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ANTIVIRAL, ANTITUMOR, AND THYMIDYLATE SYNTHETASE INHIBITION STUDIES OF 5-SUBSTITUTED STYRYL DERIVATIVES OF 2′-DEOXYURIDINE AND THEIR 5′-PHOSPHATES

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Abstract—2′-Deoxyuridine derivatives containing styryl, 3-nitrostyryl, 4-nitrostyryl, and phenylethyl groups substituted at the 5-position of the pyrimidine ring have been evaluated for their effects on vaccinia and herpes simplex virus replication (in primary rabbit kidney cell cultures) and mouse leukemia L-1210 cell culture growth. 5-Phenylethyl-2′-deoxyuridine inhibited herpes simplex (type 1 and 2) virus-induced cytopathogenicity by 50 per cent at a dose (ID_{50}) of 10–30 μg/ml. It was inactive against tumor cell growth. The corresponding styryl derivative showed an ID_{50} of 30–70 μg/ml for herpes simplex virus, 20 μg/ml for vaccinia virus, and 280 μg/ml for L-1210 cell growth. 5(E)-(3-Azidostyryl)-2′-deoxyuridine 5′-phosphate inhibited vaccinia replication with an IC_{50} of 20 μg/ml and L-1210 cell culture growth with an ID_{50} of 80 μg/ml. The nucleotides of these compounds were all potent reversible inhibitors of thymidylate synthetase (*Lactobacillus casei*) with the following K_i/K_m ratios: 3-nitrostyryl, 0.035; 4-nitrostyryl, 0.05; 3-azidostyryl, 0.06; styryl, 0.08; and phenylethyl, 0.31. The photodecomposition of the azidostyryl derivative, a photoaffinity labeling reagent for thymidylate synthetase, was examined at two wavelengths.

The major obstacle in the search of more effective chemotherapeutic agents for the treatment of cancer or viral infections is the lack of selectivity these drugs display in their cytotoxic or antiviral effects. The drugs presently available for the treatment of malignant and viral diseases exert considerable effects on the metabolism of normal cells. An attractive target in the design of more selective and/or non-toxic agents is thymidylate synthetase. A key enzyme in cellular reproduction, it catalyzes the conversion of 2′-deoxyuridine 5′-phosphate (dUMP) to thymidine 5′-phosphate (dTMP), the precursor for thymidine triphosphate which is essential for DNA replication.

Two agents currently in clinical use exert their effects at least partially by inhibition of this enzyme. 5-Fluorouracil, a precursor of the actual inhibitor (5-FdUMP), has been invaluable in the clinical control of solid tumors [1]. Use of this agent is limited, however, because of toxicity and the development of resistant cell lines. In the search for more effective agents, 5-trifluoromethyl-2′-deoxyuridine (CF_3-dUrd) was synthesized and found to be an effective antitumor and antiviral agent [2]. This agent has been most intensively investigated for the topical treatment of herpes simplex virus (HSV) infections, principally herpetic keratitis [3]. As established for 5-FdUrd, the mechanism of antitumor action of CF_3-dUrd appears to be mediated by an inhibition of thymidylate synthetase by the 5′-phosphate derivative [4].

Initial steps in the mechanism of thymidylate synthetase catalysis are reasonably well understood, principally from the elegant work leading to the isolation and characterization of a ternary complex formed from the enzyme, the cofactor 5,10-methylenetetrahydrofolic acid, and 5-FdUMP [5]. Important structural features of substituted dUrd derivatives that impart high affinity are the 5′-phosphate and an electron withdrawing group (X, Scheme 1) covalently attached to carbon-5 of the pyrimidine ring. The latter apparently serves the dual function of enhancing the acidity of the N_3 proton and increasing the electrophilic character of carbon-6 of the pyrimidine ring. For example, both 5-nitro- and 5-formyl-dUMP have these features and have been shown to have high affinity for thymidylate synthetase. Analysis of the binding constants for these and the fluorinated derivatives suggests that two types of reversible complexes contribute to the high association of the inhibitor for the enzyme. The first step (Scheme 1) leads to a non-covalent complex E·I, and the second yields the covalent complex E–I, where the catalytic cysteine residue of the enzyme forms a covalent bond to carbon-6 of the pyrimidine ring [6, 7].

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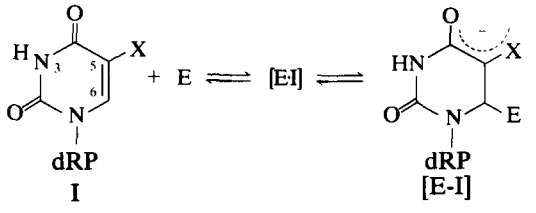


Scheme 1. Thymidylate synthetase inhibition (X = electron withdrawing group; dRP = 2′-deoxyribose 5′-phosphate).

Inherent to the design of new chemotherapeutic agents in any chemical series is the available synthetic chemistry that allows for the preparation of derivatives. Approaches to 5-substituted-dUrd derivatives and their 5′-phosphates have been hampered by multiple synthetic steps of low yielding reactions, particularly at the stages of α and β anomer separation and selective 5′-phosphorylation. Recently, Bergstrom and Ruth [8] described a unique reaction leading to carbon-5 alkylation of pyrimidine nucleosides. We have found that this reaction is applicable to the direct synthesis of 5-substituted styryl derivatives of dUrd [9]. Moreover, the reaction is useful for the direct alkylation of unprotected dUMP in protic solvents affording high yields of 5-substituted dUMP derivatives. Thus, a new series of 5-substituted derivatives of dUrd and dUMP has now become available as biochemical probes and as potential anticancer and antiviral agents.

The specific derivatives synthesized in this study were designed to examine structural factors that contribute to affinity for thymidylate synthetase. The two-stage mechanism depicted in Scheme 1 illustrates reversible binding and reactivity as the sequential requirements for high enzyme affinity in any potential inhibitor. Accordingly, enhanced reactivity toward nucleophilic addition of the enzyme to carbon-6 of the pyrimidine ring is not, by itself, sufficient to assure high affinity. Reversible binding of the enzyme and inhibitor in the non-covalently bonded complex (E·I) must occur prior to reaction leading to the covalent E–I complex. In earlier studies using 5-benzyloxymethyl-dUMP, we proposed that bulky groups at carbon-5 do not hinder enzyme binding and could, in fact, be used to advantage for selective inhibition [10]. Although this proposal was not completely acceptable on the basis of QSAR analysis data [11], subsequent studies using 5-[4-methyl-1,2,3,4-tetrahydroquinoxalyl]-methyl-dUMP (K_i/K_m = 0.23) have supported the view that large substituents at C-5 should be further explored [12]. Knowledge of the magnitude of bulk interference to binding is vital to the development of effective chemotherapeutic agents if selectivity is to be derived from differences in the tertiary structure of the enzyme in regions near the active site.

5(E)-Styryl-dUMP (compound **1b**, see Table 1) was found to be a potent reversible inhibitor of the enzyme from *Lactobacillus casei*. If **1b** forms a reversible E–I covalent complex, then the addition of electron withdrawing groups should enhance the reactivity of carbon-6 to nucleophilic addition. The subsequent shift in the equilibrium should be reflected in a lower inhibition constant (K_i). On the other hand, if there are no significant differences between the K_i values of the unsubstituted styryl derivatives and of those bearing strong electron withdrawing groups, one must conclude that the inhibition is simply a result of the equilibria expressed in the first step of Scheme 1 between the enzyme and inhibitor leading to non-covalent E·I.

The logical choice for such a substituent, that is compatible with the synthetic chemistry, is the nitro group. The substituent constants for nitro are + 0.71 for *meta* (**2b**) and + 0.78 for *para* (**3**) substitution. While both the *meta* and *para* substituents should exert an inductive effect to enhance enzyme nucleophile addition to carbon-6 of the pyrimidine ring, the *para* nitro derivative (**3**) should have slightly greater enzyme affinity (lower K_i) than **2b** and considerably more than **1b** by virtue of the resonance stabilization afforded the resulting anionic E–I covalent complex (Scheme 1).

The phenylethyl derivative **4b** was prepared for two basic reasons: (1) to verify whether resonance with the phenyl ring such as in the styryl derivative enhanced enzyme affinity, and (2) because alkyl substituents (ethyl, propyl) at C-5 have yielded selective antiviral agents.

A fifth analog, 5(E)-(3-azidostyryl)-dUMP (**5**), was prepared as a photoaffinity labeling reagent after the high enzyme affinity of compounds **1b**, **2b**, and **3b** was confirmed.

METHODS

Thymidylate synthetase purified from methotrexate resistant *L. casei* was purchased from the New England Enzyme Center, Tufts University (Boston, MA), at a specific activity of 1.1 μmoles dTMP formed per min per mg of protein, using the radioisotope assay. The crude preparation was purified according to the method of Galivan *et al.* [13] to give enzyme with a specific activity in excess of 3 μmoles of ^3H_2O formed per min per mg of protein. The enzyme was activated by dialysis for 4 days at 4° against 0.1 M potassium phosphate (pH 6.8) containing 50 mM mercaptoethanol and assayed by reported procedures [14]. The substrate [5-^{3}H]-2′-deoxyuridine 5′-phosphate, at a specific activity above 15 Ci/mmole, was purchased from Moravek Biochemicals, Industry, CA and diluted with cold substrate purchased from the Sigma Chemical Co., St. Louis, MO, to give a specific activity of 500 μCi/μmole. The cofactor, *dl*-tetrahydrofolic acid, also was purchased from the Sigma Chemical Co.

Chemical photolysis studies. The chemical photolysis studies were done in a Rayonet model RPR 100 photochemical reactor using light rated as maximum emission at 310 or 350 nm wavelength. The samples were photolyzed in 1 cm^2 silica cuvettes placed in a rotating stand at the center of the apparatus which ensured even light exposure. The temperature was maintained in the range 25–28° by air cooling.

An aqueous solution of 5(E)-(3-azidostyryl)-2′-deoxyuridine 5′-phosphate (**5**, approximately 0.1 mM) was photolyzed at 310 nm using seven lamps or at 350 nm using six lamps. The decomposition was monitored by removing the sample at appropriate times and scanning the u.v. spectrum from 400 to

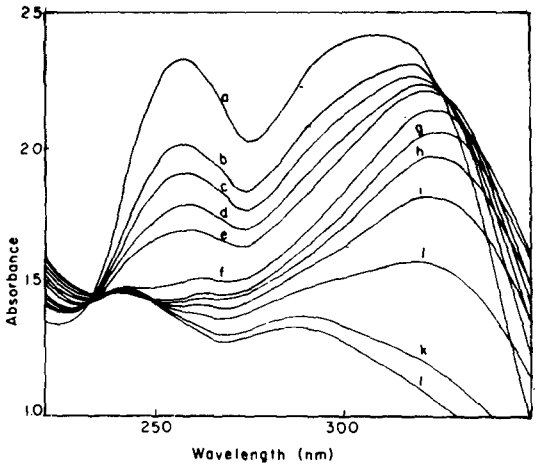


Fig. 1. Ultraviolet spectra of a neutral aqueous solution of 0.12 mM 5(E)-(3-azidostyryl)-2′-deoxyuridine 5′-phosphate (**5**) at the times indicated (min) after irradiation of the solution as described in Methods using six lights of 350 mm wavelength: a = 0, b = 0.2, c = 0.4, d = 0.7, e = 1, f = 2, g = 4, h = 8, i = 15, j = 30, k = 60 and l = 70 min.

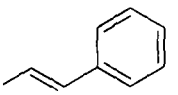
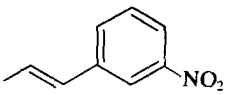
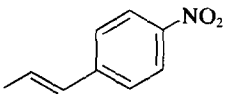
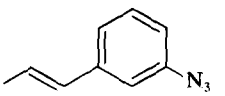
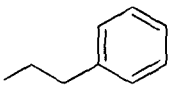
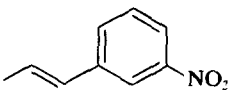
220 nm. As a control the samples were exposed to room light for 5 hr; no decomposition could be detected. The photolysis solutions were simultaneously resolved by h.p.l.c. on a Partisil strong anion exchange column using 0.005 M potassium phosphate buffer, pH 5.0, as eluent. Under these conditions compound **5** had a retention time of 9.6 min, and 5(E)-(3-aminostyryl)-2′-deoxyuridine 5′-phosphate, a reference compound, eluted at 3.5 min. The u.v. spectra of solutions of compound **5** irradiated at 350 nm are illustrated in Fig. 1.

Antiviral and antimetabolic assays. The methodology for measuring the inhibition of virus-induced cytopathogenicity in primary rabbit kidney (PRK) cell cultures, and for measuring the inhibition of incorporation of 2′-deoxythymidine or 2′-deoxyuridine into DNA of these cells, has been described previously [15, 16]. In the present experiments, however, [1′,2′-^{3}H]-2′-deoxyuridine (sp. act. 42 Ci/mmole), and not [2-^{14}C]-2′-deoxyuridine, served as radiolabeled 2′-deoxyuridine; it was added at 0.25 μCi, or 6 pmoles per 10^5 PRK cells per well (Linbro microplates).

RESULTS

The nucleotides **1b**, **2b**, **3**, **4b** and **5** were examined for inhibition of thymidylate synthetase purified from *L. casei*. Thymidylate synthetase activity was monitored by measuring the amount of ^3H_2O formed during the conversion of [5-^{3}H]-dUMP to dTMP [14]. The substrate K_m for these studies varied from 4.06 to 6.82 μM; enzyme concentrations of 10–30 nM were used in these assays. The results (Table 1) show that the styryl derivatives were potent thymidylate synthetase inhibitors with K_i values in the range of 0.21 μM for 5(E)-(3-nitrostyryl)-dUMP (**2b**), the most effective inhibitor, to 0.39 μM for the unsubstituted styryl compound **1b**. Since the K_m did show some variation in these studies, the K_i/K_m ratio

Table 1. Inhibition of thymidylate synthetase purified from amethopterin resistant *L. casei*

Compound	5-Substituent	2′-Substituent	K_i (μM)	K_i/K_m
5(E)-Styryl-dUMP (**1b**)		H	0.39	0.080
5(E)-(3-Nitrostyryl)-dUMP (**2b**)		H	0.21	0.035
5(E)-(4-Nitrostyryl)-dUMP (**3**)		H	0.34	0.050
5(E)-(3-Azidostyryl)-dUMP (**5**)		H	0.35	0.060
5-(2-Phenylethyl)-dUMP (**4b**)		H	1.63	0.31
5(E)-(3-Nitrostyryl)UMP		OH	1000	247

affords a better overall comparison of relative potency. By this measure the 3-nitro derivative **2b** was twice as potent as the unsubstituted analog **1b**, while the 4-nitro (**3**) and 3-azido (**5**) compounds exhibited intermediate activity. These values were estimated from a Lineweaver–Burk double reciprocal plot and the inhibition of enzyme by compound **5** was clearly competitive with respect to the substrate; this was true for all inhibitors used in this study. Reduction of the olefinic double bond in **1b** to give the phenylethyl derivative **4b** results in a 4-fold decrease in affinity (Table 1). Compounds **1b**, **4b** and **5** did not inactivate the enzyme on prolonged incubation in the absence of substrate and cofactor.

It was anticipated that the analogous ribose nucleotides would be poor inhibitors of thymidylate synthetase. This was verified for 5(E)-(3-nitrostyryl)-uridine 5′-phosphate which had a calculated K_i of 1 mM.

5(E)-(3-Azidostyryl)-2′-deoxyuridine 5′-phosphate (**5**) was found to be a photoaffinity labeling reagent for thymidylate synthetase*. Both rate saturation kinetics and substrate protection were evident when **5** and the enzyme were photolyzed at 350 nm. The chemistry of the photodecomposition reaction of **5** was studied by recording the changes in the u.v. spectra of **5** at various times after photolysis (Fig. 1). The same pattern was observed using light of 310 or 350 nm emission. In Fig. 1 the spectrum of **5** at time zero shows two broad maximum absorption bands at 310 and 257 nm and a minimum at 275 nm. Exposure to 350 nm light caused a rapid decrease in the 257 nm absorption maximum and a shift of the 310 nm band to 325 nm. These changes were followed by a much slower decrease in the new absorption band at 325 nm. The absorbance decreases at 257 and 310 nm shared the isosbestic absorption point at 228 nm. The appearance of a new isosbestic point at 251 nm was associated with the decrease in absorption at 325 nm. The half-life for the initial 350 nm light photodecomposition of **5**, calculated from the decrease in absorption at 257 nm, was 0.51 min; using seven lamps of 310 nm emission, the calculated half-life was 0.22 min. High pressure liquid chromatographic analysis of the initial reaction induced by 350 nm light photolysis showed a corresponding decrease in the concentration of **5** (retention time 9.6 min) with the appearance of four new peaks: three minor products of 3.5, 5.5 and 7.3 min retention time and the major product at 8.3 min. The first compound eluted had the same retention time as 5(E)-(3-aminostyryl)-2′-deoxyuridine 5′-phosphate. This material reached a maximum and steady-state concentration after 1 min of irradiation and represented less than 10 per cent of the total material.

The second photolytic reaction (ΔA_{325}) had a half-life of 18 min, using 350 nm light. High pressure liquid chromatographic analysis of the reaction at 2, 4, 8, 30, 60 and 70 min exposure to light showed a decrease of the major product of the initial photodecomposition (8.3 min peak), a corresponding

Table 2. Antiviral activity of 5-substituted 2′-deoxyuridines in PRK cell cultures

Compound	MTD* (μg/ml)	ID_{50}† (μg/ml)						
		HSV-1 KOS	HSV-1 F	HSV-1 McIntyre	HSV-2 Lyons	HSV-2 G	HSV-2 196	Vaccinia
5(E)-Styryl-dUrd (**1a**)	200	40	30	30	70	40	70	20
5(E)-(3-Nitrostyryl)-dUrd (**2a**)	100	>100	>100	>100	>100	>100	100	100
5(E)-(3-Azidostyryl)-dUMP (**5**)	200	>200	>200	>200	>200	200	200	20
5-(2-Phenylethyl)-dUrd (**4a**)	>400	15	10	30	15	15	10	>400
5-Iodo-dUrd	>400	0.1	0.1	0.1	0.2	0.2	0.2	0.2

* Minimal toxic dose or dose causing a microscopically visible alteration of cell morphology.
† Inhibitory dose required to reduce virus-induced cytopathogenicity by 50 per cent.

* E. De Clercq, J. Balzarini, C. T-C. Chang, C. F. Bigge, P. Kalaritis and M. P. Mertes, *Biochem. biophys. Res. Commun.* (in press).

Table 3. Antimetabolic activity of 5-substituted 2′-deoxyuridines in PRK cell cultures

Compound	ID_{50}* (μg/ml)	
	[Methyl-^{3}H]dThd incorporation	[1′,2′-^{3}H]dUrd incorporation
5(E)-Styryl-dUrd (**1a**)	20, 10	40, 50
5(E)-(3-Nitrostyryl)-dUrd (**2a**)	25, 30	40, 60
5(E)-(3-Azidostyryl)-dUMP (**5**)	75, 95	120, 150
5-(2-Phenylethyl)-dUrd (**4a**)	40, 30	70, 70
5-Iodo-dUrd	1, 1.2, 0.8	0.2, 0.3

* Inhibitory dose required to reduce dThd or dUrd incorporation by 50 per cent.

increase in the material with a retention time of 5.5 min, and no significant change in the minor 3.5 min peak.

Nucleosides **1a**, **2a**, **4a** and nucleotide **5** were examined as inhibitors of viral replication in primary rabbit kidney (PRK) cell cultures inoculated with vaccinia virus or herpes simplex virus (types 1 and 2). The inhibitory dose or concentration that afforded a 50 per cent reduction (ID_{50}) of virus-induced cytopathogenicity was used as a measure of antiviral potency. Table 2 lists the results of these studies. The phenylethyl derivative **4a** proved to be the most active antiherpes compound. It was equally effective against herpes simplex virus types 1 and 2 but it was ineffective against vaccinia virus. Nor was it effective against TK^- (thymidine kinase deficient) mutants of HSV-1 (data not shown). The unsubstituted styryl compound (**1a**) displayed some activity against herpes simplex virus and vaccinia virus which was unexpected, since the hydrogenated compound **4a** was inactive against vaccinia and the *meta* nitro-substituted compound **2a** was inactive against both vaccinia and herpes simplex. The azidostyryl derivative **5** was active against vaccinia but not herpes simplex. Cytotoxicity of this series of compounds was estimated by defining the minimal toxic dose (MTD) required to produce changes in cell morphology (Table 2). Only compound **4a** did not exhibit cytotoxicity at 400 μg/ml, the highest concentration tested. Thus, compound **4a** can be considered as fairly selective in its antiherpes action.

Agents that inhibit dUrd incorporation into DNA to a significantly greater extent than dThd incorporation are postulated to act by inhibition of thymidylate synthetase [16]. As shown in Table 3, none of the new compounds tested had a greater effect on dUrd incorporation than on dThd incorporation (as assayed in PRK cell cultures).

The toxic effects on L-1210 cell growth were examined at several inhibitor concentrations, and from these tests we calculated the doses causing a 50 per cent reduction in the number of viable cells (ID_{50}) (Table 4). The most effective compound was the azidostyryl nucleotide **5** which exhibited an ID_{50} of 80 μg/ml. Addition of either dThd or dUrd did not greatly alter the ID_{50} of compound **5**. As noted for PRK cells, all new compounds tested inhibited dThd incorporation into L-1210 DNA to a greater extent than dUrd incorporation. This was particularly evident for compound **5**, which reduced dThd incorporation by 50 per cent at a dose of 31 μg/ml; the equivalent reduction in dUrd incorporation required a 6-fold dose increase (193 μg/ml).

DISCUSSION

It is clear from the results in Table 1 that 5-styryl derivatives of dUMP have high affinity for *L. casei* thymidylate synthetase. The potency of these derivatives exceeded that of the 5-cyano derivative (K_i/K_m = 0.13) [17], which also is a reversible inhibitor. This contrasts with the more potent inhibition reported for the formyl [18], nitro [19, 20], fluoro [4], and trifluoromethyl [4] derivatives. The most obvious conclusion from these results is that large substituents on carbon-5 of the pyrimidine ring of dUMP are not a hindrance to high affinity, at least for the *L. casei* thymidylate synthetase.

If enzyme affinity of this series of compounds is a consequence of the equilibrium between E·I and E–I in Scheme 1, then the compounds contributing inductive (**2b**) and resonance (**3**) stabilization to the covalent E–I complex should be more potent inhibitors than the unsubstituted styryl compound **1b**. Evidence that the 5-styryl group lies in the same plane as the pyrimidine ring and should enhance enzyme addition to carbon-6 through the electronic effect is derived from the extended conjugation that is apparent in a red shift in the u.v. spectra of these derivatives. 5-Substituted dUMP derivatives with an sp^3 carbon substituent joined at this position have u.v. maxima at 270 nm or below. Substitution of an sp^2 (5-formyl-dUMP) or sp (5-cyano-dUMP) carbon leads to greater electron delocalization and a red shift in the maximum absorption band. As an example, the acidic or neutral solution spectrum of 5-formyl-dUMP has a maximum at 278 nm, while the corresponding band for 5-cyano-dUMP is at 276 nm. A pronounced inductive and resonance delocalization in the styryl derivatives is evident from their u.v. spectra. A red shift is predicted and follows the sequence: 268 nm [5-phenylethyl-dUMP, (**4b**)], 298 nm [5(E)-(3-nitrostyryl)-dUMP (**2b**)], 305 nm [5-styryl-dUMP, (**1b**)] and 363 nm [5(E)-(4-nitrostyryl)-dUMP (**3**)] [9]. An electronic effect of this nature should be reflected in a higher enzyme affinity by the *para*-nitro derivative **3**, with decreasing potency in the order styryl **1b**, *meta*-nitrostyryl **2b**, and phenylethyl **4b** substituents. From the similarity in the K_i/K_m ratios it appears that the formation of the covalent E–I complex is doubtful.

It is significant that reduction of the olefinic bond

Table 4. Inhibitory effects of 5-substituted 2′-deoxyuridines on L-1210 cell growth and metabolism

Compound	ID_{50}* (μg/ml)				
	for cell growth	for cell growth upon addition of dThd (5 μg/ml)	for cell growth upon addition of dUrd (125 μg/ml)	for [methyl-^{3}H]dThd incorporation	for [2-^{14}C]dUrd incorporation
5(E)-Styryl-dUrd (**1a**)	280	289	280	75 (40–110)	270 (210–330)
5(E)-(3-Nitrostyryl)-dUrd (**2a**)	>1000	>1000	>1000	75	>1000
5(E)(3-Azidostyryl)-dUMP (**5**)	80	94	67	31	193
5-(2-Phenylethyl)-dUrd (**4a**)	>1000	>1000	>1000	110 (75–150)	600 (440–880)
5-Iodo-dUrd	61.2	125	61.2	4.27	0.82

* Inhibitory dose required to reduce the number of (living) cells by 50 per cent or to reduce [methyl-^{3}H]dThd or [2-^{14}C]dUrd incorporation by 50 per cent. All data represent average values for four to five experiments. In some cases, the range of individual values is indicated in parentheses.

of 5-styryl-dUMP to give the phenylethyl derivative (**1b** → **4b**) leads to a 5-fold decrease in affinity. It also appears that the presence of the phenyl group in 5-phenylethyl-dUMP (**4b**) is responsible for an increased affinity: the K_i/K_m ratio for 5-ethyl-dUMP is 4.4 (*Escherichia coli* thymidylate synthetase) [21], as compared to 0.31 for 5-phenylethyl-dUMP (*L. casei* thymidylate synthetase).

If thymidylate synthetase is a key enzyme and perhaps a control point for DNA synthesis, then it must have high discrimination for the 2-deoxyribosyl substrate and not utilize ribosyl analogs which are diverted into RNA metabolism. This premise is supported by the fact that 5(E)-(3-nitrostyryl)-UMP has 7000 times less affinity (Table 1) than the 2′-deoxy derivative **2b** for *L. casei* thymidylate synthetase.

5(E)-(3-Azidostyryl)-dUMP (**5**), designed to be a photoaffinity labeling reagent, has fulfilled this premise [22,*] for thymidylate synthetase. The chemical photolysis experiments at both wavelengths show, from the changes in the u.v. spectra (Fig. 1), that two photochemical reactions are occurring. The most rapid reaction ($T_{1/2} < 1$ min) was followed by a decrease in the 257 nm absorption band and is reasonable for conversion of the arylazide to a nitrene. This chemically reactive function is known to react in both the singlet and triplet excited states to give a variety of products [23]. The second reaction was characterized as a shift in the isosbestic point from 232 to 251 nm during the reaction. The nature of the change, a slow but considerable decrease in absorbance, suggests photohydration of the 5–6 double bond of the pyrimidine ring of the initial product [24–26].

High pressure liquid chromatographic analysis of the reaction at each time point in Fig. 1 confirmed the sequential nature of the two reactions. The initial photolysis reaction through the first 4 min showed the breakdown of **5** yielding a major unidentified peak with a slightly shorter retention time and three minor products, one of which had the same retention time as 5(E)-(3-aminostyryl)-dUMP [9]. It is recognized that the photolytic decomposition of arylazides gives low yields of arylamines via hydrogen abstraction by the intermediate nitrene [27]. Analysis by h.p.l.c. of the second and much slower reaction showed an increase in the peak at 5.5 min retention time at the expense of the initial photolysis reaction product.

Based on these findings and previous studies,* it is proposed that the photolytic inactivation of thymidylate synthetase by 5(E)-(3-azidostyryl)-dUMP (**5**) is caused by reaction of the nitrene derivative in the reversible E·I complex with the enzyme to give a covalent complex.

The relationship of thymidylate synthetase inhibition and antiviral activity of 5-substituted 2′-deoxyuridines is not immediately obvious. Some patterns have emerged, however, that are useful in

* E. De Clercq, J. Balzarini, C. T-C. Chang, C. F. Bigge, P. Kalaritis and M. P. Mertes, *Biochem. biophys. Res. Commun.* (in press).

predicting antiviral selectivity.* Some of these compounds, i.e. the 5-trifluoromethyl, 5-nitro, and 5-carboxaldehyde derivatives, are non-specific inhibitors of vaccinia and herpes simplex virus replication. In fact, these compounds interfere with dUrd incorporation into DNA of the host cells, at a concentration that is lower than the effective antiviral concentration. The *in vivo* thymidylate synthetase index shows that, for these compounds, dUrd incorporation is inhibited to a greater extent than dThd incorporation. It is reasonable to conclude that their mechanism of action may, at least partially, be accounted for by thymidylate synthetase inhibition. The 5-carboxaldehyde oxime and the 5-cyano derivatives may also be considered as non-selective antiviral agents by virtue of their effect on thymidylate synthetase. The decreased antiviral potency of both compounds is paralleled by a proportional decrease in the thymidylate synthetase indexes.

5-Iodo- and 5-azidomethyl-dUrd are active against vaccinia and herpes simplex virus. Their low *in vivo* thymidylate synthetase indexes (~2), however, suggest that inhibition of this enzyme is probably not the principal mechanism for the antiviral action.

The extremely high antiherpes activity of (E)5-(2-bromovinyl)-dUrd and the uniquely selective action of 5-propyl-dUrd and 5-methylthiomethyl-dUrd and its sulfone, according to their thymidylate synthetase indexes, cannot be attributed to thymidylate synthetase inhibition. From the current study, 5-styryl-dUrd (**1a**) has proven to be a relatively weak and non-selective inhibitor of both vaccinia and herpes simplex virus replication. This antiviral activity does not appear to be due to *in vivo* thymidylate synthetase inhibition. 5-Phenylethyl-dUrd (**4a**) is an effective and rather selective inhibitor of herpes virus replication. From the evidence at hand, it resembles 5-methylthiomethyl-dUrd and 5-propyl-dUrd, which are thought not to act by inhibition of thymidylate synthetase.

For a series of nine 5-substituted (fluoro, trifluoromethyl, nitro, ethynyl, formyl, l-chlorovinyl, carboxaldehyde oxime, thiocyano, and cyano) deoxyuridines, we have recently established a strong correlation between thymidylate synthetase inhibitory potency and inhibition of L-1210 cell proliferation.† Thus, the most effective antitumor agents proved also to be the most potent *in vitro* and *in vivo* thymidylate synthetase inhibitors, and it was suggested that the thymidylate synthetase may serve as the principal, if not the sole, intracellular target for the antitumor activity of 5-substituted 2′-deoxyuridines. With one exception (**5**), it would appear from the low activity of the styryl derivatives as L-1210 cell growth inhibitors (Table 4) that *in vitro* thymidylate synthetase activity does not guarantee *in vivo* activity. Obviously, to be effective *in vivo*, the nucleoside must penetrate the cell membrane intact, be a good substrate for the enzyme(s) that catalyzes 5′-phosphorylation (thymidine kinase), and not be susceptible to glycosidic bond cleavage. Apparently these barriers are not insurmountable since several of the aforementioned thymidylate synthetase inhibitors have excellent *in vivo* activity.

The dramatic photo-dependent increase in the potency of 5(E)-(3-azidostyryl)-dUMP (**5**) as an inhibitor of vaccinia viral replication and L-1210 cells, coupled with the photo-dependent enzyme inactivation and photodecomposition studies, suggest that further studies are essential to establish that the mechanism for *in vivo* action is based on inhibition of thymidylate synthetase.* However, if true it agrees with previous studies of 5-substituted-dUrd derivatives where the nitro and formyl compounds are proposed to act by this mechanism [15, 16] and where a strong correlation has been found between inhibition of L-1210 cell growth and inhibition of thymidylate synthetase.†

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