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Unusual nucleoside triphosphate donors for nucleoside kinases: 3′-Deoxyadenosine-2′-triphosphate and 2′-deoxyadenosine-3′-triphosphate*°

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Two non-conventional analogues of ATP, 3′-deoxyadenosine-2′-triphosphate (3′-d-2′-ATP) and 2′-deoxyadenosine-3′-triphosphate (2′-d-3′-ATP), the syntheses of which are described, were examined as potential phosphate donors for the nucleoside kinases: 2′-deoxycytidine kinase (dCK), cytosolic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2). The reactions were monitored by means of a mixture of [γ-^{32}P]ATP and cold analogue, and/or with the use of ^{3}H-labelled acceptors and cold donor.

With dCK, using equimolar mixtures of ATP with each analogue, and dC as acceptor, phosphate transfer from 3′-d-2′-ATP and 2′-d-3′-ATP amounted to 34% and 14%, respectively. With each analogue used alone (each at concentration of 100 μM), phosphate transfer from 3′-d-2′-ATP was 55% that from ATP, and from 2′-d-3′-ATP 16%. With human TK2, and equimolar mixtures of [γ-^{32}P]ATP with each of the analogues, and 1 μM dT as acceptor, there was no detectable transfer from either analogue. But,

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Abbreviations: dCK, deoxycytidine kinase (EC 2.7.1.74); TK, thymidine kinase (EC.2.7.1.21), cytoplasmic (TK1) and mitochondrial (TK2); N, nucleoside; dN, 2′-deoxynucleoside; 3′-d-2′-ATP, 3′-deoxyadenosine-2′-triphosphate; 2′-d-3′-ATP, 2′-deoxyadenosine-3′-triphosphate; BAP, bacterial alkaline phosphatase; TEAB, triethylammonium bicarbonate.

when each analogue was used alone, phosphate transfer attained 11% and 5%, respectively, that for ATP alone. With the low affinity form of human TK1, and dT as acceptor, only low phosphate transfer occurred with either analogue used alone. Both compounds exhibited Michaelis-Menten kinetics (with significantly lower V_{max} than ATP), while ATP exhibited cooperative kinetics with all three kinases.

Deoxycytidine kinase (dCK, NTP:deoxycytidine-5′-phosphotransferase, EC 2.7.1.74) and thymidine kinases (ATP:thymidine-5′-phosphotransferases, EC 2.7.1.21) are key enzymes of the salvage pathway, also involved in the metabolic activation (phosphorylation) of nucleoside analogues with antitumor and/or antiviral activities.

dCK catalyzes the phosphorylation of dC to dCMP in the presence of a nucleoside-5′-triphosphate phosphate donor. The cytosolic enzyme has been isolated and purified to apparent homogeneity from human leukemic spleen [1] and leukemic human T-lymphoblasts [2]. The enzyme also phosphorylates purine deoxyribonucleosides and some nucleoside analogues [3–6].

TK catalyzes the phosphorylation of dT to dTMP. In eukaryotic cells, two thymidine kinases (TK) are known: TK1 – in cytoplasm and TK2 – predominantly localized in mitochondria. TK1 has been purified to homogeneity from HeLa cells [7] and human leukemic spleen [8]. TK2, most highly purified from human leukemic spleen [8], exhibits substrate specificity partially overlapping with dCK.

Substrate specificities of all three enzymes have been extensively studied for various phosphate acceptors [3–6, 8]. Although less attention has been devoted to the specificity of the phosphate donors, several communications describe nucleoside triphosphates other than ATP as reasonably good donors for nucleoside kinases [9–13]. Nonetheless, because of the relatively high level of ATP in cellular systems, it has been widely, and implicitly, assumed that ATP is the universal phosphate donor for both nucleoside and protein kinases, notwithstanding the existence of the aforementioned exeptions (for review, see [14]).

In the case of dCK, it was suggested some time ago [15, 16] that UTP may be the intracellular phosphate donor, subsequently supported by a comparison of the phosphate donor properties of UTP relative to ATP under conditions simulating those *in vivo* [17], and by means of enzyme kinetics [15–18]. This, in turn, directed us to other potential NTP donors, not necessarily limited to 5′-NTPs. In this context, our attention was drawn to a long-overlooked observation of Englund *et al.* [19] that the affinity of 2′-deoxynucleoside-3′-triphosphates (2′-d-3′-NTP) for DNA polymerase was comparable to that of normal 5′-triphosphates, although the former were not substrates. Independently of the foregoing, it has more recently been demonstrated in a series of reports ([20] and references cited) that 2′-d-3′-ATP, and other related analogues are potent inhibitors of adenylyl cyclases and may physiologically regulate these enzymes, leading to a reduction in levels of 3′:5′-cAMP, and consequently of cAMP-dependent protein kinases.

We here report on the affinities, and potential substrate properties, of 2′-d-3′-ATP and 3′-d-2′-ATP for three highly purified nucleoside kinases dCK, TK1 and TK2.

MATERIALS AND METHODS

Materials. [5-^{3}H]-2′-deoxycytidine (19.3 Ci/mmol), [6-^{3}H]-2′-deoxythymidine (29 Ci/mmol) and [γ-^{32}P]-ATP (about 3000 Ci/mmol) were from Amersham, U.K. [2,8-^{3}H]-2′-Deoxyadenosine (46 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, California). Unlabelled nucleosides (dC, dT, dA and 3′-dA), ATP, BSA (fraction V, for enzyme stabilisation) were from Sigma, and bacterial

alkaline phosphatase (BAP) from Boehringer (Germany).

Enzymes and assays. All three enzymes were highly purified to apparent homogeneity from human leukemic spleen: dCK 6000-fold to a specific activity of 260 nmols dCMP formed per min per mg, TK1 20000-fold to a specific activity of 9.5 μmols dTMP formed per min per mg, and TK2 approximately 20000-fold to a specific activity of 0.5 μmols dTMP formed per min per mg. Although TK1 may exist in low- and high-affinity forms *vs* dT, the latter being obtained by incubation in the presence of ATP [21], the present study was limited to the low-affinity form because ATP analogues were tested in the absence of ATP.

Activity of enzymes was routinely followed by a radiochemical procedures with ^{3}H-labelled nucleosides, described for dCK by Ives & Wang [22] with modifications reported by Kierdaszuk & Erikson [23], and in the case of thymidine kinases according to Munch-Petersen [24]. To follow phosphate transfer with a mixture of two triphosphate donors, one of which was ATP, the latter was [γ-^{32}P]ATP, and the nucleoside acceptors were unlabelled. This permitted quantitation of the relative donor properties of the two triphosphates, as described in detail elsewhere for the donor properties of each component of an ATP/UTP mixture [17].

Chemical syntheses. UV spectra were recorded on a Specord UV-VIS spectrophotometer (Carl Zeiss, Germany). Thin-layer chromatography (TLC) was carried out on F 1500 LS 254 silica gel plates (Schleicher & Schuell, Germany). Solvent systems used were: (A) chloroform/methanol 9:1 (v/v), (B) chloroform/methanol 19:1, (C) 2-propanol/25% aq. ammonia/water 11:7:2 (by vol.). Column chromatography was performed on silica gel L 40/100 μ or 100/400 μ (Chemapol, Czechoslovakia). Melting points (uncorrected) were determined with a Boetius (Germany) apparatus.

High-performance liquid chromatography (HPLC) was carried out with a Spectra Physics apparatus with a column of Supelco LC-18 T (4.6 mm × 250 mm) using a linear gradient (0–100%) of buffer E (MeOH/buffer D (1 M KH_2PO_4, pH 6.0) 1:3, (v/v)) in buffer D at a flow rate 1.3 ml/min (time of analysis was 15 min). Solutions of compounds in organic solvents were dried with anhydrous sodium sulphate for 4 h. Reactions were performed at room temperature, unless otherwise stated.

N^6-Benzoyl-3′-deoxyadenosine. Trimethylsilyl chloride (1.1 g, 1.28 ml, 10 mmol) was added to a solution of 0.5 g (2 mmol) 3′-deoxyadenosine [25] in pyridine (10 ml) and the reaction mixture was stirred for 15 min. Benzoyl chloride (1.56 g, 1.16 ml, 10 mmol) was then added and stirring continued for 2 h. The reaction mixture was cooled to 0°C and water (2.0 ml, 0°C) was added with stirring. After 5 min, 25% aq. ammonia (4 ml) was added and stirring continued for 30 min. The reaction mixture was evaporated and the residue partitioned between water (70 ml) and ethylacetate (50 ml). The organic phase was dried, evaporated and the residue crystallised from ethanol to give 0.7 g (88%) of N^6-benzoyl-3′-deoxyadenosine, m.p. 207–209°C. R_F 0.37 (A). UV (MeOH) λ_{max} (ε): 233 nm (24500), 282 nm (15100) [26], for ^{1}H NMR data see [26].

N^6-Benzoyl-2′-deoxyadenosine was obtained as described above for N^6-benzoyl-3′-deoxyadenosine, starting from 1.9 g (7.6 mmol) of 2′-deoxyadenosine to give 1.78 g (66%) of N^6-benzoyl-2′-deoxyadenosine, m.p. 114–115°C; R_F 0.29 (A). UV (MeOH) λ_{max} (ε): 280 nm (20400) (cf. data in [27]).

N^6-Benzoyl-5′-O-monomethoxytrityl-3′-deoxyadenosine was synthesized essentially as elsewhere described [25].

A mixture of N^6-benzoyl-3′-deoxyadenosine (1.0 g, 2.5 mmol) and monomethoxytrityl chloride (1.4 g, 4.6 mmol) in dry pyridine (16 ml) was stirred for 12 h and then poured into a mixture of ice and water (300 ml) with vigorous stirring. The resulting precipitate was col-

lected by filtration, washed with water and dried *in vacuo*. The product was purified on a column of silica gel (100 ml), eluting with a linear methanol gradient (0–10%, v/v, 2 × 500 ml) in chloroform to give 1.56 g (88%) of N^6-benzoyl-5′-*O*-monomethoxytrityl-3′-deoxyadenosine as an amorphous powder. R_F 0.45 (B). UV (MeOH) λ_{max} (ε): 233 nm (27400), 280 nm (19500), for ^{1}H NMR data see [26].

N^6-Benzoyl-5′-O-monomethoxytrityl-2′-deoxyadenosine was obtained as described above, starting from 1.5 g (4.22 mmol) of N^6-benzoyl-2′-deoxyadenosine to give 2.24 g (86%) of N^6-benzoyl-5′-*O*-monomethoxytrityl-2′-deoxyadenosine, m.p. 104–106°C; R_F 0.52 (A), UV (MeOH) λmax (ε): 232 nm (25700), 280 nm (19900), (cf. data in [27]).

Preparation of triphosphates was performed from the protected deoxyribonucleosides using 1 M triimidazolylphosphate in anhydrous pyridine for selective monophosphorylation of the 2′- or 3′-hydroxyl function [28], followed by condensation with bis(tributylammonium)pyrophosphate.

1 M triimidazolylphosphate in pyridine was prepared as follows: phosphorus oxychloride (0.3 g, 2 mmol) was added to a solution of imidazole (0.41 g, 6 mM) in anhydrous pyridine (2 ml) at 0°C, the mixture stirred for 2 h, centrifuged for 10 min at room temperature and the supernatant collected.

Triimidazolylphosphate in pyridine (1 M, 0.45 mmol) was added to each protected deoxyribonucleoside (0.3 mmol) and the reaction mixture stirred for 4–6 h. A mixture of 0.5 M bis(tributylammonium)pyrophosphate in anhydrous *N,N*-dimethylformamide (3 ml) and tributylamine (0.3 ml) were then added and stirring continued for 30 min. The reaction mixture was poured into 0.5 M TEAB (3 ml), stirred at 20°C for 3 h, and evaporated. The residue was dissolved in conc. NH_4OH (20 ml), kept for 20 h, evaporated and then co-evaporated with methanol. After addition of 80% acetic acid (15 ml), the mixture was stirred at 20°C for 7 h, and evaporated. The residue was chromatographed on a DEAE Sephadex A-25 (HCO_3^- form, 40 ml) column using a linear gradient (0.001–1.0 M, pH 7.6, 2 × 500 ml) of TEAB buffer. The product-containing fractions were collected and evaporated. Triphosphates were obtained in the form of Na^+ salts as amorphous powders [29].

The purity of synthesised 2′- and 3′-triphosphates was checked by HPLC and TLC, further confirmed by conversion to the corresponding nucleosides under the action of BAP [30]. This was further confirmed by NMR spectroscopy, demonstrating the absence of contamination by 5′-ATP. Detailed NMR data, including an analysis of the conformations of the two triphosphates relative to that of ATP, will be presented elsewhere.

3′-Deoxyadenosine-2′-triphosphate was obtained as described above, starting from 0.188 g (0.3 mmol) of N^6-benzoyl-5′-*O*-monomethoxytrityl-3′-deoxyadenosine to give 0.11 g (58%) of 3′-deoxyadenosine-2′-triphosphate. R_F 0.27 (C), UV (MeOH) λ_{max} (ε): 260 nm (13200), R_t 7.22 min.

2′-Deoxyadenosine-3′-triphosphate was obtained as described above, starting from 0.126 g (0.2 mmol) of N^6-benzoyl-5′-*O*-monomethoxytrityl-2′-deoxyadenosine to give

Table 1. Relative phosphate uptake (%) from 100 μM ATP, 3′-d-2′-ATP or 2′-d-3′-ATP phosphate donors catalyzed by dCK, TK1 and TK2, shown as a percentage of total transfer from ATP (100 %)[a].

	dCK	TK1[b]	TK2
ATP	100	100	100
3′-d-2′-ATP	55	7	11
2′-d-3′-ATP	16	1.5	5

[a] Concentration of phosphate acceptors was 3 × K_m for each enzyme, 2.5 μM dC for dCK, 1 μM dT for TK2 and 20 μM dT (for technical reasons slightly above K_m) for TK1.

[b] For the low affinity form of TK1 [21].

0.057 g (45%) of 2′-deoxyadenosine-3′-triphosphate. R_F 0.27 (C), UV (MeOH) λ_{max} (ε): 260 nm (12600), R_t 7.62 min.

RESULTS

The procedure adopted for the synthesis of the two ATP analogues is both novel and simple. Each of the starting nucleosides, dA and 3′-dA was converted to the 5′-*O*-benzoyl derivative, followed by monomethoxytritylation of the secondary hydroxyl. The free hydroxyl group was then phosphorylated to the monophosphate, and finally to the triphosphate by conventional procedures, and the 5′-*O*-benzoyl protecting group was then removed. Details of the syntheses, and tests for purity are given in Materials and Methods.

Both nucleoside triphosphate analogues, 2′-d-3′-ATP, and 3′-d-2′-ATP, were initially tested at 100 mM concentration for potential donor properties with all three enzymes, dC as acceptor in the case of dCK, and dT as acceptor with TK1 and TK2. As may be seen from Table 1, both triphosphates exhibited donor activities, particularly high in the case of dCK, with 3′-d-2′-ATP more effective than 2′-d-3′-ATP in all instances.

The high donor activities of the two analogues, relative to ATP, prompted us to examine their activities in the presence of ATP. With equimolar mixtures of each of the analogues with [γ-^{32}P]ATP, each component at 100 μM, transfer of phosphate from 3′-d-2′-ATP attained a value of 34%, and from 2′-d-3′-ATP 14% that from ATP. It follows that each effectively competes with ATP. In the case of TK2, under similar conditions, no detectable phosphate transfer was observed from either analogue.

The data for all kinases were further extended by an examination of the kinetics for the superior donor 3′-d-2′-ATP. In all cases V_{max} values for both analogues were significantly lowered, to 10–50% those for ATP. From Table 2 it will be seen that these differed from those for ATP, with marked differences in K_m and/or type of kinetics. This is particularly pronounced for TK2, which exhibits a 20-fold higher K_m, and Michaelis-Menten kinetics, in striking contrast to the negative cooperativity with ATP, while in the case of dCK (Table 2) K_m values are similar to those for ATP, and the main differences are in V_{max} values and type of kinetics.

Table 2. K_m (μM) and Hill coeficients (h) for ATP, 3′-d-2′-ATP and 2′-d-3′-ATP as phosphate donors in phosphorylation reactions catalyzed by dCK, TK1 and TK2, respectively.

Enzyme	Phosphate acceptor	Phosphate donor		
		ATP	3′-d-2′-ATP	2′-d-3′-ATP
TK1	dT	140 (h = 1.6)[a]	120 ± 40 (h = 1)	500[b]
TK2	dT	2.5 ± 0.3 (h = 0.4)	50 ± 6 (h = 1)	n. d.
dCK	dC	15 ± 1 (h = 0.7)	20 ± 3 (h = 1)	25 ± 5 (h = 1)

[a] Data from [31]; [b] Approximate value; n.d. not determined.

DISCUSSION

The natural occurrence of purine nucleoside 3′-di- and triphosphates has long been known (see [32] and references cited), but their properties and biochemistry have been little studied in eukaryotic systems. It has been shown recently that 2′-d-3′-ADP and 2′-d-3′-ATP are potent inhibitors and regulators of adenyl cyclase [20]. In a separate study we have found

that both 2′-d-3′-ADP and 2′-d-3′-ATP are inhibitors, but not substrates, of deoxynucleotidyl transferase (Nowak R., Siedlecki J. & Shugar D., in preparation).

The two analogues embraced in this study, and their mono- and diphosphate counterparts, are expected to be useful in studies on the mode of binding NTP donors to nucleoside and protein kinases, the specificities of nucleases, the intermediates involved in the stringent response, etc. They may also prove of interest as potential differential inhibitors of cellular and viral polymerases.

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