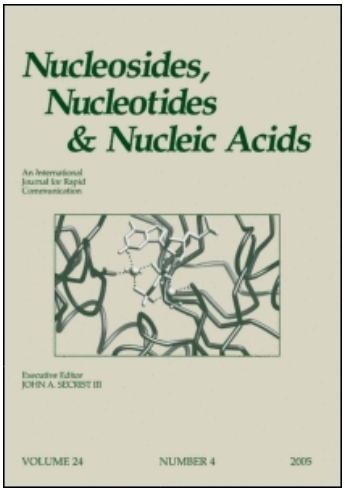

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Phosphorylation of Isocarbostyril- and Difluorophenyl-Nucleoside Thymidine Mimics by the Human Deoxynucleoside Kinases

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ABSTRACT

The thymidine mimics isocarbostyril nucleosides and difluorophenyl nucleosides were tested as deoxynucleoside kinase substrates using recombinant human cytosolic thymidine kinase (TK1) and deoxycytidine kinase (dCK), and mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK). The isocarbostyril nucleoside compound 1-(2-deoxy-β-D-ribofuranosyl)-isocarbostyril (EN1) was a poor substrate with all the enzymes. The phosphorylation rates of EN1 with TK1 and TK2 were <1% relative to Thd, where as the phosphorylation rates for EN1 were 1.4% and 1.1% with dCK and dGK relative to dCyd and dGuo, respectively. The analogue 1-(2-deoxy-β-D-ribofuranosyl)-7-iodoisocarbostyril (EN2) showed poor relative-phosphorylation efficiencies (k_{cat}/K_m) with both TK1 and dGK, but not with TK2. The k_{cat}/K_m value for EN2 with TK2 was 12.6% relative to that for Thd. Of the

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difluorophenyl nucleosides, 5-(1′-(2′-deoxy-β-D-ribofuranosyl))-2,4-difluorotoluene (JW1) and 1-(1′-(2′-deoxy-β-D-ribofuranosyl))-2,4-difluoro-5-iodobenzene (JW2) were substrates for TK1 with phosphorylation efficiencies of about 5% relative to that for Thd. Both analogues were considerably more efficient substrates for TK2, with k_{cat}/K_m values of 45% relative to that for Thd. 2,5-Difluoro-4-[1-(2-deoxy-β-L-ribofuranosyl)]-aniline (JW5), a L-nucleoside mimic, was phosphorylated up to 15% as efficiently as deoxycytidine by dCK. These data provide a possible explanation for the previously reported lack of cytotoxicity of the isocarbostyril- and difluorophenyl nucleosides, but potential mitochondrial effects of EN2, JW1 and JW2 should be further investigated.

Key Words: Deoxynucleoside kinases; Thymidine kinase; Deoxycytidine kinase; Thymidine analogues.

INTRODUCTION

Deoxyribonucleoside kinases catalyze the rate limiting phosphorylation of deoxynucleosides into their corresponding monophosphate forms, utilizing ribonucleoside triphosphates as phosphor donors. Four deoxyribonucleoside kinases have been characterized in mammalian cells, two of which, thymidine kinase (TK1) and deoxycytidine kinase (dCK), are found in the cytosol, whereas thymidine kinase (TK2) and deoxyguanosine kinase (dGK) are predominantly localized in the mitochondria.[1–4] Both TK1 and TK2 are pyrimidine specific, phosphorylating thymidine (Thd) and deoxyuridine (dUrd) to their monophosphate derivatives. In addition, TK2 uses deoxycytidine (dCyd) as a substrate. dCK has a broad substrate specificity, phosphorylating dCyd, dGuo and dAdo, whereas, the mitochondrial dGK is purine-specific, and phosphorylates dGuo and dAdo (for review, see Ref. [1]).

Deoxynucleoside kinases play a crucial role in chemotherapeutic treatment of cancer and viral infections. These enzymes catalyze the rate-limiting phosphorylation of the nucleoside-analogue pro-drugs into their cytotoxic phosphorylated forms. Interestingly, elevated levels of deoxynucleoside kinases are detected in proliferating cells such as cancer cells. These properties recruited great interest in the synthesis and biological evaluation of nucleoside analogues as possible candidate for cancer and viral therapy.[5]

Recently, the syntheses of several unnatural pyrimidine analogues as potential anticancer and antiviral therapeutic agents have been reported (Fig. 1). A group of compounds (EN1 and EN2) containing the isocarbostyril moiety (and its 7-I-derivative) as a replacement for the thymine base were designed.[6] Earlier, McMinn et al. reported that the triphosphate derivative of 7-propynylisocarbostyril nucleoside mimics is a template for replication by the Klenow fragment (KF) of *E. coli* DNA polymerase.[7] In synthetic DNA, this compound is efficiently forms a stable self-pair and the unnatural self-pairing resulted in an insufficient polymerase chain elongation, which could lead to cytotoxic effects in rapidly proliferating cancer cells.[7]

A second group of nonpolar hydrophobic isosteres of pyrimidine nucleoside mimics, based on the pioneering study of Moran et al.[8] has also been synthesized.[9] These compounds were characterized by a 2,4-difluorotoluene moiety as an analogue to thymine in Thd, where F is the isosteric replacement for O, and C–H groups replaces

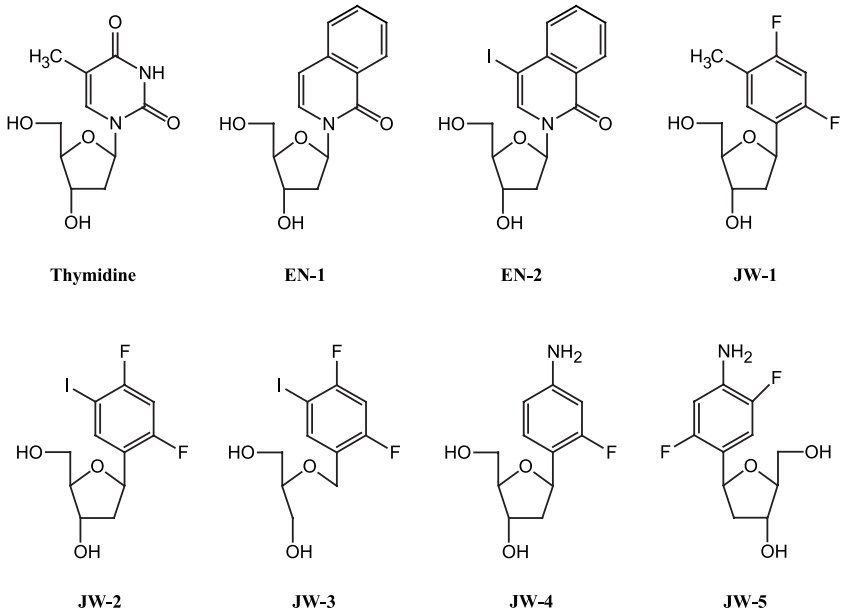

Figure 1. Chemical structures of thymidine and the nucleoside mimics EN1, EN2 and JW1–JW5.

N–H groups (compound JW1, [9]). In 1-(2-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-iodobenzene (JW2, 9), the CH_3 group was replaced with I, analogous to iodo-dUrd. The 2,4-difluorophenyl-5-iodo moiety was also attached to an acyclic 2′-deoxyribose ring similar to that found in ganciclovir (GCV) (JW3, [10]). Based on the same principle, 3-fluoro-4-[1-(2-deoxy-β-D-ribofuranosyl)] aniline (JW4) and 2,5-difluoro-4-[1-(2-deoxy-β-L-ribofuranosyl)]-aniline (JW5) were synthesized as dCyd mimics. In JW4,[10] the cytosine moiety was replaced by a 4-(3-fluoroaniline) ring system, whereas JW5 was designed to mimic L-dCyd.[11] The rationale behind the synthesis of these unusual pyrimidine mimics relate to the work of Kool and his collaborators,[8,12] who found that the triphosphate derivative of the 2,4-difluoro-5-methylphenyl isostere (dFTP) is a substrate for DNA polymerases, e.g. Klenow fragment. Steady-state measurements indicated that dFTP base pair with dATP at high efficiency and fidelity. dFTP is a non polar molecule that forms a weak hydrogen bonds with dATP sufficient to destabilize DNA duplexes.[13] The 2,4-difluoro-5-methylphenyl isostere and its derivatives could also be promising candidates for cancer and viral chemotherapy agents. Pharmacokinetic and metabolism studies of JW2 in rats present a picture of high bioavailability from oral doses, dose-dependent pharmacokinetics, hepatic conversion to glucuronide and sulphate conjugates and metabolic deiodination.[14–16]

We now report studies designed to provide information on the mechanisms of action of these unnatural nucleoside analogues. The objectives of this study were to provide insights into the failure of these compounds to exhibit any biological activity, and to develop new ideas to design mimics, which might serve as antitumor and antiviral drugs.

EXPERIMENTAL METHODS

Materials

Nucleoside mimics, the isocarbostyril deoxynucleosides EN1 and EN2,[6] and difluorophenyl deoxynucleosides JW1, JW2, JW3, JW4 and JW5, were synthesized using literature methods.[9–11,17] Natural nucleosides were from Sigma–Aldrich Corp., ST. Louis, MO, USA.

Expression and Purification of the Recombinant Human Deoxynucleoside Kinases: The recombinant enzymes TK1, TK2, dCK and dGK were expressed and purified from bacterial expression systems according to procedures described previously.[2–4] The enzymes were at least 95% pure as judged by SDS-PAGE.

Phosphoryl-Transfer Assay with Recombinant Deoxynucleoside Kinases: Natural substrates (Thd, dCyd, dGuo and dUrd) and the nucleoside analogues were dissolved in DMSO to produce stock solutions of various concentrations (100–200 mM). The assays were carried out as described previously[18] with minor modifications. The reaction mixture contained 100 μM nucleoside, 100 μM ATP, 0.03 μM [$\gamma-^{32}$P] ATP (Amersham Pharmacia Biotech, IL, USA), 50 μM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 125 mM KCl, 10 mM DTT and 0.5 mg/ml bovine serum albumin (BSA). The reaction mixture was incubated at 37°C for 20 min in the presence of 50 ng of enzyme. Following the incubation period, the enzyme was heat inactivated for 2 min at 95°C. The reaction mixture was centrifuged and a 1-μl portion was spotted on PEI-cellulose TLC plates (Merck). These were placed overnight in a solvent system containing isobutyric acid: ammonium hydroxide: water (66:1:33). The radiolabeled spots were visualized by a phospho-imager (Fuji Film, Science Lab., Image Gauge V3.3).

Kinetic Analysis

The kinetic parameters were determined by non-linear regression analysis using the Michaelis-Menten equation at a substrate concentrations range of 1–100 μM.[19] Data were analyzed by the Sigma Plot Enzyme Kinetic Module version 1.1 (SPSS Inc.).

RESULTS

Specificity of dN Kinases with Nucleoside Analogues

Thymidine and deoxycytidine analogues were evaluated at a fixed concentration (100 μM) with recombinant human deoxynucleoside kinases highly purified from bacterial expression systems.[2–4] Phosphoryl transfer assays indicated that substitution of the thymine moiety by an unnatural isocarbostyril group (EN1, EN2; Fig. 1) resulted in new properties that are not favored by the human deoxynucleoside kinases. The recombinant human thymidine kinases (TK1 and TK2) did not accept EN1 as a substrate, but, EN2, the 7-I-derivative of EN1, showed some capacity to undergo phosphorylation compared to Thd as a reference (Table 1). The relative phosphorylations of EN2 were 1.8 and 6.9% with TK1 and TK2, respectively (Table 1).

Table 1. Phosphorylation of natural and mimetic nucleosides by recombinant human nucleoside kinases.

	Enzyme			
Compound	TK1	TK2	dCK	dGK
EN1	<0.1	<0.1	1.4 ± 0.3	1.1 ± 0.4
EN2	1.8 ± 0.8	6.9 ± 0.7	<0.1	4.4 ± 0.2
JW1	6.0 ± 0.9	90.4 ± 1.4	<0.1	<0.1
JW2	9.9 ± 1.3	73.6 ± 0.7	<0.1	<0.1
JW3	<0.1	5.1 ± 0.2	<0.1	<0.1
JW4	<0.1	<0.1	<0.1	<0.1
JW5	<0.1	<0.1	14.6 ± 0.8	<0.1
dThd	100	100	–	–
dUrd	77	–	–	–
dCyd	–	–	100	–
dGuo	–	–	–	100

Phosphoryl transfer assay was performed as described in Experimental Methods, the concentrations of the compounds were 100 μM and the phosphate donor ATP was 100 μM. The values and the standard deviations are the percent phosphorylation relative to the natural substrate for each enzyme.

Although the isocarbostyril group mimics, to some extent, the pyrimidine ring, EN1 was poorly phosphorylated by dCK and dGK. The 7-I-substitution (EN2) was a marginally better dGK substrate, with a relative phosphorylation of 4.4% compared to dGuo as a reference (Table 1).

The 2,4-difluorophenyl isosteres of pyrimidine nucleosides (JW1–JW3) showed variable substrate specificity with the human recombinant kinases with respect to their configuration, the nature of the sugar ring and the sub-group substitutions within the moiety (Fig. 1). These 2,4-difluorophenyl pseudo-bases thymidine mimics were also not phosphorylated by the recombinant kinases dCK and dGK. On the other hand, JW1 and JW2 were substrates for the recombinant human thymidine kinases. TK1 formed low but significant levels of phosphorylated products with JW1 and JW2 (6 and 9.9%, respectively, relative to Thd). Interestingly, JW1 and JW2 were phosphorylated by recombinant TK2 at relatively high rates (90.4 and 73.6%, respectively) in relation to Thd as a reference. No activity (less than 0.1%) was observed with JW3, the acyclic-sugar derivative of JW2, using recombinant TK1. Low levels of phosphorylation products with recombinant TK2 were detected for JW3, but with roughly 14-fold lower compared to JW2 (5.1% with respect to Thd as a reference).

The unnatural deoxycytidine mimic analogue, 2-fluoro-4-aminophenyl (JW4) did not show any detectable phosphorylation with any of the four human deoxynucleoside kinases studied, whereas the β-L-deoxycytidine mimic (JW5) was significantly phosphorylated by recombinant dCK (14.6% relative to dCyd). This observation is in agreement with the previously reported stereo-specificity of dCK, which shows that dCK have a higher affinity for β-L-isomers than for the β-D-isomers, although the catalytic activity is higher for substrates in the natural configuration.[21]

Kinetic Properties of the Analogues

The biochemical analysis of the interactions of deoxynucleoside kinases with these Thd mimics was extended to include kinetics parameters, which gives a better understanding of the nucleoside/enzyme interaction. The kinetic parameters with recombinant TK1 and TK2 were determined using the phosphoryl transfer assay, in which the formation of radioactive monophosphate products was monitored using [$\gamma-^{32}$P]-ATP as a phosphate donor.

The apparent K_m values of the isocarbostyril nucleoside compound 1-(2-deoxy-β-D-ribofuranosyl)-7-iodoisocarbostyril (EN2) with TK1 and TK2 was 38- and 4- fold higher than Thd, respectively. Thus, the calculated relative phosphorylation efficiencies were 0.8% and 12.6% relative to Thd using both TK1 and TK2, respectively. When dGK was used, EN2 showed 9 fold higher apparent K_m and 10 fold lower k_{cat} values relative to dGuo, as a result the k_{cat}/K_m relative efficiency was 100 fold lower than that of dGuo.

The difference in the capacity of TK1 and TK2 to use JW1 and JW2, as substrates was apparent when the K_m and k_{cat} values were determined. The K_m values for JW1 and JW2 with TK1 were 2- and 6- fold higher than those with TK2 (Table 2). The relative efficiency, k_{cat}/K_m, for JW1 and JW2 with TK1 were 3.2% and 4.5%, relative to that for Thd. In the case of TK2, JW1 had 3.5 fold higher apparent K_m and two fold higher k_{cat} values relative to that for Thd, resulting in an over all efficiency of 44.6% relative to Thd. JW2 showed a two-fold higher apparent K_m value and no differences in the k_{cat} value compared to that for Thd; i.e. the k_{cat}/K_m value was 44.1% of that for Thd (Table 2).

Table 2. Michaelis–Menten kinetic parameters for phosphorylation of nucleoside mimics by the recombinant human deoxynucleoside kinases.

Nucleoside	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m Relative to natural nucleoside
dThd	TK1	2.3 ± 0.5	3.5 ± 0.3	100
EN2	TK1	88.8 ± 27	1.2 ± 0.2	0.8 ± 0.1
JW1	TK1	147.4 ± 47	7.7 ± 1.3	3.2 ± 0.5
JW2	TK1	259.9 ± 75	19.0 ± 3.2	4.5 ± 0.7
dThd	TK2	18.2 ± 0.8	0.31 ± 0.03	100
EN2	TK2	74.4 ± 4.6	0.16 ± 0.08	12.6 ± 2.4
JW1	TK2	69.9 ± 6.3	0.53 ± 0.02	44.6 ± 1.0
JW2	TK2	41.1 ± 4.1	0.31 ± 0.01	44.1 ± 0.4
dGuo	dGK	2.0 ± 0.71	0.07 ± 0.01	100
EN2	dGK	18.8 ± 3.3	0.007 ± 0.005	1.0 ± 0.04
dCyd	dCK	1.0 ± 0.3	0.11 ± 0.02	100
JW5	dCK	1.3 ± 0.6	0.02 ± 0.01	13.1 ± 1

The parameters were calculated using Michaelis–Menten equation. The concentration of the compounds was varied from 1 to 100 μM. The k_{cat} values were calculated using the equation $V_{max} = k_{cat} \times [E]$, where [E] is the total enzyme concentration and is based on one active site/monomer.

The kinetic parameters for JW5, determined over a 1–100 μM concentration range, showed an apparent K_m value of 1.3 μM, similar to that for dCyd, whereas the apparent k_{cat} was very low, about 5.5 fold lower than that for dCyd. The phosphorylation efficiency (k_{cat}/K_m) was 13.1% relative to that for dCyd.

DISCUSSION

A group of unnatural pyrimidine analogues (Fig. 1) with diverse structures have been evaluated for their potential as substrates for the human deoxynucleoside kinases in phosphoryl transfer assays using recombinant enzyme preparations. The biochemical properties of this group of compounds were determined to understand their substrate activity relationships and to evaluate such biochemical approaches to identify and design new anticancer drugs.

Earlier, these novel classes of isocarbostyril-thymine substitutes (EN-1 and EN-2) and 2,4-difluorophenyl pseudo-bases exhibited weak cytotoxic effects.[6,9–11,17] The concentration of these compounds that killed 50% of several different cancer cell lines (CC_{50}) were in the range of 10^{-3} to 10^{-5} M, which is high as compared to cytotoxic nucleosides like 5-fluoro-2′-deoxyuridine ($CC_{50} = 10^{-11}$ to 10^{-12} M).[6] The current findings provide a possible explanation for the low toxicity, as we observed that the isocarbostyril-thymine mimics are phosphorylated to their 5′-monophosphate derivatives at low rates by the deoxynucleoside kinases. Although the mitochondrial enzymes (TK2 and dGK) showed low activity with EN-2 as a substrate, kinetic analysis indicated that EN2 is a substrate for TK2 but not for dGK. The phosphorylation efficiency of EN2 with TK2 was 12.6% relative to Thd, indicating a possible biological role for EN2 in the mitochondrial DNA metabolism. On the other hand, the 2,4-difluorophenyl nucleosides were relatively poor substrates for the cytosolic enzymes and thus are probably not activated in proliferating cells. Exceptions were shown for compounds JW1 and JW2, which were efficiently phosphorylated by TK2. However, the transport of these analogues into the mitochondria is unknown but it is possible that they may give mitochondrial toxicity by affecting the dNTP pools and/or DNA replication. Although the triphosphate derivative of the 2,4-difluoro-5-methylphenyl isostere (dFTP) is a substrate for the Klenow fragment of *E. coli* DNA polymerase I,[8,12,13] it is possible that the 2,4-difluoro-5-methylphenyl-nucleoside is not phosphorylated into di- and tri-phosphate under physiological conditions.

It is well established that the substrate specificities of TK1 and the mitochondrial TK2 differ significantly, and also that the substrate tolerance with TK2 is more flexible.[20] Thus, TK1 and TK2 can accept minor modifications at the thymine base without loss of substrate activity.[1] In accordance, our results indicate that thymidine kinases can tolerate to some extent the isosteric replacement of halogens at positions 2 and 4. In addition, the existence of a methyl group (JW1) or iodine (JW2) at the 2,4-difluorotoluene moiety mimics modifications at the 5-position of the thymine base. These substitutions are still accepted by the thymidine kinases. In fact, TK1 and TK2 are reported to tolerate substitutions at the 5-position that are in size and physicochemical properties comparable to a methyl or ethyl group.[1] On the other hand, the existence of intact sugar moiety is critical for TK1 activity, but not for TK2 activity, which can to some extent tolerate acyclic compounds as in the case with compound JW3.

In case of TK2, the substitution of a bulky isocarbostyril group (EN1) resulted in poor substrate characteristics. However, the existence of the iodine atom at the 5-position (EN2) that has the potential to interact via charge or hydrogen bonds with the enzyme resulted in a relatively effective substrate.

In contrast, dCK exhibits a high specificity toward pyrimidine nucleosides, with little flexibility toward modification at the cytosine base.[22] dGK is a purine-specific enzyme, phosphorylating dGuo, dAdo, and dIno, but not Thd or its derivatives.[1,2]

In summary, the isocarbostyril- and difluorophenyl- 2′-deoxyribonucleosides were generally poor substrates for the recombinant human enzymes TK1, TK2, dCK and dGK. Exceptions were noticed for the difluorophenyl nucleosides JW1 and JW2, which were moderate substrates of TK1, and JW2, which was a good substrate for the mitochondrial enzyme TK2. On the other hand, JW1 and JW2 are most likely weak competitive substrates in the presence of Thd (data not shown). These results, in combination with the previously reported weak cytotoxicity of this group of compounds and their kinase by-pass pro-drugs (for JW2), do not support a biochemical model involving either bioactivation through phosphorylation, nor of incorporation into cellular DNA under cell culture conditions. Surprisingly, the isocarbostyril nucleoside EN2 showed good substrate specificity with TK2, indicating a possible biological role in mitochondrial cytotoxicity. Thus, this study encouraged further investigations of the cellular effects of EN2.

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