Synthesis of 5-Selenium-Substituted Uracil Derivatives. Inhibition of Thymidylate Synthetase by 5-Hydroseleno-2′-deoxyuridylate

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5-Selenium-substituted derivatives (diselenides) of uracil, 2′-deoxyuridine, and 2′-deoxyuridylic acid were synthesized via the addition of methyl hypobromite to the 5,6 double bond, followed by reaction of the adducts with sodium diselenide. The physical and chemical properties of these compounds (including their facile reduction by dithiothreitol and rapid reoxidation) were similar to those of the corresponding 5-sulfur analogues. 5-Hydroseleno-2′-deoxyuridylic acid was as potent as 5-mercapto-2′-deoxyuridylate in inhibiting thymidylate synthetase from *L. casei* ($k_i \approx 6 \times 10^{-8}$ M) but the nucleoside III was considerably less active than 5-mercapto-2′-deoxyuridine in the inhibition of growth of the leukemia L1210 cell in culture.

Selenium is a close isostere of sulfur, and the selenium analogues of biologically active sulfur compounds often resemble the latter in their biological effects.[1] However, they usually show some quantitative differences in their activities in various test systems. Thus, several selenopurine derivatives were reported to be either more or less active than the corresponding thiopurine derivatives against different types of cells.[2-5] In view of the continuing interest of our laboratory in the chemical and biological properties of 5-mercaptopyrimidine nucleoside and nucleotide derivatives[6] as potent antiviral and antineoplastic agents in the monomeric form,[7] as well as at the polynucleotide level,[8,9] we decided to synthesize the 5-selenium analogues of 5-mercaptouracil (MU), 5-mercapto-2′-deoxyuridine (MUdR), and 5-mercapto-2′-deoxyuridine 5′-phosphate (MdUMP).

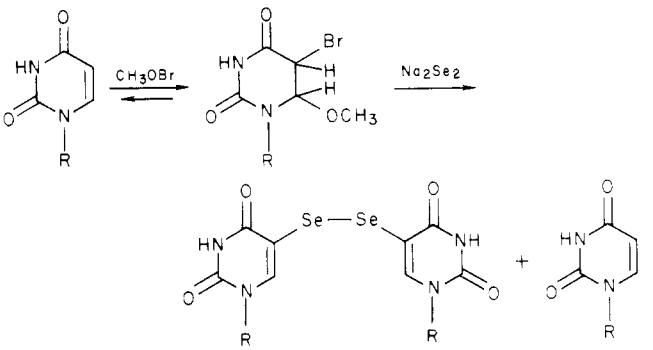
Chemistry. Several of the synthetic schemes which were successful in the case of the analogous 5-mercaptopyrimidine derivatives failed to prove satisfactory when the sulfur reagent was replaced with the corresponding selenium compound. Thus, our initial attempt to react 5-diazouracil with selenourea under the conditions used in the original synthesis of 5-mercaptouracil[10] led to decomposition of the selenium reagent. The reactions of 5-diazouracil with selenocyanate and of uracil with selenocyanogen[11] also failed to give satisfactory results in our preliminary experiments. On the other hand, acceptable yields of the 5-seleno derivatives were obtained by a synthetic route based on the general method of Szabo et al. for the synthesis of 5-mercaptopyrimidine nucleotides.[12] Thus, the methyl hypobromite adducts of uracil and its N_1-substituted derivatives reacted with sodium diselenide in dimethyl sulfoxide at room temperature to give the corresponding uracil 5-diselenides, as shown in Scheme I.

The sodium diselenide reagent was prepared essentially according to the method of Klaymen and Griffin[13] by reacting selenium powder with sodium borohydride in ethanol, in stoichiometric amounts based on the following equation:

$$2NaBH_4 + 3Se + 6C_2H_5OH \rightarrow Na_2Se_2 + H_2Se + 6H_2 + 2B(OC_2H_5)_3$$

In the original procedure, the reaction was driven to completion by the expulsion of the gaseous byproducts with a slow stream of nitrogen, and the remaining solution of sodium diselenide was used without separation from the trimethyl borate side product. In the case of our present procedure, it was necessary to evaporate the ethanol completely in order to change to an aprotic solvent (dimethyl sulfoxide) for the subsequent reaction step, and, thus, the low-boiling triethyl borate was also removed in the process. The residue containing the unstable solid sodium diselenide was placed under nitrogen and used immediately in the subsequent reactions with the uracil derivatives.

Scheme I



I, R = H; II, R = CH_3; III, R = 2-deoxyribosyl; IV, R = 2-deoxy-5-phosphonoribosyl

The 5-bromo-6-methoxy adducts of 1-methyluracil and 2′-deoxyuridine 5′-phosphate were readily obtained in high yields, as we previously reported.[12] In contrast, the adducts of uracil and 2′-deoxyuridine were difficult to isolate in pure form. In the case of uracil, the addition of methyl hypobromite to the 5,6 double bond is an unfavorable equilibrium reaction and did not go to completion; after separation from the unreacted starting materials, all attempts to purify the adduct by chromatography or by crystallization were unsuccessful and resulted in a slow reversal of the reaction, with the regeneration of the 5,6 double bond (as indicated by a gradual increase of the UV absorption at 260 nm). In the case of 2′-deoxyuridine, the addition reaction proceeded in a quantitative manner; however, the adduct appeared to be quite unstable. It is probable that the decomposition of this adduct is accelerated by anchimeric assistance of the 5′-hydroxyl group which is known to participate in some of the reactions of uracil nucleosides at the 5,6 double bond.[14] Therefore, the adducts of both uracil and 2′-deoxyuridine were used without purification in the subsequent reaction step.

The reactions of the 5-bromo-6-methoxy-5,6-dihydrouracils with the sodium diselenide reagent were carried out in dimethyl sulfoxide solution at room temperature under nitrogen atmosphere. In the case of the uracil and 1-methyluracil derivatives, the poor solubilities of the diselenides I and II in most organic solvents, together with their relative instabilities, rendered their purification by either chromatography or recrystallization very difficult; therefore, these compounds were purified by repeated precipitation from weakly basic aqueous solutions with carbon dioxide. On the other hand, the diselenides of the

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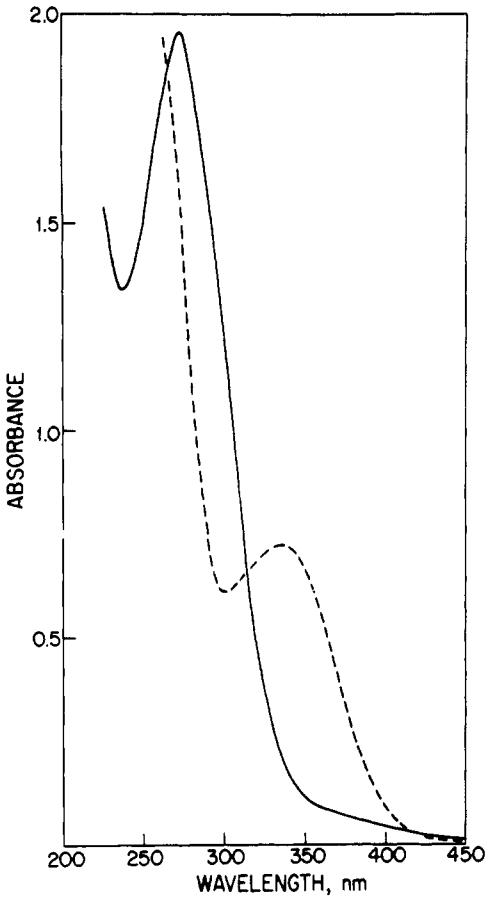
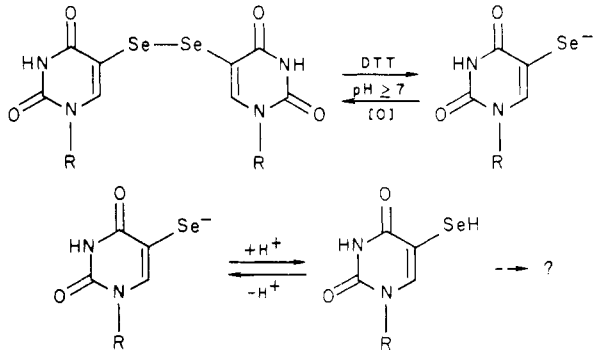


Figure 1. Ultraviolet spectrum of IV in the absence (solid line) and presence (broken line) of dithiothreitol at pH 7.

2′-deoxyriboside (III) and 2′-deoxyribotide (IV) were separated from some 5-unsubstituted 2′-deoxyuridine and 2′-deoxyuridylic acid, respectively (which were regenerated from the adducts and obtained as byproducts in the second reaction step), by chromatography on DEAE-cellulose and Sephadex G-10 columns.

The ultraviolet spectra of the diselenides I–IV were



almost identical with those of the corresponding disulfides,[12,15] and they also showed essentially the same changes upon the addition of dithiothreitol (see Figure 1). In the latter case, the new λ_{max} appearing at neutral or alkaline pH in the 330–340-nm region, which corresponds to the absorption of the "selenolate" anions formed upon reduction with dithiothreitol, show a slight bathochromic shift (by 4 to 8 nm) as compared to those of the "thiolate" ions of the corresponding 5-mercaptopyrimidines.

Spectrophotometric titration[15] of compound IV in the presence of dithiothreitol at 345 nm yielded for the reduced compound an estimated pK_a of approximately 4. Thus, as was expected, the 5-selenols are even more acidic than the corresponding 5-mercapto compounds (pK_a = 5.3) and should exist in their ionized form under physiological conditions.

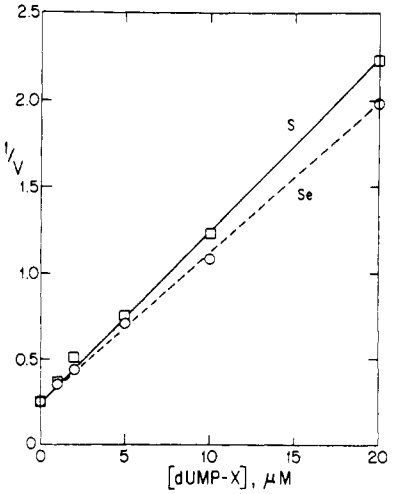


Figure 2. Dixon plots of the kinetics of inhibition of *L. casei* thymidylate synthetase by IV (○, X = Se) and 5-mercapto-2′-deoxyuridylate (□, X = S). Substrate concentration used: 2.5 × 10^{-4} M; $v = \Delta OD_{340}$/min.

On standing in dilute, aqueous neutral or alkaline solutions, the pyrimidine-5-selenols appear to undergo autoxidation[16] even more readily than the corresponding 5-mercaptopyrimidine derivatives. The 5-mercaptopyrimidines were stable to oxidation in the un-ionized form and could be isolated as such by crystallization from acidic solutions.[15] Unfortunately, the pyrimidine-5-selenols, like many other organoselenium compounds, are slowly decomposed by acid and, therefore, their purification in the reduced state presented considerable difficulties. Because of the presence of free sulfhydryl groups (reduced cysteine residues) in most cells, our previous experiences with the 5-mercapto compounds indicated that the latter could be used interchangeably with the corresponding disulfides in most of the biological assay systems.[17] We expected that the diselenides would be reduced in situ in a manner similar to the disulfides and, thus, would adequately serve our purpose of comparing the biological activities of the pyrimidine-5-selenols with those of their 5-mercapto analogues.

The infrared spectra of compounds I–IV were almost superimposable with those of the corresponding disulfides. In the NMR spectra, the singlet assigned to the C-6 proton showed a small downfield shift (by 0.2 ppm) in the case of the selenium analogues. This is in agreement with the observed slightly greater deshielding effect of selenium, as compared to sulfur, on a "conjugated" β proton (e.g., in selenophene vs. thiophene).[18]

Biological Activity. It was of interest to determine the effect of the substrate analogue, 5-hydroseleno-2′-deoxyuridylate (the reduced form of compound IV), on the activity of thymidylate synthetase, since the corresponding 5-mercapto compound was found to be a very potent inhibitor of this enzyme. In the presence of excess thiols, strong inhibition of *L. casei* thymidylate synthetase[19] by compound IV was observed. Kinetic studies indicated that the inhibition was reversible and competitive with deoxyuridylate. An apparent K_i value of 6 × 10^{-8} M was obtained for the 5-selenol derivative, similar to that of the related analogue 5-mercaptodeoxyuridylate,[20] which gave a K_i value of 4.8 × 10^{-8} M in this system. The Dixon plots of the respective 5-substituted selenium and sulfur derivatives of the substrate deoxyuridylate are shown in Figure 2.

However, preliminary testing of the nucleoside III for cytotoxicity against leukemia L1210 cells in culture showed no significant inhibition of cell growth at 10^{-4} M concentration, while the corresponding sulfur analogue MUdR caused half-maximal inhibition at 5×10^{-6} M. It is conceivable that the rate of phosphorylation of the nucleoside III is the factor limiting its intracellular activity, since MUdR was previously found to be a relatively poor substrate for thymidine kinase.[20] Further loss of substrate activity due to increased acidity of the 5-substituent may explain the low cytotoxicity observed in this system. The chemotherapeutic activity of III in various assay systems is currently under investigation.

Experimental Section

All solvents used (methanol, ethanol, and Me_2SO) were dried by distillation over calcium hydride shortly before use. All melting points were taken in open capillary tubes in a Mel-Temp apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer 197 using KBr pellets and were calibrated against the 1601 cm^{-1} band of polystyrene. Ultraviolet spectra were obtained using a Cary 118C spectrophotometer. The molar absorbance values of the selenopyrimidines in the absence of dithiothreitol were based on the molecular weights of the *dimeric* diselenides, but those obtained after the addition of dithiothreitol were calculated on the basis of the corresponding *monomeric* hydroselenides. Optical rotations were determined in a 1.0-dm tube on a Perkin-Elmer 141 polarimeter at 589 nm. Nuclear magnetic resonance spectra were recorded on a Varian Associates T-60A NMR spectrometer. Chemical shifts are expressed in parts per million relative to Me_4Si as internal standard. Spin multiplicities are indicated by the symbols s (singlet) and d (doublet). Elemental analyses were performed by Atlantic Microlab, Inc., or Galbraith Laboratories, Inc. Selenium analyses were determined by Gould's iodometric method.[21,22]

Methyl hypobromite was prepared by the method of Duschinsky et al.[23] The required weight of Br_2 in MeOH was chilled in dry ice and CCl_4 and then added to 2.2 mol-equiv of Ag_2CO_3. The resulting suspension, after stirring for 1 h at −15 °C, was ready for use.

Sodium diselenide was prepared by a slight modification of the method of Klayman and Griffin:[13] selenium powder and sodium borohydride in a molar ratio of 3:2 were stirred in ethanol under nitrogen at room temperature for about 15 min. The brownish red solution was then refluxed for 1.5 h while a slow stream of nitrogen was being passed into the solution and then into a trap containing a 5% lead acetate solution. The brownish red solution was then evaporated to dryness in a flash evaporator. The reddish pink residue of sodium diselenide was used without further purification.

1-Methyluracil[24] was prepared by an unpublished procedure developed in this laboratory by Dr. L. Novak. Uracil (10.0 g, 89.4 mmol) and a few milligrams of ammonium sulfate were refluxed in 43 mL of hexamethyldisilazane (HMDS) at 120–125 °C for 1.5 h. Excess HMDS was evaporated in a flash evaporator. The resulting oil was distilled under diminished pressure to yield 20.1 g (88.0%) of the silylated uracil. This intermediate was refluxed with 16 mL of methyl iodide for over 5 h. Excess MeI was removed under reduced pressure and the remaining residue was washed with ethanol, followed by ether. The white powder obtained was recrystallized from water to yield colorless needle-shaped crystals of analytically pure 1-methyluracil,[24] yield 8.6 g (76.0%).

Bis(uracil-5-yl) Diselenide (I). Methyl hypobromite (25.3 mmol) freshly prepared from bromine (2.02 g, 12.7 mmol) and Ag_2CO_3 (8.37 g, 30.4 mmol) in methanol was filtered directly into uracil (0.95 g, 8.43 mmol). The resulting yellow suspension was stirred in an ice bath for 2 h. Unreacted uracil (0.41 g) was removed by filtration. The filtrate yielded, on addition of chilled ether, 0.84 g of white precipitate after drying in vacuo. This was crude 5-bromo-6-methoxy-5,6-dihydrouracil. It was allowed to react with 2.65 mmol of freshly prepared Na_2Se_2 in Me_2SO under nitrogen at room temperature for over 12 h. Some black residue (elemental selenium) was removed by filtration. The yellow filtrate yielded, on addition of ethanol–ether, a pale yellowish precipitate. This was purified via repetitive precipitation with CO_2 from a weakly basic aqueous solution: yield 0.39 g (39.5% based on moles of uracil reacted); mp 100 °C dec gradually; UV λ_{max} (pH 1) 260 nm (ϵ 8300), λ_{max} (pH 7.2) 267 nm (ϵ 11 300), λ_{max} (pH 12) 293 nm (ϵ 21 500), after the addition of DTT λ_{max} (pH 7.2) 331 nm (ϵ 2140); IR (KBr) ν 3500 and 3080 (NH), 1715 and 1670 (C=O), 1600 (C=C) cm^{-1}; NMR (Me_2SO-d_6) δ 7.87 (s, 2 H). Anal. ($C_8H_6N_4O_4Se_2 \cdot 0.2H_2O$), C, H, N, Se.

Bis(1-methyluracil-5-yl) Diselenide (II). 1-Methyl-5-bromo-6-methoxy-5,6-dihydrouracil was prepared according to our previously described procedure[12] in a yield of 2.40 g (80.3%). The reaction of this analytically pure adduct with Na_2Se_2 and the workup of the reaction mixture was conducted in the same manner as above. The pure product II, 0.206 g (20.2% overall yield), decomposed on heating above 150 °C: UV λ_{max} (pH 1) 277 nm (ϵ 8300), λ_{max} (pH 7.2) 277 nm (ϵ 12 500), λ_{max} (pH 12) 272 nm (ϵ 10 600), after addition of DTT λ_{max} (pH 7.2) 339 nm (ϵ 3500); IR (KBr) ν 3175 and 3045 (NH), 1730 and 1675 (C=O), 1600 (C=C), 1418 (CH_3) cm^{-1}; NMR (Me_2SO-d_6) δ 3.29 (s, 6 H), 8.13 (s, 2 H). Anal. ($C_{10}H_{10}N_4O_4Se_2$) C, H, N, Se.

Bis[N_1-(2-deoxy-β-D-ribofuranosyl)uracil-5-yl] Diselenide (III). Methyl hypobromite was prepared from bromine (1.87 g, 11.7 mmol) and Ag_2CO_3 (7.10 g, 25.7 mmol) in methanol and then filtered directly into 1.96 g (8.58 mmol) of 2′-deoxyuridine. After stirring in an ice bath for 30 min, the yellow solution was evaporated to dryness to afford 2.73 g (93.7% yield) of crude N_1-(2-deoxy-β-D-ribofuranosyl)-5-bromo-6-methoxy-5,6-dihydrouracil. This material was allowed to react with 6.4 mmol of Na_2Se_2 in Me_2SO. By following the same procedure as in the workup and purification of the nucleotide IV (see below) (in this case an ionic concentration gradient of 0.01 to 0.1 M of unbuffered ammonium acetate was used instead of triethylammonium bicarbonate buffer), 0.82 g of the starting material 2′-deoxyuridine and 0.102 g of the analytically pure product III were obtained: UV λ_{max} (pH 7.2) 270 nm (ϵ 12 000), after addition of DTT λ_{max} 330 nm (ϵ 2110); IR (KBr) ν 3500–3100 (OH), 1700–1675 (C=O), 1600 (C=C) cm^{-1}; NMR (D_2O) δ 8.20 (s, 2 H); $[\alpha]^{25}_D$ +138° (*c* 0.05, H_2O). Anal. ($C_{18}H_{20}N_4O_{10}Se_2$) C, H, N, Se.

Bis[N_1-(5-*O*-phosphono-2-deoxy-β-D-ribofuranosyl)-uracil-5-yl] Diselenide Disodium Salt (IV). N_1-(2-Deoxy-β-D-ribofuranosyl)-5-bromo-6-methoxy-5,6-dihydrouracil 5′-phosphate monosodium salt was prepared according to our previously described procedure[12] and obtained in nearly quantitative yield. This material, 0.990 g (2.25 mmol), was then allowed to react with 1.69 mmol of Na_2Se_2 in Me_2SO. After stirring under nitrogen for over 12 h, some black residue formed and was removed by filtration. The yellow filtrate yielded, upon addition of ethanol–ether, a yellow precipitate. This material was dried in vacuo for several hours and then chromatographed on a 3 × 40 cm column of an anion-exchange cellulose (Cellex D, OH^- form) and eluted with an ionic concentration gradient of 0.05–0.25 M of triethylammonium bicarbonate solution buffered at pH 7.4. Two UV-absorbing fractions (A and B) were collected separately and freeze-dried in a lyophilizer. The residue from the first fraction (A) was dissolved in 50 mL of methanol, and 100 mL of a 0.2 M solution of NaI in acetone was added. Some white precipitate was collected and dried in vacuo for 1 h. The residue obtained was then chromatographed on a 3 × 30 cm Sephadex G-10 column and eluted with water to remove any contaminating salts present. The UV-absorbing fractions were collected and freeze-dried to afford 0.316 g of starting material, 2′-deoxyuridine 5′-phosphate disodium salt. Similar treatment of the second fraction (B) afforded 0.425 g of analytically pure product: overall yield 40.5%; UV λ_{max} (pH 8) 272 nm (ϵ 20 000), λ_{min} 236 nm (ϵ 13 200), after addition of DTT λ_{max} (pH 2) 270 nm (ϵ 10 500), λ_{min} 237 nm (ϵ 7100), λ_{max} (pH 8) 338 nm (ϵ 3600), λ_{min} 302 nm (ϵ 3050); NMR (D_2O) δ 8.00 (s, 2 H); $[\alpha]^{25}_D$ +109.1° (*c* 0.208, H_2O). Anal. ($C_{18}H_{20}N_4O_{16}P_2Se_2Na_4 \cdot 4H_2O$) H, N, Se; C: calcd, 23.19; found, 23.62.

Enzyme Assay. 2-Mercaptoethanol, dithiothreitol (DTT), and disodium 2′-deoxyuridylate were obtained from Calbiochem.

Thymidylate synthetase of dichloromethotrexate-resistant *Lactobacillus casei* was purified essentially as described by Leary and Kisliuk.[19] Enzyme activity was assayed using the method of Wahba and Friedkin[25] as described[26] by following the absorbance change at 340 nm in a GCA/McPherson spectropho-

tometer thermostated at 30 °C. The regular assay mixture contained the following: Tris–acetate, 70 mM, pH 7.4; mercaptoethanol, 80 mM; $MgCl_2$, 20 mM; d,l-L-tetrahydrofolate, 0.3 mM; CH_2O, 12 mM; EDTA, 0.3 mM; 2′-deoxyuridylate, 1.0 mM; and sufficient enzyme to produce an absorbance change of 0.015–0.025 OD unit/min at 340 nm in the absence of inhibitors.

Inhibitory constants (apparent K_i values) were calculated from the data represented in Figure 2, using an apparent K_m value of 5×10^{-6} M, independently determined for the substrate 2′-deoxyuridylate.

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Design of Species- or Isozyme-Specific Enzyme Inhibitors. 1. Effect of Thymidine Substituents on Affinity for the Thymidine Site of Hamster Cytoplasmic Thymidine Kinase

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5-(Ethylamino)- and 5-acetamido-2′-deoxyuridine 5′-triphosphates were synthesized; the extent and concentration dependence of their inhibitory action on the title enzyme resembled that of the feedback inhibitor TTP. This and other findings provide a tentative indication that bulk tolerance near C-5 of the thymine ring may be more extensive at the TTP site than at the thymidine site. Enzyme–inhibitor dissociation constants (K_i values) were determined for thymidine derivatives monosubstituted at various positions. Competitive inhibition with respect to thymidine (indicative of substituent tolerance in the enzyme–thymidine complex) was produced by 3-amylthymidine (K_i = 65 μM), *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine diastereoisomers (K_i = 180 and 310 μM), 5′-*C*-(acetamidomethyl)- and 5-*C*-(propionamidomethyl)thymidine epimers (K_i range 65–1100 μM), 3′-acetamido- and 3′-(ethylthio)-3′-deoxythymidines (K_i = 2.5 mM and 12 μM, respectively), and certain 5′-(alkylamino)- and 5′-(alkylthio)-5′-deoxythymidines (K_i range 180–1200 μM). Evidence indicates that bulk tolerance at some, if not most, of the above atoms of thymidine is found in the enzyme–thymidine complexes of human and other mammalian thymidine kinases; attachment of suitable substituents to such atoms could, in principle, lead to thymidine site directed isozyme-specific inhibitors of human cytoplasmic thymidine kinase, which is a candidate target in the design of antineoplastic drugs.

The TMP[1] required for cell multiplication can be furnished either from dUMP via the de novo pathway of biosynthesis or from exogenous thymidine. Several drugs, e.g., methotrexate, are effective in blocking the de novo pathway. The alternate route to TMP involves transfer of phosphate from a nucleoside 5′-triphosphate to thymidine, a reaction catalyzed by thymidine kinase. In neoplastic tissue and rapidly proliferating cells, the activity of thymidine kinase is elevated to a level which is sufficiently high to permit this enzyme to play a major role in TMP production in vivo.[2,3] Two isozymes of thymidine kinase, associated with the mitochondrial and cytoplasmic fractions, respectively, of mammalian cells, have been recognized.[4–11] The mitochondrial isozyme is the preponderant form in human adult spleen, liver, and fibroblasts,[5,6] whereas the cytoplasmic isozyme is reported to be the sole or predominant form in KB and HeLa human tumor cell lines,[6] in human fibroblasts transformed by SV40 virus,[5,6] in human fetal liver,[5,6] and in the following tumor tissues of clinical origin: Wilm's tumor,[5] rhabdomyosarcoma,[5] bladder adenocarcinoma,[5] and cervical carcinoma.[12] A nonmalignant uterine fibroma, on the other hand, contained principally the mitochondrial isozyme.[5] These data suggest that effective antineoplastic chemotherapy might be obtainable by the coadministration of a drug which can block de novo TMP biosynthesis with a drug which can inhibit cytoplasmic thymidine kinase without appreciably inhibiting mitochondrial thymidine kinase. In this context, the present work has been aimed at the attempted design of thymidine site directed selective inhibitors of the cytoplasmic isozyme. Baby hamster kidney cells grown in culture served as a convenient source

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