] The abbreviations used are : FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; TLC, thin-layer chromatography; DMF, *N,N*-dimethylformamide; NMR, nuclear magnetic resonance.

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Savant electrophoresis flat plate using 0.02 M disodium hydrogen phosphate (pH 7.5) as buffer on Whatman No. 1 paper at 40 V/cm for 1 hr. UV-absorbing compounds were detected using a Mineralight lamp. Optical rotations were determined with a Perkin-Elmer Model 141 polarimeter. UV data were obtained on a Cary Model 14 recording spectrophotometer. NMR spectra were obtained using a Varian A-60A spectrophotometer. Tetramethylsilane was used as internal standard with $CDCl_3$ and 2,2-dimethyl-2-silapentane-5-sulfonic acid was used as external standard with D_2O. DMF was dried over Linde Molecular Sieves, 4A. *N,N*-Dimethylformamide dineopentyl acetal was a product of Aldrich Chemical Co., Milwaukee, Wis. Elemental analyses were carried out by M-H-W Laboratories, Garden City, Mich.

1-(2-Deoxy-α-D-erythro-furanosyluronic Acid)-5-fluorouracil (*3b*). The platinum-catalyzed oxidation (from 4 g PtO_2; Engelhard Industries, Murray Hill, N. J.) of *2b* (2.0 g, 8.1 mmoles) in water (400 ml) containing Na_2CO_3: $NaHCO_3$ (pH 8) was heated at 60° with high-speed stirring accompanied by a continuous flow of O_2 into the reaction mixture. After 6 hr, electrophoresis of an aliquot indicated complete conversion into a single, acidic product. The catalyst was removed by filtration, the solution was lyophilized to ~200 ml, and the sodium ions were removed by stirring with excess Dowex 50-X2 (200 to 400 mesh; pyridinium) for 30 min. The resin was removed by filtration and the solution was lyophilized several times with distilled water to give the free acid that was recrystallized from water to give white needles: yield 1.6 g (76%); m.p. 206–207°; $[\alpha]_D^{25}$ 0.00°, $[\alpha]_{436}^{25}$ −24.19°, $[\alpha]_{365}$ −99.3°, (*c* 1.0, water); λ_{max} (ethanol) nm 269 (ε 8000), λ_{min} 233 (ε 1333); NMR (D_2O + NaOH) δ 2.31 (*m*, 2, *H-2'*), 4.63 (*m*, 2, *H-3',4'*), 6.25 (*d* of *q*, 1, $J_{1'\cdot 2'}$ = 7.3 Hz, $J_{1'\cdot 2''}$ = 2.8 Hz, $J_{F\cdot H\text{-}1'}$ = 1.7 Hz).

$C_9H_9FN_2O_6$

Calculated: C 41.54, H 3.49, N 10.77
Found: C 41.32, H 3.25, N 10.44

(S)-1-[3(H)-Dihydrofuran-2-yl]-5-fluorouracil (*4b*). The carboxylic acid *3b* (0.94 g, 3.6 mmoles) was evaporated twice in a vacuum with DMF (2 × 10 ml) to remove traces of water; 20 ml of DMF and 3.9 ml (14 mmoles) of *N,N*-dimethylformamide dineopentyl acetal were added and the reaction mixture was heated at 80° for 2 hr, at which time electrophoresis indicated the absence of starting material. The reaction mixture was evaporated and the syrupy residue was chromatographed on 2 loose layers of silica gel. Elution of the major band and evaporation of the filtrate gave solid, chromatographically pure *4b*, m.p. 165–169°, which was recrystallized from ethanol; m.p. 180–180.5°; yield 0.53 g (74%); $[\alpha]_D^{25}$ + 174.3°, $[\alpha]_{436}^{25}$ + 375.3°, $[\alpha]_{365}^{25}$ + 652.3° (*c* 1.0, CH_2Cl_2); λ_{max} (ethanol) nm 268 (ε 6500), λ_{min} 235 (ε 1650); NMR ($CDCl_3$) δ 7.30 (*d*, 1, *H-6*), 6.70 (*d* of *q*, 1, *H-1'*), 6.52 (*q*, 1, *H-4'*), 5.20 (*q*, 1, *H-3'*), 2.90 (*m*, 2, *H-2'*).

$C_8H_7FN_2O_3$

Calculated: C 48.48, H 3.56, N 14.14
Found: C 48.32, H 3.41, N 14.21

(S)-(1-Tetrahydrofuran-2-yl)-5-fluorouracil (*1b*). The olefin *4b* (250 mg, 1.3 mmoles) was hydrogenated over 10% palladium on $BaSO_4$ (300 mg) in 120 ml ethanol at atmospheric pressure and room temperature for 3 hr. The catalyst was filtered through a Celite pad and washed with ethanol, and the filtrates were evaporated to a syrup that crystallized immediately. Recrystallization from benzene gave chromatographically homogeneous *1b* as white needles; m.p. 173–174°; yield 219 mg (87%); $[\alpha]_D^{25}$ −56.2°; $[\alpha]_{436}^{25}$ −160.2°, $[\alpha]_{365}^{25}$ −357.2° (*c* 0.5, CH_2Cl_2); λ_{max} (ethanol) nm 270 (ε 7300), λ_{min} 235 (ε 1500); NMR ($CDCl_3$) δ 7.46 (*d*, 1, *H-6*), 6.00 (*m*, 1, *H-1'*), 4.12 (*m*, 2, *H-4'*), 2.10 (*m*, *H-2',3'*).

$C_8H_9FN_2O_3$

Calculated: C 48.00, H 4.53, N 14.00
Found: C 47.84, H 4.23, N 13.68

Biological

Human Cell Cultures. Primary cultures of human fibroblasts were initiated from foreskins and grown as monolayer cultures in Ham's F10 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum (BioQuest, Cockeysville, Md.) and penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells used for this study were grown and maintained in Eagle's medium supplemented with Earle's salts plus 10% calf serum.

Cytotoxicity (Loss of Cloning Ability). Cells (10^2 to 10^3) were plated into 60-mm plastic culture dishes (10 to 12 per individual determination) and allowed ~10 hr to attach. The compound to be tested was introduced into the culture medium in the dishes from stock solutions, freshly dissolved in water, and sterilized by Millipore filtration. The cells were refed every other day with fresh culture medium containing the appropriate concentration of the freshly prepared compound until they developed into clones of macroscopic size (about 2 weeks). Clones were then fixed in 100% methanol, stained in 2% methylene blue, and counted. The cytotoxicity of the compound was determined by comparing the number of colonies in the experimental dishes with the number in the control dishes, taking into account the total number of cells plated per dish.

Effect on Cell Multiplication. Cells (10^4) were plated per 35-mm dish (4 per individual determination) and allowed to attach. After 12 hr, the number of cells per dish was determined by electronic counting (Coulter Electronic, Hialeah, Fla.), and the compounds to be tested were introduced into the dishes at various concentrations from fresh stock solutions prepared as above. After 48 hr the culture medium containing freshly prepared solutions of the compounds was renewed. After an additional 48 hr, the number of cells per dish was determined by Coulter counting. The increase in cell number from the original number present at the time of administration of the compound was calculated and expressed as percentage of control.

Tumor Cells. L1210 cells were maintained in culture using Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cells were collected, suspended in fresh

medium at a concentration of 5 to 7 × 10^6 cells/ml, and used for studies of the effects of R- and S-Ftorafur on DNA synthesis.

Studies on DNA Synthesis. Incorporation of [2-^{14}C]deoxyuridine into DNA was measured as previously described (8). A suspension of L1210 cells (7 × 10^6/ml) at 37° was mixed with the drug solutions for 10 min. Labeled deoxyuridine was added to give a final concentration of 0.50 mM (0.02 μCi/ml of cell suspension) and the incubations were terminated after 10 min. The suspensions were chilled, and the cells were collected by centrifugation, washed several times with 0.3 M $HClO_4$, and then washed with ethanol. The acid-insoluble material was solubilized and radioactivity was measured by liquid scintillation counting. In another type of study, cell cultures (0.5 × 10^6/ml) were treated with drugs for 18 hr. The cells were then collected and the rate of incorporation of [2-^{14}C]deoxyuridine into DNA was measured as described here.

RESULTS AND DISCUSSION

The platinum-catalyzed oxidation of α-FUdR [*2b; cf.* Chart 1], obtained by deblocking (6) the corresponding 3',5'-di-*O*-(*p*-toluoyl) derivative, afforded the uronic acid (*3b*) in 60% yield. Treatment of *3b* with *N,N*-dimethylformamide dineopentyl acetal in DMF at 80–90° for 2 hr gave (*S*)-1-[3-(*H*)-dihydrofuran-2-yl]-5-fluorouracil (*4b*) in 50% yield. Catalytic hydrogenation of *4b* provided the S form of Ftorafur (*1b*).

The inhibitory activity of *1a* and *1b* on incorporation of deoxyuridine into DNA by L1210 cells was not detectable at levels of 200 μg/ml and intervals of exposure ranging from 10 to 60 min (Table 1). Only after 18 hr were both isomers observed to effect 50% inhibition at 50 μg/ml. By contrast, both FU and FUdR showed inhibition of 2'-deoxyuridine incorporation at low levels. Contamination of Ftorafur by 0.1% FU would have been detected by this procedure.

As can be seen in Chart 2, there is no significant difference in cytotoxic effect (*i.e.*, inhibition of cell cloning) between the R or the S isomer and Ftorafur. The corresponding unsaturated precursors (*4a* and *b*) are less active in preventing cloning of the cells than are the saturated isomers but do exhibit a cytotoxic effect. For purposes of comparison, FU and FUdR were also tested for their cytotoxic effects. FU is 25 times more cytotoxic than either

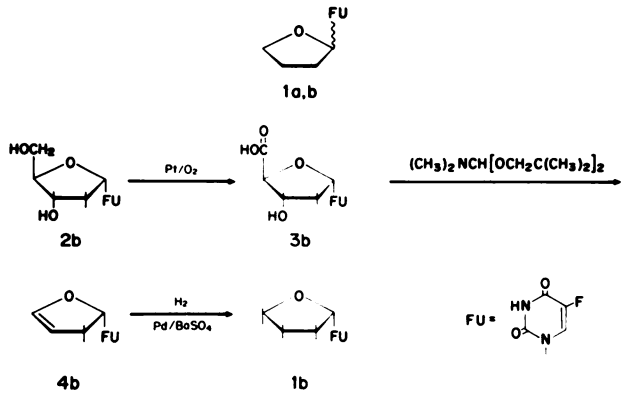

Chart 1. Synthetic sequence leading to S-Ftorafur.

Table 1

Effect of R- and S-Ftorafur and some related compounds on DNA synthesis by L1210 cells

Compound	ID_{50} (μg/ml)[a]
R-Ftorafur	>200[b]
S-Ftorafur	>200[b]
α-FUdR	400
β-FUdR	3 × 10^{-4}
FU	3

[a] Period of exposure, 10 min. ID_{50}, inhibitory dose of 50%.

[b] No inhibition of growth was observed at levels of 200 μg, even after 60 min of exposure. After 18 hr, an ID_{50} of 50 μg was obtained.

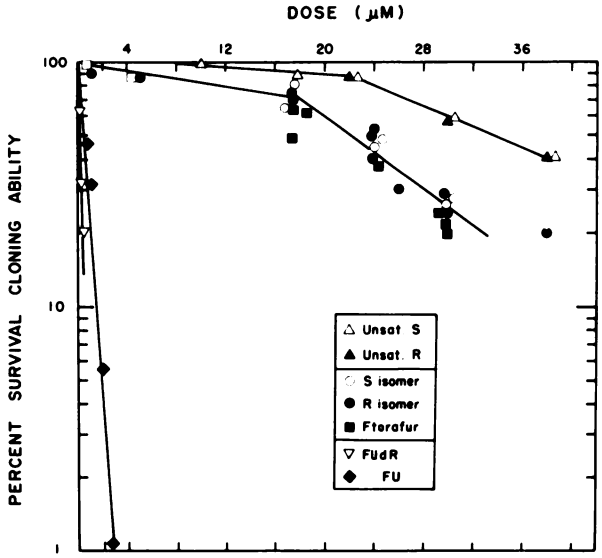

Chart 2. Cytotoxicity (loss of cloning ability) in human fibroblasts as a function of the dose of various compounds tested. Each determination represents the average of 10 to 12 replicates. The size of the symbol includes the amount of variance observed. The average number of clones found in the experimental dishes compared with the number in the untreated control dishes, taking into account the total number of cells plated per dish, is expressed as percentage of the controls. *Unsat.*, unsaturated.

isomer or Ftorafur. Thus, a dose of 2 μM FU reduces survival of the cloning ability to 5.5%, whereas it requires a concentration of 48 to 50 μM of the latter 3 compounds to produce such toxicity in the human cells in culture. 5-Fluoro-2'-deoxyuridine was about 5 times more toxic than was FU in this system.

The ability of the R and S isomers to prevent increase in cell number was then determined and compared with the growth-inhibiting effect of Ftorafur and of an equimolar mixture of the 2 isomers (*1a,b*). The results are presented in Chart 3. Each individual determination represents the average of 4 replicates. Again, it is clear that there is no significant difference in activity between the R (*1a*) and the S (*1b*) or the racemic mixtures. The 2 unsaturated precursors (*4a* and *b*) are less active than are their saturated derivatives. FU is about 30-fold more active than is the R or S isomer. A dose of about 2 μM FU inhibits the increase in cell numbers down to 37% of the untreated control cultures, while a dose of about 60 μM of the 2 isomers or the racemic

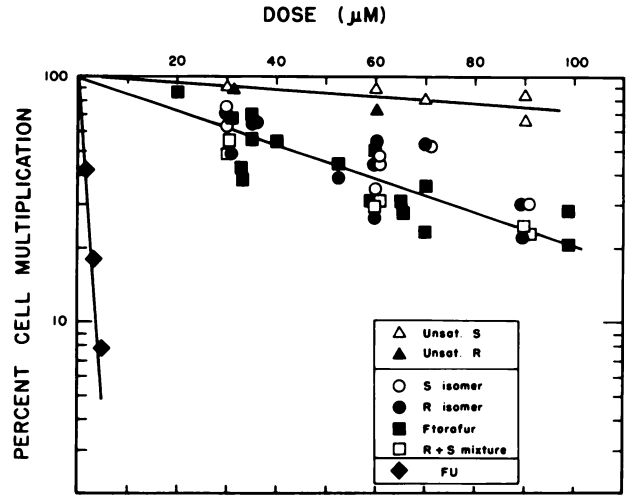

Chart 3. Inhibition of multiplication of human fibroblasts as a function of the dose of the various compounds tested. Each determination represents the average of 4 replicates. The size of the symbol includes the amount of variance observed. The increase in cell number after 96 hr from the original number determined by electronic counting at the time of administration of the compound was calculated and expressed as percentage of the untreated controls. *Unsat.*, unsaturated.

mixtures is needed to produce a similar level of inhibition of cell multiplication.

The activity of the saturated R and S isomers is equal in this tissue culture system and the activity of Ftorafur is contributed equally by both isomers. From our data, it is not clear whether the activity of Ftorafur (or its isomers) is a property of the compound itself or a result of hydrolysis of the compound to FU. However, it is clear from Charts 1 and 2 that hydrolysis of as little as 3% of Ftorafur or its isomers to FU could account for the observed cytotoxicity.

The slight activity observed with the unsaturated precursors might be a property of these compounds, or it might result from hydrolysis of as little as 1% of these compounds to FU when in solution.

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