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ACCELERATED COMMUNICATION

Non-Nucleoside Inhibitors of Mitochondrial Thymidine Kinase (TK-2) Differentially Inhibit the Closely Related Herpes Simplex Virus Type 1 TK and *Drosophila melanogaster* Multifunctional Deoxynucleoside Kinase

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ABSTRACT

5′-*O*-Trityl derivatives of thymidine (dThd), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), and their acyclic analogs 1-[(*Z*)-4-triphenylmethoxy-2-butenyl]thymine (KIN-12) and (*E*)-5-(2-bromovinyl)-1-[(*Z*)-4-triphenylmethoxy-2-butenyl]uracil (KIN-52) have been synthesized and evaluated for their inhibitory activity against the amino acid sequence related mitochondrial dThd kinase (TK-2), herpes simplex virus type 1 (HSV-1) TK, and *Drosophila melanogaster* multifunctional 2′-deoxynucleoside kinase (Dm-dNK). Several compounds proved markedly inhibitory to these enzymes and represent a new generation of nucleoside kinase inhibitors. KIN-52 was the most potent and selective inhibitor of TK-2 (IC_{50}, 1.3 μM; K_i, 0.50 μM; K_i/K_m, 0.37) but was not inhibitory against HSV-1 TK and Dm-dNK at 100 μM. As found for the alternative substrate BVDU, the tritylated compounds competitively inhibited the three enzymes with respect to dThd. However, whereas BVDU behaved as a noncompetitive inhibitor (alternative substrate) of TK-2 and HSV-1 TK with respect to ATP as the varying substrate, the novel tritylated enzyme inhibitors emerged as reversible purely uncompetitive inhibitors of these enzymes. Computer-assisted modeling studies are in agreement with these findings. The tritylated compounds do not act as alternative substrates and they showed a type of kinetics against the nucleoside kinases different from that of BVDU. KIN-12, and particularly KIN-52, are the very first non-nucleoside specific inhibitors of TK-2 reported and may be useful for studying the physiological role of the mitochondrial TK-2 enzyme.

In mammalian cells, there are four different 2′-deoxynucleoside kinases with partially overlapping substrate specificities (Arnér and Eriksson, 1995; Johansson et al., 2001). The cytosolic thymidine (dThd) kinase (TK-1) recognizes only dThd and 2′-deoxyuridine (dUrd) as a substrate for phosphorylation. In contrast, TK-2 is located in the mitochondria and phosphorylates, besides dThd and dUrd, 2′-deoxycytidine (dCyd) as a natural substrate. The cytosolic/nuclear dCyd kinase converts dCyd, but also purine deoxynucleosides, such as 2′-deoxyguanosine (dGuo) and 2′-deoxyadenosine (dAdo), to their 5′-monophosphate derivative. Finally, dGuo kinase (dGK) represents the second mitochondrial deoxynucleoside kinase phosphorylating dGuo

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ABBREVIATIONS: dThd, 2′-deoxythymidine; TK, thymidine kinase; dUrd, 2′-deoxyuridine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; dAdo, 2′-deoxyadenosine kinase; dGK, 2′-deoxyguanosine kinase; dNK, 2′-deoxynucleoside kinase; Dm, *Drosophila melanogaster*; BVDU, (*E*)-5′-(2-bromovinyl)-2′-deoxyuridine; HSV, herpes simplex virus; BVU, (*E*)-5-(2-bromovinyl)uracil; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; THF, tetrahydrofuran; KIN5, (*E*)-5-(2-bromovinyl)-1-(2′-deoxy-5′-*O*-triphenylmethyl)uridine; KIN6, 5′-*O*-triphenylmethyl-thymidine; KIN12, 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine; KIN39, (*R,S*)-1-[3-hydroxy-4-(triphenylmethoxy)butyl]thymine; KIN-52, (*E*)-5-(2-bromovinyl)-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]uracil.

and dAdo. Both TK-2 and dGK are constitutively expressed, in contrast with TK-1, which is highly S-phase specific. However, in proliferating cells, intracellular TK-2 levels are much lower compared with TK-1, but in nonproliferating and resting cells, TK-2 is virtually the only pyrimidine deoxynucleoside kinase that is physiologically active (Wintersberger et al., 1992; Hengstschlager et al., 1994; Johansson and Karlsson, 1997).

There is a high sequence homology between TK-2, dCK, and dGK, but also with herpes simplex virus type 1 (HSV-1) TK and the recently discovered multifunctional 2′-deoxynucleoside kinase (dNK) from *Drosophila melanogaster* (Dm-dNK) (Munch-Petersen et al., 1998; Johansson et al., 1999). The crystal structures of HSV-1 TK and Dm-dNK but not TK-2 have been resolved in complex with their natural substrate dThd and several alternative nucleoside substrates, such as (*E*)-5-(2-bromovinyl)-dUrd (BVDU), ganciclovir, and acyclovir (for HSV-1 TK) (Wild et al., 1997; Champness et al., 1998; Johansson et al., 2001). Whereas HSV-1 TK, like TK-2, recognizes the natural pyrimidine deoxynucleosides dThd, dUrd, and dCyd as a substrate for phosphorylation, Dm-dNK can convert all five natural purine/pyrimidine 2′-deoxynucleosides to their 5′-monophosphates (Munch-Petersen et al., 1998, 2000; Johansson et al., 1999).

Only a few inhibitors of the deoxynucleoside kinases have been reported. A series of potent and selective inhibitors of HSV-1 TK consist of guanine, guanosine, or thymidine analogs that contain a lipophilic entity (i.e., halogeno/methoxy-substituted phenyl) at the 5′-position of the deoxyribose moiety (Hildebrand et al., 1990). These anti-HSV TK drugs did not recognize other TKs such as TK-1 or TK-2. Instead, 2′-alkylether or -ester derivatives of 1-β-D-ribofuranosylthymidine and 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil were reported to be selective inhibitors of TK-2 but not to inhibit HSV-1 TK (Balzarini et al., 2001; Manfredini et al., 2001). Finally, several 5-substituted ribonucleosides [e.g., (*E*)-5-(2-bromovinyl)uridine] and 3′-substituted thymine riboside analogs were found to be inhibitory against TK-2 but not TK-1 (Balzarini et al., 2000). Also, Kierdaszuk et al. (1999) have reported on substrate/inhibitor properties of TK-2 toward *O*′-alkyl sugar-modified nucleoside analogs. Their inhibitory potential against the enzyme is caused by competition of the inhibitor with thymidine as an alternative substrate, which is endowed [at least in case of (*E*)-5-(2-bromovinyl)uridine and analogs] with a very low V_{max} value. We now report on a novel class of acyclic thymine and (*E*)-5-(2-bromovinyl)uracil (BVU) analogs that contain a bulky lipophilic (trityl) moiety linked to the pyrimidine base through an aliphatic butenyl or 2-hydroxybutyl moiety as potent inhibitors of TK-2. Interestingly, the BVU trityloxybutenyl derivative turned out to selectively inhibit TK-2 and was not measurably recognized by TK-1, HSV-1 TK, and Dm-dNK. Our enzyme kinetic studies revealed that this is the first example of a reversible non-nucleoside, nonsubstrate inhibitor of TK-2 that is competitive with respect to dThd and uncompetitive with respect to ATP.

Experimental Procedures

Compounds

The synthesis and structural characterization of KIN-6, KIN-12, and KIN-39 (Fig. 1) have been described recently (Hernández et al., 2002). BVDU was from the Rega Institute (Leuven, Belgium). Thymidine and ATP were from Sigma Chemical (St. Louis, MO). The synthesis of the BVU derivatives KIN-5 and KIN-52 was as follows:

(*E*)-5-(2-Bromovinyl)-1-(2′-deoxy-5′-*O*-triphenylmethyl)uridine (KIN-5). Trityl chloride (107 mg, 0.30 mmol) was added to a stirred solution of BVDU (100 mg, 0.30 mmol) in dry pyridine (4 ml) at 0°C. The mixture was stirred at room temperature for 24 h. Then, it was diluted with EtOAc (50 ml) and treated with saturated aqueous $NaHCO_3$ (20 ml). The aqueous phase was extracted with EtOAc (20 ml). The combined organic extracts were washed with brine (10 ml), dried (anhydrous Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by flash-column chromatography (EtOAc/hexane, 1:2) and repurified by centrifugal circular thin-layer chromatography in the Chromatotron (CH_2Cl_2:MeOH) to yield (90 mg, 52%) of KIN-5 as an amorphous solid. Mass spectroscopy (electrospray, positive mode) 597, 599 $(M+Na)^+$ (isotopes). ^{1}H-NMR (acetone-d_6) δ: 2.35 (m, 2H, H-2′), 3.87 (t, 2H, J = 3.1 Hz, H-5′), 4.00 (m, 1H, H-4′), 4.56 (m, 1H, H-3′), 6.33 (m, 1H, H-1′), 6.81 (d, J = 13.5 Hz, 1H, 5-CH), 7.30–7.50 (m, 16H, Ph, CHBr), 8.26 (s, 1H, H-6), 10.26 (br s, 1H, 3-NH). Analysis for $C_{30}H_{27}BrN_2O_5$: C, 65.62; H, 4.73; N, 4.87. Found: C, 65.78; H, 4.89; N, 4.74. No traces of BVDU were detected in the KIN-5 preparation. Because of their very different polarity, BVDU could be easily separated from KIN-5.

(*E*)-5-(2-Bromovinyl)-1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]-uracil (KIN-52). A solution of diisopropyl azodicarboxylate (0.05 ml, 0.3 mmol) in dry THF (1 ml) was slowly added to a suspension containing (*Z*)-1-hydroxy-4-(triphenylmethoxy)-2-butene (49.6 mg, 0.15 mmol) (Hernández et al., 2002), polystyrene-triphenylphosphine (3 mmol/g, 126.7 mg, 0.38 mmol) (Fluka, Buchs, Switzerland), and N^3-benzoyl-(*E*)-5-(2-bromovinyl)uracil [100 mg, 0.3 mmol; prepared from (*E*)-5-(2-bromovinyl)uracil following the method described for the N^3-benzoylation of uracil and thymine] (Cruickshank et al., 1984) in dry THF (1.5 ml). The mixture was stirred at room temperature overnight. The reaction mixture was filtered, the residue washed with THF (2 × 5 ml), and the combined filtrates were evaporated to dryness. The residue was treated with saturated NH_3/MeOH solution (10 ml) and stirred overnight. Then, EtOAc (10 ml) and brine (5 ml) were added. The aqueous phase was further extracted with EtOAc (3 × 10 ml). The combined organic extracts were dried (anhydrous Na_2SO_4), filtered, and evaporated. The residue was purified by centrifugal circular thin-layer chromatography in the Chromatotron (hexane/EtOAc, 1:1) to yield (30 mg, 38%) of KIN-52 as an amorphous solid. Mass spectroscopy (electrospray, positive mode) 551, 553 $(M+Na)^+$ (isotopes). ^{1}H-NMR ($CDCl_3$) δ: 3.67 (d, J = 6.4 Hz, 2H, CH_2O), 4.19 (d, J = 7.1 Hz, 2H, CH_2N), 5.38, 5.92 (m, 2H, CH = CH), 6.16 (d, J = 13.6 Hz, 1H, 5-CH), 6.96 (s, 1H, H-6), 7.16–7.41 (m, 16H, Ph, CHBr), 8.49 (br s, 1H, 3-NH). Analysis for $C_{29}H_{25}BrN_2O_3$, C, 65.79; H, 4.76; N, 5.29. Found: C, 65.52; H, 4.81; N, 5.34. No traces of BVU were detected in the KIN-52 preparation. Because of their very different polarity, BVU could be easily separated from KIN-52.

Construction, Expression, and Purification of HSV-1 TK

HSV-1 TK was expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion protein as follows. The HSV-1 TK coding sequence was amplified by PCR using primers 5′-GAGGAATTCATGGCTTCGTACCCCGGCCATC and 5′-CTCGTCGACTCAGTTAGCC TCCCCCATCTCC (Kebo Lab, Stockholm, Sweden) with the pMCTK plasmid (kindly provided by Dr. D. Ayusawa, Yokohama University, Japan) as a template, and ligated between the *Eco*RI and *Sal*I sites of the pGEX-5X-1 vector (Amersham Biosciences, Uppsala, Sweden). The plasmid vector was checked by automated fluorescence sequencing (ALFexpress; Amersham Biosciences) and transfected into *E. coli* BL21(DE3)pLysS. Bacteria were grown overnight in 2× yeast/tryptone medium containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and then diluted in fresh medium. After further growth of the bacteria at 27°C (for 1 h), isopropyl-β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 0.1 mM to induce the production of the GST-TK fusion protein.

After 15 h of further growth at 27°C, cells were pelleted (7700*g* for 10 min at 4°C) and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiotreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.15 mg/ml lysozyme). Bacterial suspensions were sonicated (on ice) and ultracentrifuged (20,000*g* for 15 min at 4°C). GST-TK was purified from the supernatant using Glutathione Sepharose 4B (Amersham Biosciences) as described by the manufacturer. Protein content of the purified fractions was assessed using Bradford reagent (Sigma).

Construction, Expression, and Purification of TK-2 and Dm-dNK

The cDNA sequences of TK-2 and Dm-dNK were cloned into the pGEX-4T-1 plasmid vector (Pharmacia, Peapack, NJ) to expressed the cDNA encoded proteins fused to glutathione *S*-transferase. The expression plasmid vectors were transformed into the *E. coli* BL21(DE3)pLysS (Stratagene, La Jolla, CA) and the proteins were expressed and purified as described previously (Johansson and Karlsson, 1997; Johansson et al., 1999). The purity of the recombi-

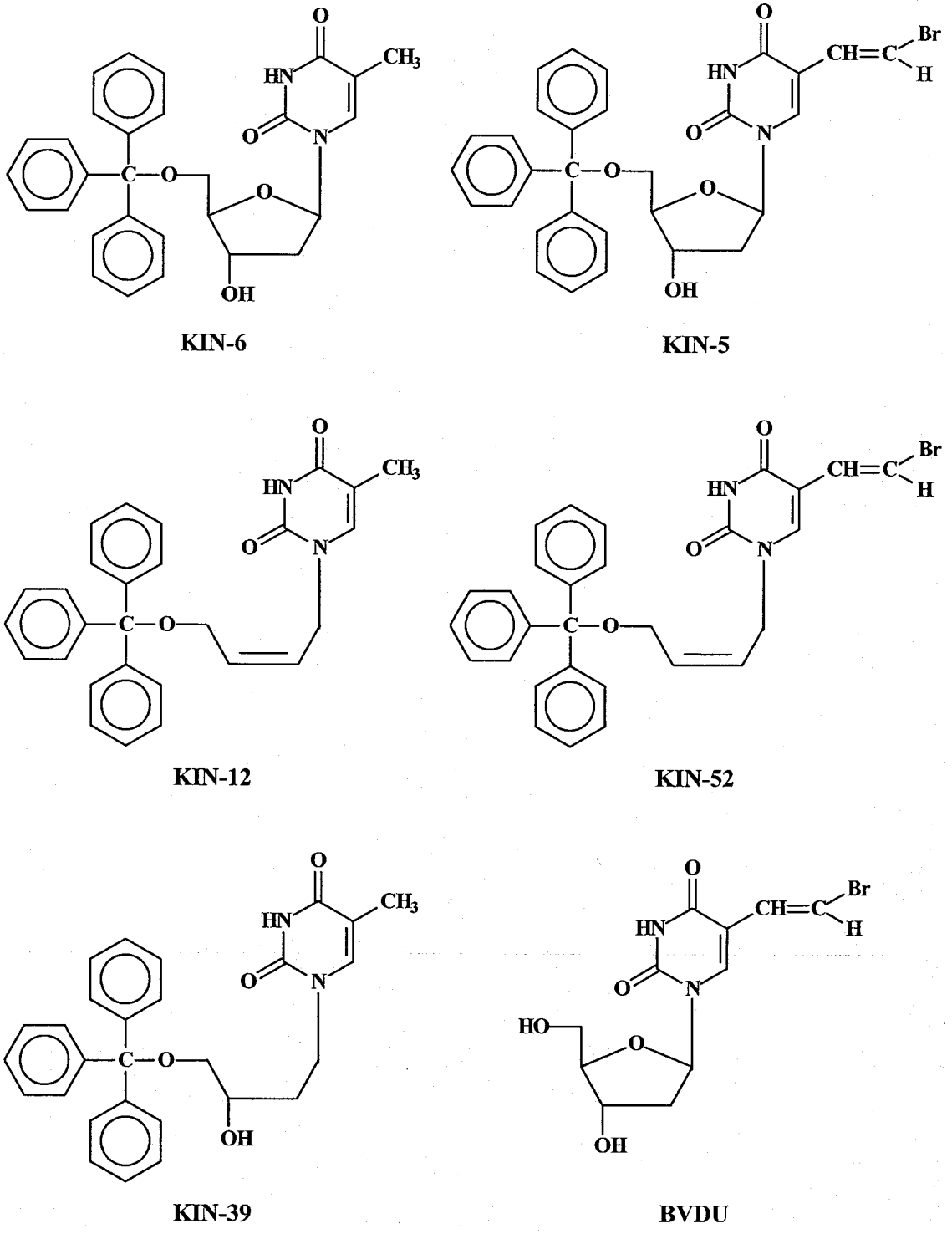

Fig. 1. Structural formulae of TK inhibitors.

nant proteins were determined by SDS-polyacrylamide gel electrophoresis (Phast system, Amersham Biosciences). The protein concentrations were determined with Bradford protein assay (Bio-Rad, Hercules, CA), and bovine serum albumin was used as the concentration standard.

Radiochemicals

The radiolabeled substrate [CH_3-^{3}H]dThd (70 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA).

TK Assay Using [CH_3-^{3}H]dThd as the Substrate

The activity of purified TK-1 [from human T lymphocyte (CEM) cells] and recombinant TK-2, HSV-1 TK, and Dm dNK and the 50% inhibitory concentration of test compounds were assayed in a 50-μl reaction mixture containing 50 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM CHAPS, 3 mg/ml bovine serum albumin, 2.5 mM ATP, and 1 μM [*methyl*-^{3}H]dThd and enzyme. The samples were incubated at 37°C for 30 min in the presence or absence of different concentrations (5-fold dilutions) of the test compounds. Aliquots of 45 μl of the reaction mixtures were spotted on Whatman DE-81 filter paper disks. The filters were washed three times for 5 min each in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity was determined by scintillation counting.

The K_m values (for dThd or ATP) and the K_i values (for the inhibitors) using varying concentrations of dThd (ranging between 0.4 and 5 μM) at saturating concentrations of ATP (2.5 mM) or using varying concentrations of ATP (ranging between 5 and 100 μM) at saturating concentrations of dThd (20 μM) were determined and derived from Lineweaver-Burk plots. The kinetics of BVDU against HSV-1 TK in the presence of different ATP concentrations were carried out at a fixed concentration of 2 μM dThd.

In the assay to reveal whether the inhibitors interact with TK-2 in a reversible or irreversible manner, serial dilutions of TK-2 were added to the reaction mixtures in the presence of 1 μM KIN-12 or KIN-52 (approximately the IC_{50} value in the presence of 1 μM dThd). The highest enzyme concentration tested was 75 ng of protein, and the lowest enzyme concentration was 4.5 ng of protein in 50 μl of the reaction mixture.

Construction of a Computer-Assisted Model of HSV-1 TK and TK-2

Model building was carried out using HyperChem 7.0 and the structures were optimized using the Amber96 force field. The solvent was approximated using a linear distance-dependent dielectric. Energies of the in silico generated structures were minimized using the conjugate gradient algorithm.

The different molecules were positioned in the HSV TK binding site by choosing the same orientation for the thymidine ring moiety as in the complex with BVDU (Protein Data Base code 1KI8; Champness et al., 1998).

The high degree of sequence conservation between HSV-1 TK and TK-2 (>50% identity around the binding site) implied that the known three-dimensional structures of the former (Protein Data Base codes 1KI8 and 1VTK; Wild et al., 1997) could be used as templates for the modeling. The TK-2 structure was modeled by replacing the side chains of residues in the ATP and dThd binding sites of HSV-1 TK by their counterparts in TK-2. The backbone conformation was not altered and the orientation of the side chains was preserved where possible.

Results

Inhibitory Activity of the Test Compounds against Nucleoside Kinases. 5′-*O*-Trityl dThd (KIN-6) proved inhibitory to HSV-1 TK and Dm dNK at IC_{50} values of 7.8 and 12 μM, respectively (Table 1). The compound was 3- to 4-fold less inhibitory to the closely related TK-2 enzyme. Given the high affinity of BVDU to all three enzymes as an alternative substrate, the 5′-*O*-trityl derivative of BVDU (KIN-5) was synthesized to be evaluated for its inhibitory activity against the nucleoside kinases. It had a similar inhibitory activity spectrum as KIN-6 (Table 1). When the acyclic dThd derivative of KIN-6, in which the corresponding 1′, 2′, 3′, and 4′ positions of the deoxyribose part of KIN-6, including the 3′-hydroxyl group, were preserved (KIN-39), was examined for its inhibitory potential against the nucleoside kinases, it surprisingly gained 20-fold higher anti–TK-2 activity, 5-fold higher anti–HSV-1 TK activity, whereas its inhibitory capability against Dm dNK was similar to that of KIN-5. The (*Z*)-4-triphenylmethoxy-2-butenyl derivatives of thymine (KIN-12) and BVU (KIN-52) were also prepared and compared with their corresponding KIN-6 and KIN-5 derivatives. Both compounds further gained a remarkable inhibitory activity against TK-2 (IC_{50}, 1.3–1.5 μM), which was at least 20- to 25-fold and 40- to 50-fold more pronounced than found for KIN-6 and KIN-5, respectively. Instead, both compounds lost considerable anti–HSV-1 TK activity (9- to >20-fold). Surprisingly, whereas KIN-12 was as potent an inhibitor against Dm-dNK as against TK-2, KIN-52 completely lost inhibitory potential against Dm dNK (Table 1). Consequently, KIN-52 emerged as a potent and highly selective inhibitor of TK-2 (IC_{50}, 1.3 μM); it was not inhibitory at 100 μM against any of the other enzymes tested, including the cytosolic TK-1 and the closely related HSV-1 TK and Dm dNK. It was ascertained by HPLC analysis that the inhibitors described in this study were entirely stable in the reaction mixture and released neither thymine, BVU, dThd, nor BVDU upon potential hydrolysis of the trityl or triphenylmethoxybutenyl moiety from the molecule. It should be mentioned that a KIN-12 derivative that lacked the 5′-trityl group in the molecule was at least 100-fold less inhibitory against TK-2 and Dm-dNK than KIN-12 (Hernández et al., 2002). These observations

TABLE 1
Inhibitory activity of test compounds against nucleoside kinase-catalyzed phosphorylation of thymidine
IC_{50} is the 50% inhibitory concentration (i.e., the compound concentration required to inhibit 1 μM [CH_3-^{3}H]dThd phosphorylation by 50%).

Compound	IC_{50}			
	TK-1	TK-2	HSV-1 TK	Dm-dNK
			μM	
KIN-5	>100	64 ± 21	5.6 ± 2.7	13 ± 2.0
KIN-6	>100	33 ± 20	7.8 ± 0.3	12 ± 1.0
KIN-12	>100	1.5 ± 0.16	45 ± 1.0	3.3 ± 1.0
KIN-39	>100	3.6 ± 0.40	1.2 ± 0.7	12 ± 4.0
KIN-52	>100	1.3 ± 1.1	>100	>100
BVDU	>100	0.34 ± 0.10	2.8 ± 1.5	2.5 ± 0.63

suggest that potential loss of the 5′-trityl group of KIN-12 or KIN-52 will not make the compounds more inhibitory to TK-2, but instead will inactivate the compounds as potential inhibitors of TK-2.

Reversible Inhibition of TK-2 by KIN-12 and KIN-52. Both KIN-12 and KIN-52 were examined for the (ir)reversibility of their inhibitory effect against TK-2 (Fig. 2). For this purpose, the inhibitors were added, at concentrations equal to their IC_{50} values, to a variety of serial TK-2 enzyme dilutions. In the case of reversible inhibition, the velocity-versus-enzyme-concentration plot in the presence of the inhibitors should dissect in the intersection of the *x*- and *y*-axes, as should the control (without inhibitor). However, in case of irreversible inhibition, the velocity-versus-enzyme-concentration plot in the presence of the inhibitors should be parallel to the control curve and should dissect on the *x*-axis. As is clear from Fig. 2, KIN-12 and KIN-52 reversibly inhibit TK-2. At all TK-2 dilutions in the reaction mixtures, the enzyme was inhibited by ~ 50% in the presence of the inhibitors that were applied at a concentration that was very close to their IC_{50} values. If TK-2 had been irreversibly inactivated, the proportion of active enzyme should have been decreased at lower TK-2 concentrations, and this was clearly not the case (Fig. 2). Note also that the inhibitor concentration was far in excess of the number of enzyme molecules at a molar basis; thus, if irreversible inhibition should have occurred in stoichiometric concentrations of enzyme and inhibitor, the enzyme would have been completely inactivated at the highest inhibitor concentrations tested.

K_i and K_i/K_m Values of the Test Compounds for Nucleoside Kinases in the Presence of Various Concentrations of dThd as the Varying Substrate. The nucleoside kinase inhibitors have been investigated on their mode of kinetic interaction with the enzymes. The K_i value for each compound was determined in the presence of a variety of dThd concentrations as the variable substrate (in the presence of saturating concentrations of ATP). All inhibitors, as also observed for the alternative substrate BVDU, showed purely competitive inhibition of the three enzymes against dThd as the variable substrate. KIN-12 and its corresponding BVU derivative KIN-52 were endowed with the lowest K_i values (0.50 and 0.78 μM, respectively) against TK-2 and the inhibition values were at the same order of magnitude as those of the alternative substrate BVDU (K_i = 0.22 μM) (Table 2). Their K_i/K_m values were virtually lower than 1. The corresponding nucleoside derivatives KIN-6 and KIN-5 had 20- to 40-fold higher K_i values (Table 2). Whereas KIN-39 had K_i values intermediary between those of KIN-12 and KIN-6 for TK-2 and also for Dm-dNK, it proved by far most inhibitory against HSV-1 TK (K_i = 0.46 μM) (compare with 16 and 6.1 μM for KIN-12 and KIN-6, respectively). Its K_i/K_m value was also less than 1 and virtually comparable with the K_i/K_m value of BVDU (Table 2).

K_i and K_i/K_m Values of the Test Compounds in the Presence of Various Concenrations of ATP as the Varying Substrate. The nature of inhibition of TK-2 and HSV-1 TK using varying concentrations of ATP in the presence of saturating concentrations of dThd (20 μM) was also determined for KIN-12, KIN-52, and BVDU against TK-2 and KIN-12 and BVDU against HSV-1 TK (Table 3, Fig. 3). Whereas BVDU resulted in noncompetitive inhibition of both enzymes, KIN-12 and KIN-52 behaved as uncompetitive inhibitors of TK-2 (KIN-12; KIN-52) and HSV-1 (KIN-12) (Fig. 3). Their K_i and K_i/K_m values are shown in Table 3.

Discussion

Mitochondrial TK-2 and dGK play an important role in the homeostasis of mitochondria, including the maintenance of the mitochondrial dNTP pools that are separated from the cytosolic pool because of impermeability of the mitochondrial inner membrane to charged molecules. Mutations in both the mitochondrial enzymes TK-2 and dGK have been recently identified in persons who developed devastating myopathy (TK-2) or a hepatocerebral form of mitochondrial DNA-depletion syndrome (dGK), caused by depletion of mitochondrial DNA (Mandel et al., 2001; Saada et al., 2001). It has also been shown that TK-2 and dGK are (co)responsible for phosphorylation of antiviral (i.e., fialuridine) or anticancer (i.e., araG) nucleoside analogs, and their metabolic activation in the mitochondrial compartment has been linked to long-term (delayed) cytotoxicities associated with such nucleoside ana-

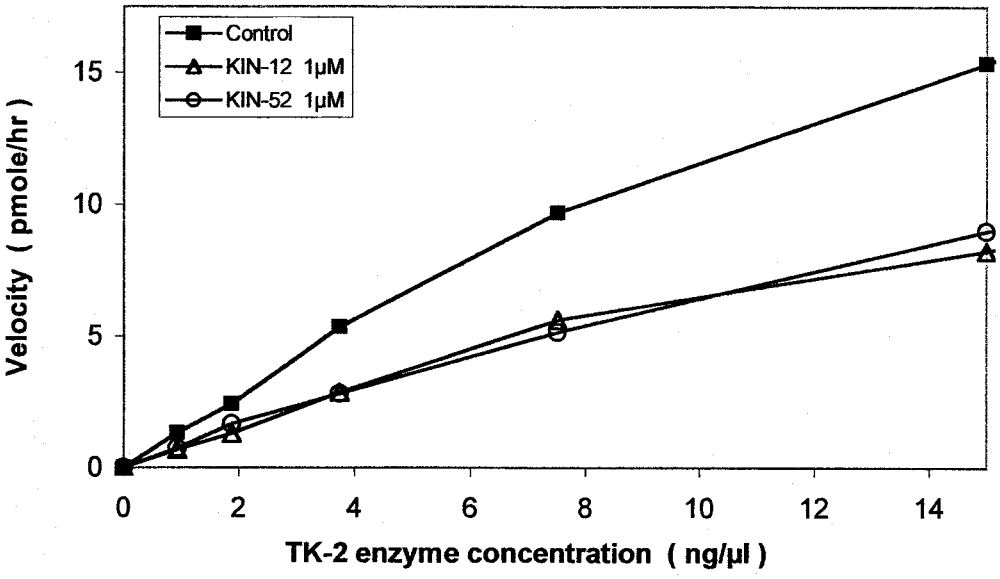

Fig. 2. Inhibition of TK-2 by 1 μM KIN-12 and KIN-52 in the presence of serial dilutions of the enzyme.

logs (Wang et al., 1993; Parker and Cheng, 1994; Curbo et al., 2001).

Development of specific inhibitors of TK-2 can be useful in investigating the role of TK-2 in the phosphorylation (activation) of nucleoside analogs with biological application, the molecular basis of delayed toxic side effects of such nucleoside analogs caused by decreasing the mitochondrial DNA content, the role of TK-2 in mitochondrial DNA repair and dNTP homeostasis, and also the contribution of the differential activity of TK-1 and TK-2 in different cell types. In fact, we could demonstrate that KIN-52, KIN-12, and KIN-39 could virtually completely suppress remaining TK activity in extracts of human lymphocytic CEM/TK$^-$ cells that were deficient for TK-1 but kept mitochondrial TK-2 activity, whereas the TK-2 inhibitors hardly affected TK activity in extracts from wild-type CEM/0 cells that were competent in both TK-1 and TK-2 activities (data not shown). Also, KIN-12 and related derivatives were shown to be able to enter intact human osteosarcoma cells and were not cytotoxic at 20 μM (Hernández et al., 2002). However, it is unclear whether these compounds were also able to enter the mitochondrial compartment in intact mammalian cells. Crystallography of such TK-2–specific inhibitors in complex with the TK-2 enzyme may also reveal the functional role of specific amino acids in the substrate/inhibitor binding pocket of TK-2 compared with their counterparts in structurally related enzymes such as HSV-1 TK, Dm-dNK, and dGK.

The advantage and uniqueness of the specific tritylated TK-2 inhibitors identified in this study is their pure inhibitory nature. They do not act as an alternative substrate for TK-2, in contrast with previously identified inhibitors, such as the 2′-*O*-acyl/alkyl-substituted 1-β-D-ribofuranosylthymidine and 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil nucleoside analogs, and several 3′-substituted ribofuranosylnucleosides (Balzarini et al., 2000, 2001). The tritylated TK-2 inhibitors behaved as purely competitive inhibitors of the enzyme (TK-2, HSV-1, Dm-dNK) with respect to dThd. Thus, the TK-2 inhibitors are mutually exclusive with the natural substrate dThd, and act to increase the apparent K_m for the substrate, keeping the V_{max} of the enzyme for the natural substrate constant. The alternative substrate BVDU acts kinetically in exactly the same manner as the tritylated inhibitors with respect to inhibition of dThd phosphorylation by TK-2. However, when the nature of the inhibition of TK-2 and HSV-1 TK by the inhibitors was investigated with respect to ATP, the cosubstrate of dThd for both enzymes, BVDU and the novel tritylated inhibitors behaved strikingly differently. BVDU behaved as a classic noncompetitive inhibitor of TK-2 and HSV-1 TK and had no effect on the efficiency of cosubstrate (ATP) binding. Thus, BVDU clearly binds independently from ATP at different sites on the enzyme; thus, it is not mutually exclusive with ATP. The resulting enzyme-substrate-inhibitor complex is catalytically inactive. In sharp contrast, the TK-2 and HSV-1 TK inhibitors KIN-12, KIN-52, and KIN-39 behave invariably as classic uncompetitive inhibitors with respect to ATP, binding reversibly to the enzyme-substrate complex and yielding an inactive enzyme-substrate-inhibitor complex. The inhibitors do not bind free enzyme (unlike noncompetitive inhibitors like BVDU); instead, they bind to the enzyme only after binding of the ATP cosubstrate. Such uncompetitive inhibitors decrease V_{max} and K_m to the same extent, in contrast with the noncompetitive inhibitor BVDU that does not affect the apparent K_m of the enzyme for ATP.

Therefore, the newly described tritylated inhibitors may reversibly interact with the TK-2 (and HSV-1 TK) enzymes by competitive inhibition of dThd binding in the substrate-binding site of the enzyme only after the enzyme has bound ATP and afforded a conformational change of the substrate-binding site to allow the inhibitor to bind to this site (as suggested by the uncompetitive nature of the enzyme inhibition with respect of ATP).

An advantage and unique property of the newly described TK-2 inhibitors is their structurally non-nucleosidic nature;

TABLE 2

Kinetic analysis of BVDU and TK inhibitors against nucleoside kinases using thymidine as the varying substrate

K_i values derived from Lineweaver-Burk plots. Data are derived from two to three independent experiments. K_m values for dThd are 1.17 ± 0.11 μM (TK-2), 1.52 ± 0.23 μM (HSV-1 TK), and 3.64 ± 1.27 μM Dm-dNK.

Compound	TK-2		HSV-1 TK		Dm-dNK	
	K_i	K_i/K_m	K_i	K_i/K_m	K_i	K_i/K_m
	μM		μM		μM	
KIN-5	22 ± 5	20	3.3 ± 0.5	2.6	10 ± 5	4.3
KIN-6	13 ± 3	10	6.1 ± 0.8	3.7	15 ± 2	2.8
KIN-12	0.78 ± 0.08	0.65	16 ± 0.8	11	1.7 ± 0.2	0.57
KIN-39	3.1 ± 0.1	3.0	0.46 ± 0.19	0.25	7.1 ± 2.5	1.7
KIN-52	0.50 ± 0.03	0.37	N.A.		N.A.	
BVDU	0.22 ± 0.02	0.14	0.29 ± 0.10	0.10	0.64 ± 0.04	0.50

N.A., not applicable: no measurable inhibition.

TABLE 3

Kinetic analysis of BVDU and TK inhibitors against TK-2 and HSV-1 TK using ATP as the varying substrate

K_i values are derived from Lineweaver-Burk plots. K_m values for ATP are 23 μM (TK-2) and 6.8 μM (HSV-1 TK).

Compound	TK-2			HSV-1 TK		
	K_i	K_i/K_m	Nature of Inhibition	K_i	K_i/K_m	Nature of Inhibition
	μM			μM		
KIN-12	17 ± 0.2	0.81	U.C.	33	6.4	U.C.
KIN-52	24 ± 4.5	1.1	U.C.	N.A.	N.A.	N.A.
BVDU	5.3 ± 0.6	0.21	N.C.	14 ± 1.0	1.7	N.C.

U.C., uncompetitive; N.C., noncompetitive; N.A., not applicable: no measurable inhibition.

they lack the intact (deoxy)ribose moiety, which is, instead, (preferentially) replaced by a trityloxybutenyl functionality. It should be noted that the trityl group occupies a totally different position for KIN-5 and KIN-6, on the one hand, and for KIN-12 and KIN-52, on the other hand, because of the cyclic (KIN-5 and -6) or acyclic (KIN-12 and -52) nature of the compounds. However, the presence of the pyrimidine entity (thymine or BVU) in the structure of the (acyclic and cyclic) tritylated inhibitors seems to be crucial to afford their inhibitory potential and is most likely required for recognition of the inhibitor in the substrate-binding site through hydrogen binding and correct positioning of the molecule in the active site of the enzyme. Indeed, the crystallographic structure of dThd or BVDU complexed with HSV-1 TK revealed hydrogen binding of the imino and carbonyl groups at the 3 and 4 positions of the pyrimidine ring with Gln-125, and efficient positioning of the pyrimidine base by Tyr-172 and Met-128 (Wild et al., 1995, 1997; Champness et al., 1998; Bennett et al., 1999; Vogt et al., 2000). Introduction of a methyl at the N^3 position of thymine in the KIN-12 inhibitor completely annihilated the inhibitory potential of the compound (data not shown) and is in line with our hypothesis that the pyrimidine moiety of the inhibitors fulfills the same role as the thymine base in dThd for efficient recognition of the natural substrate by the enzyme.

In conclusion, we discovered a unique class of potent and

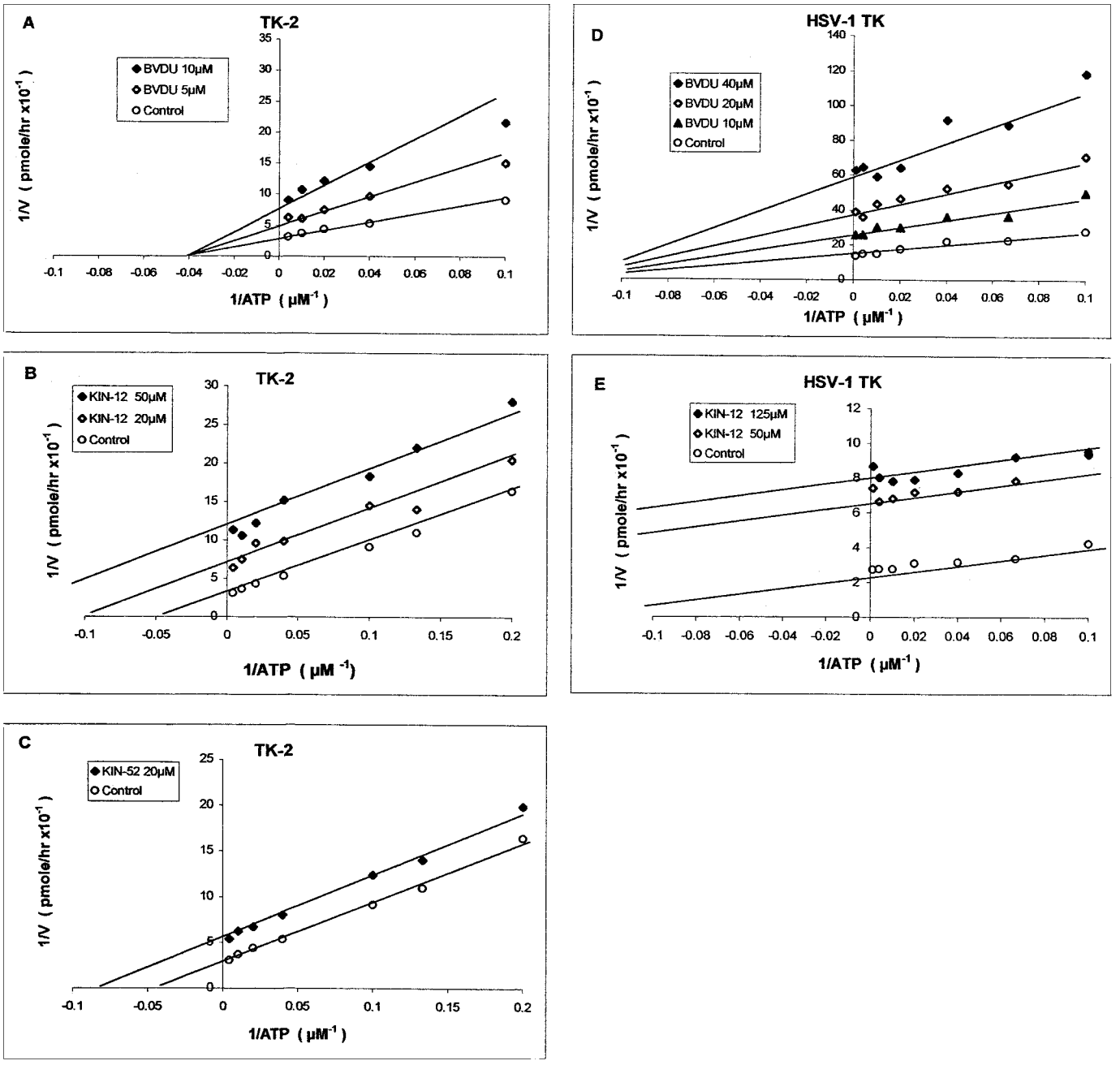

Fig. 3. Lineweaver-Burk plots of TK-2 (A, B, C) and HSV-1 TK (D, E) inhibition by BVDU and two nucleoside kinase inhibitors [KIN-12 (B, E) and KIN-52 (C)] in the presence of varying concentrations of the cosubstrate ATP.

pure inhibitors of TK-2, with a non-nucleosidic structure and with different kinetics than the currently known (alternative substrate) inhibitors. Some members of this class of compounds are highly specific for TK-2 and may represent a new lead for selective TK-2 inhibitors that will be useful for the study of the physiological role of TK-2.

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