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Design, Synthesis and Enzymatic Activity of Highly Selective Human Mitochondrial Thymidine Kinase Inhibitors

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Abstract—Highly selective arabinofuranosyl nucleosides, which inhibit the mitochondrial thymidine kinase (TK-2) without affecting the closely related herpes simplex virus type 1 thymidine kinase (HSV-1 TK), varicella-zoster virus thymidine kinase (VZV-TK), cytosolic thymidine kinase (TK-1) or the multifunctional *Drosophila melanogaster* deoxyribonucleoside kinase (*Dm*-dNK), have been obtained. SAR studies indicate a close relation between the length of the substituent at the 2′ position of the arabinofuranosyl moiety and the inhibitory activity.

The majority of nucleoside analogues in clinical use is phophorylated by human deoxyribonucleoside kinases (dNKs) and biochemical studies showed that there are four distinct dNKs in the human salvage pathway,[1] with different subcellular location: the thymidine kinase 1 (TK-1) cytosolic enzyme, the cytosolic/nuclear constitutively expressed deoxycytidine kinase (dCK), and deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK-2) both being constitutive mitochondrial enzymes. Among the deoxyribonucleoside kinases, TK-2 is the less characterized and up to now no selective nucleoside analogues recognizing TK-2, have been synthesized except for AraT. TK-2 is an interesting deoxyribonucleoside kinase for several reasons:

i. It phosphorylates pyrimidine deoxyribonucleosides such as dThd, dUrd, dCyd[2] and also the antiviral nucleoside analogues AZT and FIAU but at much lower rates than the natural substrates[2,3]
ii. The mitochondrial localization of TK-2 may be important for the mitochondrial toxicity observed for several pyrimidine nucleoside anti-viral analogues and may also represent a target to induce DNA depletion.
iii. TK-2 can be useful for gene therapy studies: the dNKs are investigated as suicide gene in anti-cancer chemotherapy.

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During a study aimed at discovering new potential inhibitors for dNKs, we have identified a new class of highly selective arabinofuranosyl nucleosides that interact with mitochondrial TK-2 without affecting the closely related HSV-1 TK, VZV TK, cytosolic TK-1 or *Dm*-dNK.[4] SAR studies indicate a close relation between the length of the substituent at the arabinofuranosyl moiety and their inhibitory activity. This is the first time that such highly effective compounds are described thus opening new perspectives in the design of selective mitochondrial TK-2 inhibitors. A new synthetic route, the biological activity, and the results of a molecular modeling study on the obtained 2′-*O*-acyl and -alkyl derivatives of the arabinofuranosyl nucleosides will be briefly reported.

Chemistry

The 2′-acyl derivatives **5a**–**c** and **6c**–**e** of BVaraU (**1**) and AraT (**2**), were obtained by reaction of the corresponding acyl halide on the 3′ and 5′ protected nucleosides (**3** and **4**). The deprotection at position 3′ and 5′ of the acylated derivatives, gave the final **7a**–**c** and **8c**–**e** in 30–90% yield (Scheme 1).

The 2′-octyl- and -benzyl-ethers were synthesized by a different synthetic strategy. The alternative synthetic pathway required the preparation of the parent 1-β-D-ribofuranosyl-thymine (**9**)[5] and the protection of 3′,5′-hydroxyl functions (**13**) with 1,2-dihydropyrane (DHP),

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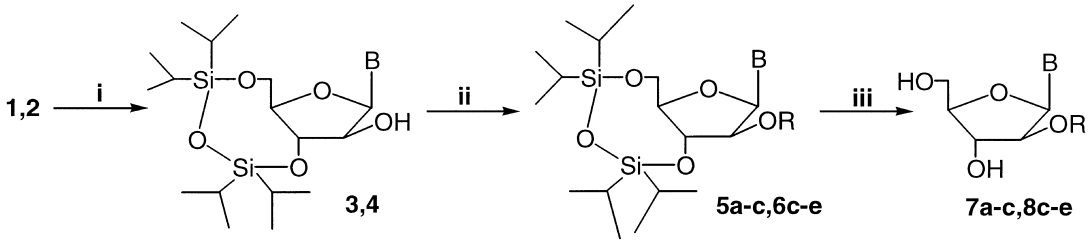
which was carried out on 2,2′-anhydro-1-(β-D-arabinofuranosyl)thymine (**11**), obtained from **9** as described by Schinazi et al.[6] (Scheme 2).

Finally, the 3′,5′ THP protected derivative **13** gave, by treatment with 0.1 M KOH in 95% ETOH, compound **15** (Scheme 2).[7] The 2′-octyl derivative of 3′,5′-protected AraT (**17**) was obtained using octyl iodide in the presence of NaH (Scheme 3). A milder procedure with benzylbromide at room temperature was adopted to obtain compound **19** because of the formation of side products. The next deprotection step was carried out with *p*-toluenesulfonic acid (TsOH) in MeOH to give, in 40% yield, the compounds **21** and **23**. The 2′-alkyl modification of the sugar moiety has been extended also to AraC. The preparation of compound **24** required a modified synthetic approach. Therefore, the synthetic steps leading to the 2′-alkyl-derivative of AraC were carried out starting on uridine (**10**) (Scheme 2). Compound **18** was then converted to the AraC derivative **24** by the procedure of Divakar and Reese[8] (Scheme 3).

Biology

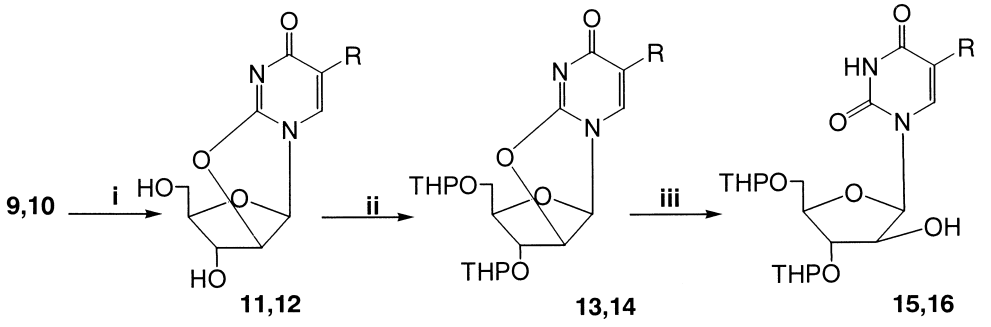
AraT is recognized as a good substrate by the nucleoside kinases encoded by HSV-1 and VZV and the mitochondrial TK-2 showed a lower sensitivity to the inhibitory activity of AraT than HSV-1 and VZV TK (Table 1).

The introduction of bulky acyl groups (i.e., decanoyl and dodecanoyl esters **8c** and **8e**) at the 2′-OH position of AraT increased the inhibitory effect of the derivatives on mitochondrial TK-2 by 10-fold, whereas the inhibition of HSV-1 and VZV TK by these compounds, was decremented by >50-fold. TK-1 was insensitive to AraT and the 2′-*O*-acyl AraT derivatives. Thus, introduction of a bulky acyl group at the 2′ position of AraT increased its selectivity by more than 500-fold for TK-2 versus the related viral thymidine kinases. Shortening the 2′-*O*-acyl chain to a pentanoyl (**8d**) resulted in a complete loss of its inhibitory activity against TK-2. The inhibitory activity of the 2′-*O*-alkyl-AraT derivatives (**21** and **23**) was lower than that found for the 2′-*O*-acyl ester derivatives (Table 1). 2′-*O*-Octanoyl and -decanoyl BVaraU derivatives (**7a** and **7c**) markedly increased the inhibitory effect of BVaraU on TK-2 7-fold (Table 1). As observed for the AraT derivatives, the 2′-*O*-acyl BVaraU derivatives lost their affinity for HSV-1 and VZV TK. The less bulky 2′-*O*-acyl derivative of BVaraU lost 10- to 20-fold activity against TK-2, HSV-1 TK and VZV TK. Again, TK-1 remained insensitive to all substituted BVaraU derivatives (Table 1). The 2′-*O*-alkyl-AraC (**24**) derivative did not show any increase in selectivity in comparison to AraC or the other 2′-*O*-acyl/alkyl derivatives. Despite the structural



1 = 1-β–D–arabinofuranosyl-5-(*E*)-(2-bromovinyl)-uracil; **2** = 1-β-D-arabinofuranosyl-thymine
3,5,7: B = 5-(*E*)-(2-bromovinyl)-uracil; **4,6,8:** B = thymine
a :R= $-CO(CH_2)_6CH_3$, **b** :R= $-COCH_2OCH_3$, **c** :R= $-CO(CH_2)_8CH_3$,
d :R= $-CO(CH_2)_3CH_3$, **e** :R= $-CO(CH_2)_{10}CH_3$

Scheme 1. (i) $TPDSCl_2$, pyr, rt; (ii) RCl, pyr, rfx; (iii) NH_4F, MeOH, Dowex H^+ form 50×2–100, rt.



9 = 1-β–D–arabinofuranosyl-thymine; **10** = uridine; **11,13,15:** R = CH_3 **12,14,16:** R = H

Scheme 2. (i) $(PhO)_2CO$, $NaHCO_3$, DMF, 150 °C; (ii) DHP, $TsOH{\cdot}H_2O$, CH_3CN, rt; (iii) KOH 0.1 M, EtOH, rt.

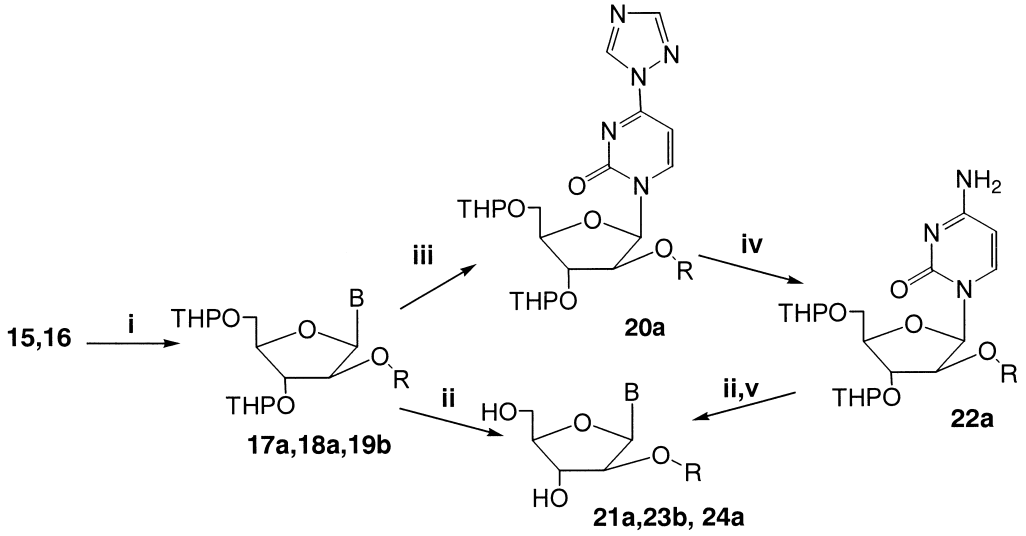
similarities between TK-2 and *Drosophila melanogaster* dNK,[9] no selectivity was achieved on this latter enzyme.

Molecular Modeling

Molecular modeling studies have been started on HSV-1 TK, *Dm*-dNK and TK-2 with the aim to develop a general model suitable for the description and prediction of the interaction of substrates with the different kinases. Because only the crystallographic data of HSV-1 TK are available,[10] the structure of *Dm*-dNK and TK-2 have been re-built by homology model techniques using the HSV-1 TK as template. The three enzymes show several structural analogies, and the amino acids involved in binding interactions are highly conserved. As reported in Table 2, the results of compounds docking into the active site of HSV-1 TK are in good agreement with the observed IC_{50}s thus validating our approach aimed at developing a possible general model for the interaction on structurally related nucleoside kinases. Ongoing studies will extend these results on *Dm*-dNK and TK-2.

Conclusions

In the present study, a synthetic approach devoted to obtaining 2′-*O*-acyl- and *N*-alkyl-substituted pyrimidine arabinosyl nucleosides has been developed with the aim of exploring substrate specificity requirements for dNKs. Inhibitory activity was investigated for cytosolic



B: **17,19,21,23** = thymine; **18** = uracil; **24** = cytosine

R: $CH_2(CH_2)_6CH_3$= **a**, Bn=**b**

Scheme 3. (i) NaH, $I(CH_2)_7CH_3$, THF, reflux or NaH, BnBr, THF, rt; (ii) MeOH, $TSOH{\cdot}H_2O$, rt; (iii) $POCl_3$, 1,2,4-triazole, TEA, 0 °C to rt; (iv) 30% NH_4OH, rt; (v) Dowex OH^- form 1×2–400.

Table 1. Inhibitory effects of AraT and BVaraU derivatives on 2′-deoxynucleoside kinases from different origins

	Compounds			IC_{50}[a] (μM)				
	X	R	Y	TK-1[b]	TK-2[c]	HSV-1 TK[d]	*Dm*-dNK	VZV TK[e]
BVaraU	–CH=CHBr	–H	–CO	>500	43±5.8	4.3±0.36	31.6±10.5	3.3±1.1
7a	–CH=CHBr	$-CO(CH_2)_6CH_3$	–CO	>1000	6.3±0.5	≥1000	178	718±59
7b	–CH=CHBr	$-COCH_2OCH_3$	–CO	>1000	402±234	84±1.8	—	35±1.5
7c	–CH=CHBr	$-CO(CH_2)_8CH_3$	–CO	>1000	6.8±0.7	>1000	163	845±30
AraT	CH_3	–H	–CO	>1000	285±94	24±3.1	65±28	17±8.7
8c	CH_3	$-CO(CH_2)_8CH_3$	–CO	>1000	27±2.3	≥1000	872	>1000
8d	CH_3	$-CO(CH_2)_3CH_3$	–CO	>1000	>1000	>1000	>1000	>1000
8e	CH_3	$-CO(CH_2)_{10}CH_3$	–CO	>1000	28±2	≥1000	>1000	>1000
21	CH_3	$-(CH_2)_7CH_3$	–CO	>1000	120±14	>1000	>1000	>1000
23	CH_3	$-CH_2(C_5H_6)$	–CO	>1000	801±71	>1000	>1000	>1000
AraC	–H	–H	$-NH_2$	>1000	>1000	>1000	5319	>1000
24	–H	$-(CH_2)_7CH_3$	$-NH_2$	>1000	>1000	>1000	—	>1000

[a]50% inhibitory concentration.
[b]Cytosolic thymidine kinase.
[c]Mitochondrial thymidine kinase.
[d]Herpes simplex virus type 1 thymidine kinase.
[e]Varicella-zoster virus thymidine kinase.

Table 2. Docking data[a]

Compounds	Position	el	el, sf	el, sf, en	IC_{50}[a] (mM)
BVDU	In	8,783,229	−4,657,439	−3,404,947	2.87±1.54[b]
BVaraU	In	8,529,401	−4,835,129	−3,613,954	4.3±0.36
7a	Out	10,877,780	−4,768,179	−2,627,095	>1000
7b	In	10,314,959	−5,372,671	−3,931,787	84±1.8
7c	Out	10,943,267	−3,770,147	−1,637,087	>1000
AraT	In	8,599,677	−3,594,713	−2,360,739	24±3.1
8c	In	11,780,963	−6,416,770	−4,760,073	>1000
8d	Out	10.323,786	−2,474,657	−0,707,513	>1000
8e	Out	11,706,303	−8,093,062	−5,723,524	>1000
21	Out	10,762,047	−3,862,358	−1,724,202	>1000
23	In	9,378,132	−5,187,049	−3,886,204	>1000
AraC	In	7,974,090	−3,749,570	−2,574,724	>1000
24	Out	11,242,538	−4,668,734	−2,687,980	>1000

[a]Binding energy was calculated using different terms: el, electrostatic energy; sf, surface term; en, entropic free energy.
[b]Data taken from ref 9.

thymidine kinase (TK-1), mitochondrial thymidine kinase (TK-2), herpes simplex virus type 1 thymidine kinase (HSV-1 TK), varicella-zoster virus thymidine kinase (VZV TK) and *D. melanogaster* deoxynucleoside kinase (*Dm*-dNK). Very interestingly, we have observed that substitution of pyrimidine arabinosyl nucleoside derivatives at the 2′-OH position of the sugar moiety by a bulky lipophilic acyl moiety markedly enhances their affinity for the mitochondrial enzyme (TK-2). In addition, it dramatically increases the selectivity of these compounds for TK-2 and shifts the capacity of these novel compounds from an efficient substrate (i.e., BVaraU) to an effective (competitive) inhibitor of the enzymatic reaction (i.e., 2′-*O*-acyl BVaraU).[5]

However, substitution of the acyl chains for an alkyl chain proved detrimental for the activity. Moreover, despite structural similarities between TK-2 and *Dm*-dNK, no selectivity was observed toward this latter enzyme thus precluding any possible application of the compounds on this interesting target for gene therapy. Anyway, this result is, in our opinion, of potential usefulness in the understanding of the structural requirements for the substrate selectivity among the thymidine kinase classes of enzymes. Moreover, the results of the docking of the study compound into the active site of HSV-1 TK are in good agreement with the observed IC_{50}s thus validating our approach aimed at developing a possible general model for the interaction on structurally related nucleoside kinases.

In summary, this study provides an interesting report on potent arabinofuranosyl nucleoside inhibitors of mitochondrial TK-2 that opens perspectives for the rational design of highly selective mitochondrial TK-2 inhibitors and, in general, of selective ligands for different kinases.

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