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Interactions of 2′-fluoro-substituted dUMP analogues with thymidylate synthase

Przemysław Ziemkowski [a], Krzysztof Felczak [a], Jarosław Poznański [a], Tadeusz Kulikowski [a], Zbigniew Zieliński [b], Joanna Cieśla [b], Wojciech Rode [b,*]

[a] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5A Pawińskiego Street, 02-106 Warszawa, Poland*
[b] *Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Street, 02-093 Warszawa, Poland*

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Abstract

A series of 2′-fluoro-substituted dUMP/FdUMP analogues were synthesized, their interaction with human recombinant thymidylate synthase investigated, and structural ^{1}H and ^{19}F NMR study of the corresponding nucleosides performed. While 2′-F-dUMP (fluorine in the "down" configuration), in striking contrast to 2′-F-*ara*-UMP (fluorine in the "up" configuration) and 2′,2′′-diF-dUMP, showed substrate activity, 2′-F-*ara*-UMP and 2′,2′′-diF-dUMP were classic inhibitors, and 2′,5-diF-*ara*-UMP behaved as a strong slow-binding inhibitor, suggesting the 2′-F substituent in the "up" position to interfere with the active center cysteine thiol addition to the pyrimidine C(6) and the pyrimidine C(5)-F to prevent this interference. In support, the direct through space heteronuclear coupling J_{HF} was observed for the fluorine "up" derivatives, 2′-F-*ara*-U and 2′,5-diF-*ara*-U, causing the splitting of the H(6) resonance lines. The absence of such splitting in 2′,2′′-diF-dUrd, indicating an unusual orientation of the base in relation to the furanose, was associated with an exceptionally weak interaction with the enzyme.

Keywords: Thymidylate synthase; 2′-Fluoro-substituted dUMP analogues; NMR

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the dUMP (2′-deoxyuridine 5′-monophosphate) methylation reaction involving a concerted transfer and reduction of the one-carbon group of N^5,N^{10}-methylenetetrahydrofolate (CH_2FH_4), with concomitant production of thymidylate and dihydrofolate [1]. As the sole intracellular source of *de novo* synthesized TMP, the reaction is a target in cancer chemotherapy [2,3] and its inhibitors, between them dUMP analogues, 5-fluoro-dUMP (5-F-dUMP) and 5-trifluoromethyl-dUMP (5-CF_3-dUMP), are active forms of drugs, such as 5-F-Ura, 5-F-dUrd, 5-F-Cyt and 5-CF_3-dUrd [4].

Tumor fluoropyrimidine sensitivity may be influenced by pyrimidine phosphorylases-catalyzed phosphorolysis of pyrimidine/fluoropyrimidine (deoxy)nucleosides, yielding pyrimidine base and (deoxy)ribose-1-phosphate [5]. As 2′-fluoro-substituted fluoropyrimidine (deoxy)nucleosides with the substituent the *ara* ("up") configuration appear to be resistant to the phosphorolysis, presumably through strengthening the *N*-glycosyl linkage [6,7], influences of differently oriented 2′-fluoro substituents in dUMP and its analogues on interaction with TS are of interest. With respect to the latter, while the 2′-fluoro substituent in the *ribo* ("down") configuration introduced into dUMP molecule did not prevent substrate activity in the TS-catalyzed reaction [8], the 2′-fluoro substituent in the *ara* configuration introduced into 5-F-dUMP molecule did not prevent time-dependent inactivation of TS by 2′-deoxy-2′,5-difluoro-1-β-D-arabino-UMP (2′,5-diF-*ara*-UMP), similar to that observed with 5-F-dUMP [9] but present in 2′-deoxy-2′-fluoro-5-phenyl-dUMP allowed only a strong classic competitive inhibition of TS-catalyzed reaction by the latter [10].

* Corresponding author. Fax: +48 22 822 5342.
E-mail address: rode@nencki.gov.pl (W. Rode).

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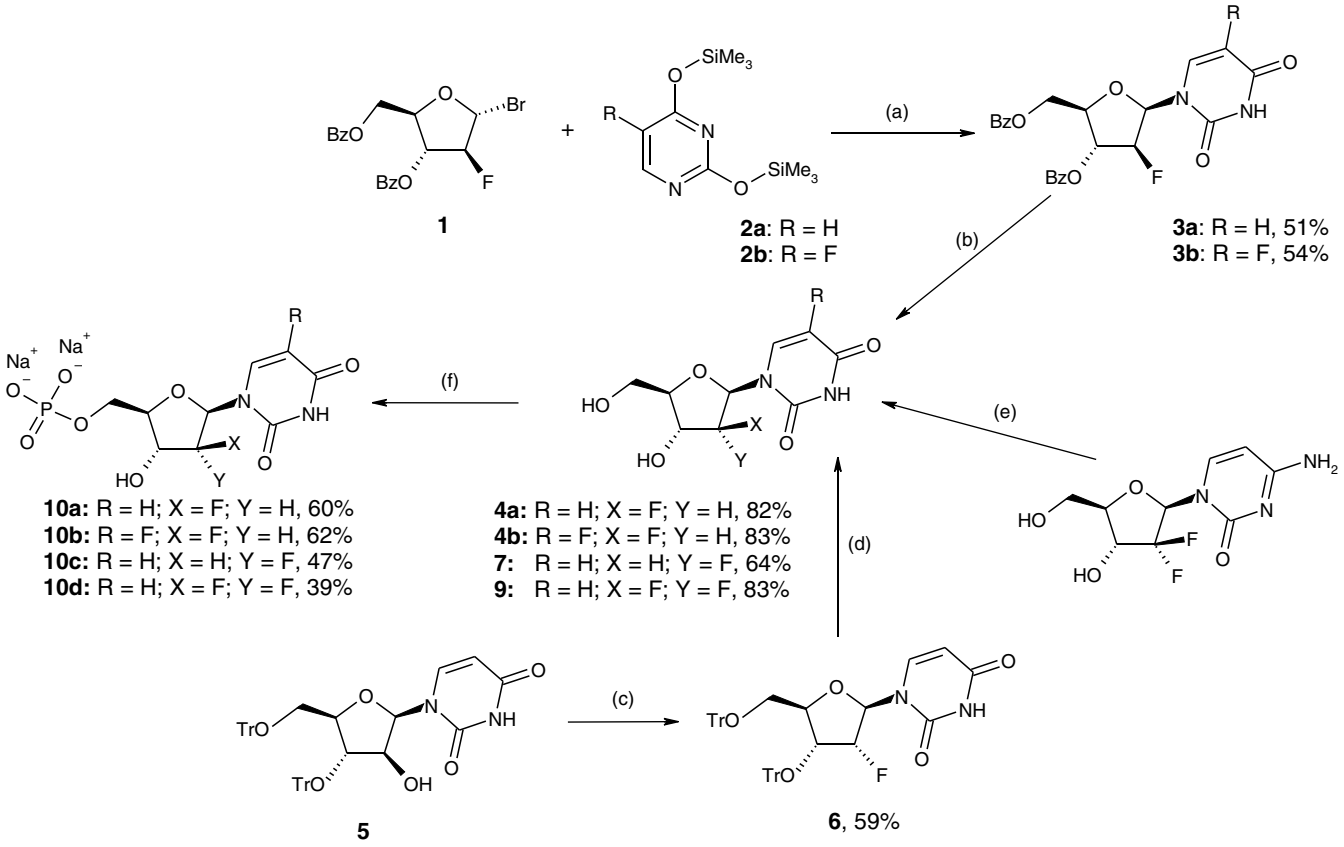

Fig. 1. Synthesis of 5′-phosphate-2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyluracil (**10a**), 5′-phosphate-2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-fluorouracil (**10b**), 5′-phosphate-2′-deoxy-2′-fluoro-1-β-D-ribofuranosyluracil (**10c**) and 5′-phosphate-2′-deoxy-2′,2′′-difluoro-1-β-D-ribofuranosyluracil (**10d**). Reagents and conditions are as follows: (a) MeOH, reflux; (b) *i*-$BuNH_2$, MeOH, reflux; (c) DAST, CH_2Cl_2, r.t.; (d) Dowex 50W H^+, MeOH, r.t.; (e) isoamyl nitrite, 0.1 N HCl – H_2O – 1,4-dioxane (1:1:1; v/v), 70 °C; (f) $POCl_3$, $PO(OEt)_3$, 0 °C.

In order to learn more about 2′-fluoro substitution influence on interaction of dUMP analogues with TS, a series of dUMP/5-F-dUMP derivatives, including three 2′-monofluoro analogues, with the 2′-fluoro substitution either in the *ara* (2′-F-*ara*-UMP and 2′,5-diF-*ara*-UMP) or *ribo* (2′-F-dUMP) configuration, and one 2′,2′′-difluoro analogue (2′,2′′-diF-dUMP) were synthesized (Fig. 1) and their interaction with human recombinant TS followed. In parallel, structural ^1H and ^{19}F NMR analyses of the corresponding nucleosides were performed.

Materials and methods

Chemistry. All details concerning organic synthesis and chemicals used are precisely listed in Supplementary Material available on-line.

NMR experiments and data analysis. The ^1H and ^{19}F spectra were recorded on 400 MHz Varian UX-NMR spectrometer in ~2 mM D_2O solution, pK ~ 7, at 298 K using 16k and 8k resolution, and 4 and 3 kHz band width, respectively. For ^1H spectra the presaturation of the resting water resonance was applied. The spectra were then processed with the aid of MestRe-C program [11] using 32k zero filling and Lorenzian filter resulting in 0.2 Hz line broadening. When required, for the precise estimation of small coupling constants, pure sine-bell, instead of Lorenzian, filter was used. Line positions were determined by parabolic interpolation with an error of ~0.05 Hz.

Chemical shifts and coupling constants (see Table 3 in Supplementary Material) were estimated by the locally made program ISSSS [12], based on the LAOCOON II [13] strategy using the determined positions of the individual resonance lines. For C(2′)-F substituted compounds the ^{19}F and ^1H data were analyzed simultaneously to decrease the biases in the determined heteronuclear $^3J_{HF}$ coupling constants.

The analysis of the furanose ring puckering was done with the aid of pseudorotation model implemented in PSEUROT 6.3 program [14] using the parameterization extended for the heteronuclear $^3J_{HF}$ coupling analysis [15]. This parameterization was then adapted for new topology records describing 2′-F-*ara*- and 2′,2′′-diF-2′-deoxy-*ribo* nucleosides. In order to decrease the calculation accuracy the average pseudorotation amplitude was used instead of the individual values determined separately for N and S pucker forms. As it was demonstrated previously [16], the pseudorotation amplitudes, τ_N and τ_S are strongly correlated, and the two values, X_S, $\tau = \frac{\tau_N+\tau_S}{2}$ sufficiently reconstruct the pattern of vicinal couplings in sugar moiety.

Structural analysis and molecular graphics were done with the aid of MolMol program [17].

Thymidylate synthase. Human TS was expressed in TS-deficient *Escherichia coli* TX61- strain (kindly supported by Dr. W.S. Dallas) as previously described [18] with the use of the plasmid construct pET17xb/hTS(LVAG) described by Pedersen-Lane et al. [19], and purified using the methods described for rat TS [20].

Enzyme activity assays. The [5-^{3}H]dUMP tritium release activity assay was performed as previously described [21]. For substrate activity, each analogue was substituted for dUMP in the enzyme reaction and absorbance at 338 nm monitored [22]. The dUMP analogues were added to the reaction mixtures as neutral aqueous solutions.

Kinetic studies. To identify the type of inhibition involved, the effects of the dUMP analogues on the dependence of reaction rate on dUMP concentration were analysed as previously reported [23].

Quantitative analyses of thymidylate synthase inhibition by 2′,5-diF-*ara*-UMP leading to time-dependent inactivation of the enzyme were performed as earlier described [21].

Statistically evaluated results. These are presented as means ± SEM or means ± % difference between the mean and each of the two results, followed by the number of experiments (*N*) in parentheses.

Results and discussion

*Synthesis of 2′-F-ara-U (**4a**) and 2′,5-diF-ara-U (**4b**)*

Recently published by Alauddin et al. [24] optimised procedure for condensation of 2,4-*bis*-*O*-(trimethylsilyl)-uracil **(2)** with 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide **(1)** provides protected 2′-F-*ara*-U **(3a)** with desired β configuration in high yield. We applied these conditions but $CHCl_3$ was used as solvent in nucleoside condensation. These conditions resulted in a very similar result with high yield and stereoselectivity. Debenzoylation of **3a** and **3b** was effected with the aid of *i*-$BuNH_2$ instead of *n*-$BuNH_2$ as it was described [25] for synthesis of L-analogue.

*Synthesis of 2′-F-dUrd (**7**)*

In general there are two possible routes for direct introduction of fluorine into 2′(R) position of ribonucleoside. The first one reported by Codington et al. [26] in 1961 is based on the nucleophilic opening of 2,2′-anhydro-1-*β*-D-arabinofuranosyluracil with anhydrous HF in 40–50% and modified [27] with the application of more convenient reagent Py HF. The second approach is based on direct fluorination of 3′,5′-di-*O*-protected *ara*-U [28]. We modified this protocol applying DAST and received similar results as previously reported [29]. After deprotection of **6** with Dowex 50W (H^+) in MeOH the desired nucleoside **7** was prepared in high yield.

*Synthesis of 2′,2″-diF-dUrd (**9**)*

The most convenient and straightforward method for synthesis of compound **9** is the deamination of commercially available Gemcitabine®. This goal was achieved after multistep purification with moderate yield using glacial acetic acid according to Hertel et al. [30]. Isoamyl nitrite is convenient, commercially available reagent which has been applied in our laboratory for deamination of heterocyclic amines for many years. So we tried to extend its application for deamination of cytosine nucleosides. To find the optimal condition for this process we check reaction with cytidine as substrate first. When this reaction was performed in 90 °C complete conversion to Urd resulted in 12 h but substantial amount of uracil was detected by TLC. To protect the glycosidic bond against cleavage we have lowered the temperature to 50 °C and complete deamination resulted after 72 h. In the case of gemcitabine we have found that reaction was slower. Due to increased glycosidic bond stability in 2′,2″-difluorosubstituted nucleoside [7] deamination was performed at 70 °C. As result **9** was received after 18 h in 83% yield without uracil contamination.

*Synthesis of nucleoside 5′-monophosphates (**10a–d**)*

5′-Monophosphates of the fluorinated nucleosides (**10a–d**) were prepared *via* phosphorylation according to the Yoshikawa protocol [31] in 39–62% yield.

Resonance assignment and spectral parameters

The resonance signals were assigned basing on the well known resonance pattern characteristic for the ribose moiety, and verified in details by the comparison of *in silico* and experimental spectra. All the spectral parameters estimated for the analyzed series of 2′ substituted ribose analogues of Urd and *ara*-U, and their parent, unsubstituted compounds, are collected in Table 3 (Supplementary Material). The reported values enable the perfect reconstruction of the recorded spectra (Figure 4 in Supplementary Material). The only problem arose with 2′,2″-diF-dUrd, for which an exchange process was observed, as evidenced by significant broadening of 2′F resonance line in the ^{19}F spectrum (Fig. 2). This effect is most probably connected with slow changes in the solvation of the fluorine atom, what is putatively coupled with the relatively slow *syn-anti* exchange.

Conformation of the sugar moiety

This was analyzed in terms of the equilibrium between two-state equilibrium of dominating puckered forms, C(2′)*endo* (S) and C(3′)*endo* (N) [32–34]. Their relative populations, pseudorotation angle and average puckering amplitudes were determined directly from homonuclear $^3J_{HH}$ and heteronuclear $^3J_{HF}$ vicinal coupling constants, using the Altona's parameterization implemented in PSEUROT program. The estimated conformational parameters are collected in Table 1 (the puckering diagram changes upon substitution of oxygen atom by the fluorine is presented in Figure 5 in Supplementary Material). The strong stabilization of the furanose form which is keeping the fluorine atom just in the plane of the ring is observed. This effect is assumed to be electrostatically-driven as the result of the interaction of fluorine with endocyclic oxygen, $O_{4'}$. Consequently, the fluorine substitution in the *ribo* configuration (in 2′-F-dUrd) causes the decrease of the S population, due to the boundaries exerted by the H(2′) proton exposed towards the base. Analogously, the fluorine in the *ara* position (in 2′-F-*ara*-U) is moved out of the base, causing stabilization of the S conformation. The double fluorine substitution (2′,2″-diF-dUrd) results in a balance of the electrostatic interactions. This effect is presented in Fig. 3, the τ/XN diagram depends on where the fluorine atom is substituted on C(2′), driving the conformational

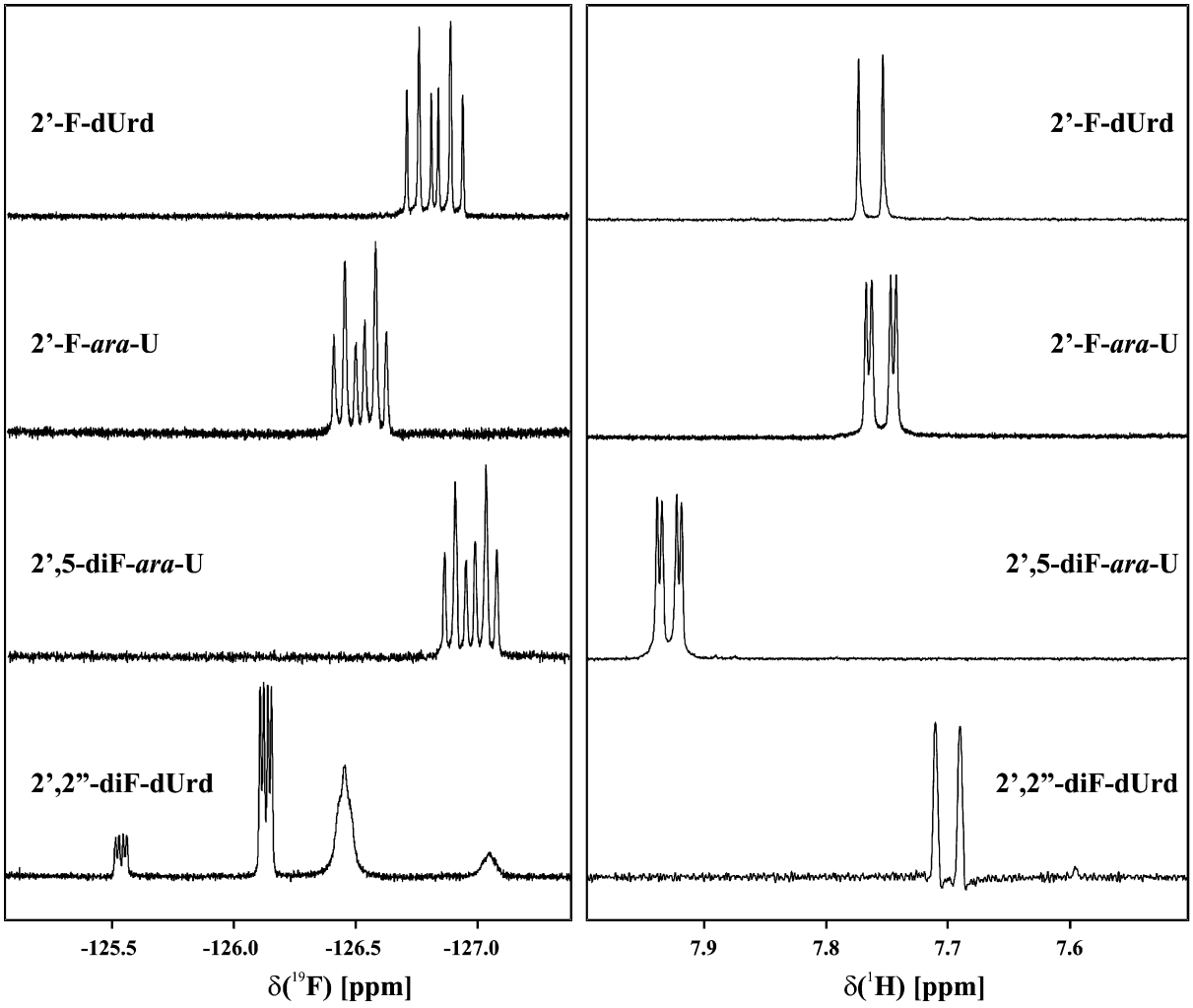

Fig. 2. Fragments of ^{19}F (left) and ^{1}H (right) NMR spectra indicative for the trough-space interaction of C(2′)-Fluorine and H(6) nuclei. The splitting of ^{1}H resonance lines in 2′-F-*ara*-U and 2′,5-diF-*ara*-U indicates that both atoms are in the close contact, while the absence of the splitting expected in 2′,2″-diF-dUrd accompanied by the significant broadening of the "up" fluorine resonance points towards the unusual conformation on the glycosidic bond.

Table 1
NMR-derived parameters describing the conformational equilibrium experienced by the sugar moiety in a series of C(2′)-fluoro-substituted Urd derivatives

	X_S	τ	g^+	t	g^-	Ng^+	Nt	Sg+	St	Sg^-	F12	F34
Urd	0.56	39.5	0.61	0.33	0.06	0.29	0.15	0.32	0.19	0.06	2.00	1.70
5-F-Urd	0.35											
2′-F-dUrd	0.31	40.4	0.67	0.34	0.00	0.46	0.23	0.21	0.11	0.00	1.96	1.96
dUrd	0.61	31.4	0.49	0.38	0.12	0.24	0.15	0.25	0.23	0.12	1.60	1.08
5-F-dUrd	0.61	33.7	0.44	0.33	0.23	0.26	0.13	0.18	0.20	0.23	2.04	0.87
2′,2″-diF-dUrd	0.58	52.4	0.62	0.34	0.03	0.27	0.14	0.35	0.20	0.03	1.90	1.75
ara-U	0.39	39.6	0.46	0.45	0.09	0.33	0.27	0.12	0.18	0.09	1.21	0.70
2′-F-*ara*-U	0.57	40.1	0.37	0.49	0.13	0.22	0.21	0.15	0.28	0.13	1.02	0.55
2′,5-diF-*ara*-U	0.55	40.1	0.38	0.47	0.14	0.24	0.21	0.15	0.26	0.14	1.10	0.56

changes in the opposite directions for *ara*- and *ribo*-nucleosides.

Exocyclic C(4′)–C(5′) rotamers

The original absolute assignment of the H(5′) and H(5″) protons in ^{1}H NMR spectra was based on the deshielding effect of the phosphate group on H(5′) and H(5″) in 3′-monophosphates of uridine and pseudouridine [35], and was later confirmed by stereospecific deuteration of adenosine and its derivatives [36]. In general, majority of nucleosides and nucleotides display a characteristic spectral pattern: $\delta(H5') > \delta(H5'')$ and $J_{4',5'} < J_{4',5''}$ [33]. Generally, despite the small differences, all the compounds present

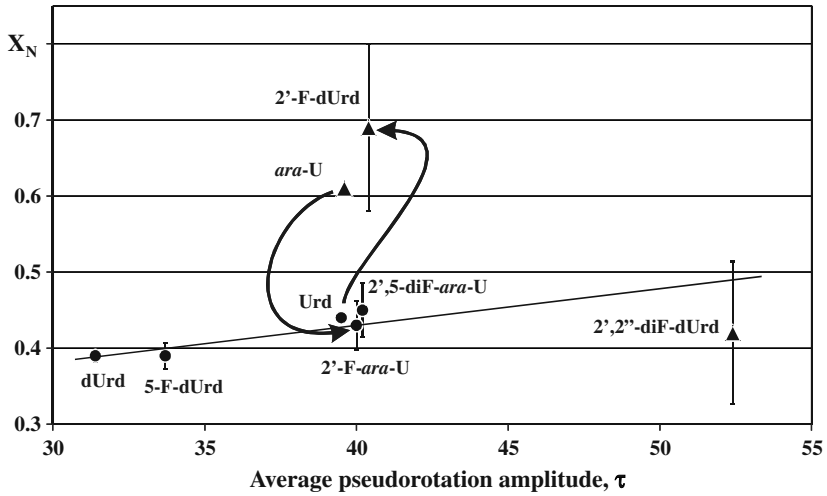

Fig. 3. The relation between the average pseudorotation amplitude and the population of S-type conformation. The observed correlation is resulting from the steric interactions between O(4′) and pyrimidyne ring undergoing for S-puckered sugars. The reverse effects of C(2′)-fluorine substitution in Urd and *ara*-U are marked by black arrows.

the equilibrium of three possible rotamers of the exocyclic group, $C_{5'}$(OH), and each population of a given rotamer falls in the range characteristic for the pyrimidine nucleoside *anti* configuration of glycoside bond. The intermolecular interactions of C(5′) exocyclic group with the proximal pyrimidine base result in uniform correlations between the separation of H(5′) and H(5″) resonance lines, $\Delta\delta(5'\,5'')$, and both vicinal $^3J_{4'5'}$ and geminal $^2J_{5'5''}$ scalar couplings.

Anti vs syn conformation of the glycoside bond

All spectral and conformational parameters point for the glycoside bond in the *anti* orientation. The distribution between populations of the three possible rotamers of the exocyclic group shows that the pyrimidine in *syn* conformation is negligibly populated. The dominance of the *anti* form is also pointed by the τ/XN relation. Moreover, for the fluorine *ara*- derivatives, 2′-F-*ara*-U and 2′,5-diF-*ara*-U, the direct through space heteronuclear coupling J_{HF}, resulting in the splitting of the H(6) resonance lines (Table 3, Supplementary Material; Fig. 2, right panel) is observed. This effect is absent, as expected, in the case of 2′-F-dUrd and, unexpectedly, also from 2′,2″-diF-dUrd. For the latter, the lack of the splitting clearly indicates the unusual orientation of the base in relation to the furanose moiety. This decrease in the steric restrictions results in an unusually high pseudorotation amplitude, elevated up to ~52°.

Finally, it must be concluded that for all analyzed compounds the fluorine substitution in sugar moiety changes the conformational equilibrium, but these effects are not so strong to preclude the molecules to adapt any structural form required for the efficient binding to the enzyme.

Interaction with recombinant human TS

Of the four analogues studied, only 2′-F-dUMP, in striking contrast to 2′-F-*ara*-UMP and 2′,2″-diF-dUMP, showed substrate activity, as observed by spectrophotometric monitoring at 340 nm of the reaction mixture with 2′-F-dUMP, $N^{5,10}$-methylenetetrahydrofolate and thymidylate synthase, characterized by maximal velocity (V_{max}) value of 0.87 ± 0.16 μmol/min/mg protein ($N = 4$), similar to that observed (under conditions of the same assay) with dUMP (0.87 μmol/min/mg protein ± 19% ($N = 2$), and K_m value of 12.2 ± 3.7 μM ($N = 4$), higher than that observed with dUMP [5.25 μM ± 31% ($N = 2$)].

Inhibition of human recombinant TS by competition vs dUMP, of 5-F-dUMP, 2′-F-*ara*-UMP, 2′,2″-diF-dUMP, and 2′,5-diF-*ara*-UMP, was examined with the use of [5-^{3}H]dUMP tritium release activity assay varying the dUMP concentration with different concentrations of inhibitor, added simultaneously to the reaction mixture. Competitive inhibition, reflected by intersection at the ordinate of a Lineweaver–Burk plots (not shown) and described by the apparent *K*i values presented in Table 2, was observed.

Only 2′,5-diF-*ara*-UMP, when preincubated with TS, in the presence of CH_2FH_4, caused time-dependent inactivation of the enzyme (not shown), consistent with the behavior as a slow-binding inhibitor [37]. The inactivation rate did not change during preincubation (Table 2).

Although 2′-F-dUMP (2′-fluoro substituent in the *ribo*-configuration), in accord with previously published results [8], showed a good TS substrate activity, its 2′-F-*ara*-U and 2′,2″-diF-dUrd congeners (2′-F-*ara*-UMP and 2′,2″-diF-dUMP) were not substrates, suggesting the 2′-fluoro substituent in *ara*-configuration to prevent the catalysis. However, 5-fluoro substitution of the pyrimidine ring of 2′-F-*ara*-UMP (in 2′,5-diF-*ara*-UMP) resulted in a compound capable of a strong slow-binding inhibition [9], similar to that exerted by 5-F-dUMP on human leukaemia CCRF-CEM enzyme-catalyzed reaction [38]. As the latter indicated 2′,5-diF-*ara*-UMP to interact with the enzyme in a way based on the mechanism of the reaction catalyzed by

Table 2
Inhibition of human recombinant thymidylate synthase by 2′-F-*ara*-UMP, 2′,2′′-diF-dUMP, and 2′,5-diF-*ara*-UMP (K_i^{app} values determined with dUMP as variable substrate)

Inhibitor	K_i^{app} (μM)	K_i/K_m	Inhibition type	Dependence of inhibition on time; slow-binding inhibition parameters, if time-dependent inhibition observed
2′-F-*ara*-UMP	10.3 ± 18% (2)	1.4	Competitive	No time dependence of inhibition observed
2′,2′′-diF-dUMP	432.8 ± 25.2 (3)	57	Competitive	No time dependence of inhibition observed
2′,5-diF-*ara*-UMP	1.37 ± 0.56 (4)	0.18	Competitive	Time-dependent inhibition $K_i = 4.77 \pm 1.32$ nM (3), $k_2 = 0.81 \pm 0.04$ min^{-1} (3)

TS, the pyrimidine 5-fluoro substituent presence appeared to activate that step of the interaction of the analogue with the enzyme that was prevented by the 2′-fluoro substituent in the *ara*-position. Of interest is that the effect of the pyrimidine C(5) fluorine, potentiating interaction of dUMP and its analogues with TS, results from the fluorine producing a local strain in the pyrimidine C(6) region, thus sensitizing C(6) to the Michael addition of the enzyme cysteine thiol [39,40]. In view of the foregoing, it seems probable that the 2′-fluoro substituent in the *ara*-position in 2′-F-*ara*-UMP or 2′,2′′-diF-dUMP interferes with the active center cysteine thiol addition to the pyrimidine C(6) and the fluorine substituent at pyrimidine C(5) prevents this interference. In this context of interest is the direct through space heteronuclear coupling J_{HF}, observed for *ara*-2′-fluorine derivatives, 2′-F-*ara*-U and 2′,5-diF-*ara*-U, causing the splitting of the H(6) resonance lines (Table 3, Supplementary Material and Fig. 2, right panel). Interestingly, the absence of such splitting in 2′,2′′-diF-dUrd, apparently indicating an unusual orientation of the base in relation to the furanose moiety, is associated with exceptionally low potential of 2′,2′′-diF-dUMP (over 40-fold lower than that of 2′-F-*ara*-UMP) to compete with dUMP for thymidylate synthase, pointing to the role of conformation of the sugar moiety and its orientation in relation to the base for the interaction with the enzyme.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.097.

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