Biochemical Pharmacology, Vol. 37, No. 4, pp. 673–677, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

INHIBITION OF HUMAN LEUKAEMIC THYMIDYLATE KINASE AND L1210 RIBONUCLEOTIDE REDUCTASE BY DINUCLEOTIDES OF ADENOSINE AND THYMIDINE AND THEIR PHOSPHONATE ANALOGUES*

ROSANNE M. ORR,† LAWRENCE C. DAVIES, JOHN A. STOCK, GORDON A. TAYLOR, RAYMOND L. POWLES‡ and KENNETH R. HARRAP

Drug Development Section, The Institute of Cancer Research, Sutton, Surrey SM2 5PX, and ‡ Division of Medicine, Royal Marsden Hospital, Sutton, Surrey SM2 5PT, U.K.

(*Received* 22 *May* 1987; *accepted* 21 *August* 1987)

Abstract—Dinucleotides of adenosine and thymidine in the Ap_nT series ($n = 3,4,5$ and 6) and their corresponding phosphonate analogues, where a methylene group replaces the oxygen between the alpha and beta phosphorus atoms adjacent to thymidine, have been evaluated as inhibitors of human leukaemic thymidylate kinase (dTMP kinase, EC 2.7.4.9) and ribonucleotide reductase (EC 1.17.4.1) from L1210 cells. Ap_3T, Ap_4T, Ap_2cpT and Ap_3cpT were poor inhibitors of both enzymes. Ap_5T, Ap_6T and their phosphonate analogues were potent inhibitors of dTMP kinase, possibly acting as bisubstrate analogues (IC_{50} values: Ap_5T, 7.9 μM; Ap_4cpT, 5.8 μM; Ap_6T, 5.4 μM; Ap_4cpT, 4.0 μM). For CDP reductase, where these compounds may bridge activity/effector sites on the M1 subunit of the enzyme, Ap_5T and Ap_6T were inhibitors with IC_{50} values of 14.4 μM and 20.3 μM respectively. The phosphonate series of compounds was far less active. The thymidine moiety of the compounds was essential for inhibition since Ap_5A was inactive against both enzymes. dTTP, although a poor inhibitor of thymidylate kinase was a potent negative effector of CDP reductase (IC_{50}, 19.3 μM). Significantly, Ap_5T was not hydrolysed to release dTTP under the conditions of the assay. These studies show that the activities of both enzymes may be modulated by nucleotide analogues.

Thymidylate, which can be produced by either salvage or *de novo* synthesis, is dependent upon the activity of thymidylate kinase (dTMP kinase, EC 2.7.4.9) for further phosphorylation. This enzyme has been shown to be elevated in both rodent and human tumours [1, 2]. Thus inhibition of dTMP kinase should severely deplete intracellular thymidine nucleotide pools and hence prohibit further DNA synthesis. This enzyme catalyses the conversion of thymidylate to the corresponding diphosphate using ATP as a phosphate donor in the presence of Mg^{2+}. By analogy with adenylate kinase (EC 2.7.4.3), where Ap_5A§ is a potent bisubstrate analogue inhibitor [3], compounds in the series Ap_nT (where n = number of phosphoryl groups) should prove to be effective inhibitors of dTMP kinase. Indeed, studies by Bone *et al.* [4] have shown that a chain length of 5 and 6 phosphoryl groups in the Ap_nT series confers the best inhibitory potency on dTMP kinase.

Ribonucleotide reductase (EC 1.17.4.1) is tightly linked to normal and neoplastic cell proliferation [5, 6]. Its activity is low compared with other enzymes involved with DNA synthesis [7–9], suggesting that it may be rate-limiting in this process. The evidence that ribonucleotide reductase activity is greater in tumour cells than normal cells possessing the same replicative rate [5], coupled with the implication that increased levels can adversely affect cellular differentiation [10], strongly suggest that this enzyme is a favourable target for anticancer chemotherapy. Ribonucleotide reductase consists of two non-identical subunits [11]: an M1 subunit which binds nucleotide substrates and effectors and an M2 subunit which contains a non-haem iron moiety and a tyrosine moiety capable of free radical formation. Metal chelating agents and free radical scavengers such as thiosemicarbazones, imidazopyrazoles, hydroxyurea and benzohydroxamic acid derivatives inactivate the M2 subunit of ribonucleotide reductase [12–15]. The M1 subunit is under stringent allosteric regulation and for the calf thymus enzyme it has been proposed that the M1 subunit contains at least two classes of effector binding sites, a specificity site which binds ATP, dTTP or dGTP and regulates substrate specificity, and an activity site which binds ATP and the negative effector dATP [16]. AraATP, fluoroAraATP, 5′-deoxyInox (the product resulting from periodate oxidation of 5′-deoxyinosine), dATP,

* This work was supported by grants to The Institute of Cancer Research: Royal Cancer Hospital from the Cancer Research Campaign and the Medical Research Council.

† To whom reprint requests should be addressed.

§ Abbreviations used: Ap_5A, P^1,P^5-(diadenosine-5′)-pentaphosphate; Ap_3T, P^1-(adenosine-5′)-P^3-(thymidine-5′)-triphosphate; Ap_4T, P^1-(adenosine-5′)-P^4-(thymidine-5′)-tetraphosphate; Ap_5T, P^1-(adenosine-5′)-P^5-(thymidine-5′)-pentaphosphate; Ap_6T, P^1-(adenosine-5′)-P^6-(thymidine-5′)-hexaphosphate; Ap_2cpT, P^1-(thymidine-5′) - P^3 - (adenosine - 5′) - P^1,P^2 - methylenetriphosphate; Ap_3cpT, P^1-(thymidine-5′)-P^4-(adenosine-5′)-P^1,P^2-methylenetetraphosphate; Ap_4cpT, P^1-(thymidine-5′)-P^5-(adenosine-5′)-P^1,P^2-methylenepentaphosphate; Ap_5cpT, P^1-(thymidine-5′)-P^6-(adenosine-5′)-P^1,P^2-methylenehexaphosphate.

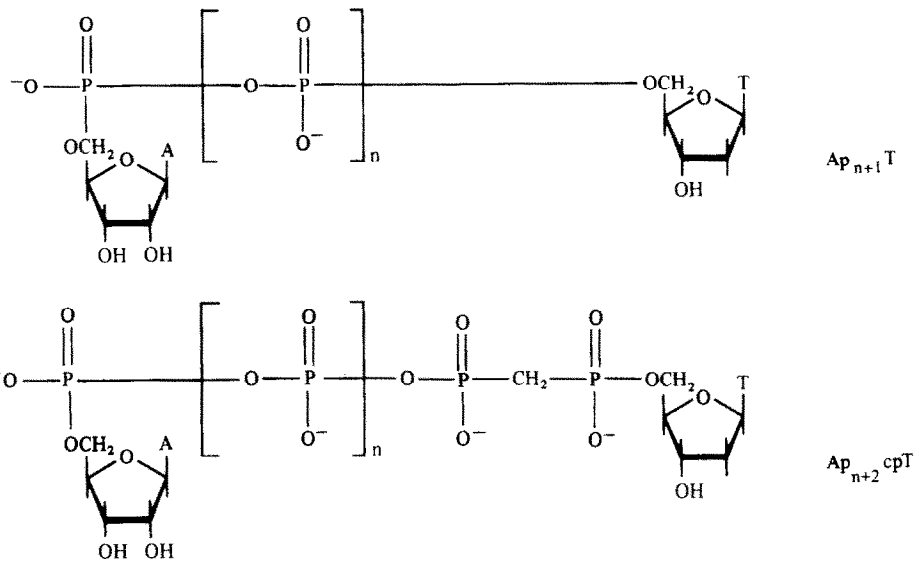

Fig. 1. Structures of the dinucleotides and phosphonate analogues where T = thymine, A = adenine and n = number of phosphoryl groups.

dTTP and dGTP are effective inhibitors of this subunit [17, 18].

We have prepared Ap_3T, Ap_4T, Ap_5T, Ap_6T and the corresponding phosphonate analogues where a methylene group replaces the oxygen between the alpha and beta phosphorus atoms adjacent to thymidine (Ap_ncpT, where n = 2,3,4 and 5 phosphoryl groups) (Fig. 1). These compounds have been examined for their inhibitory potencies against human leukaemic dTMP kinase as bisubstrate analogues and against L1210 CDP reductase where these compounds may bridge activity/effector sites on the M1 subunit of the enzyme.

MATERIALS AND METHODS

Materials

Nucleotides, dithioerythritol, bovine liver nucleoside 5'-diphosphate kinase, rabbit muscle creatine phosphokinase, bovine serum albumin, phosphocreatine, snake venom (*Crotalus atrox*) and Dowex-1×8-200 were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). [Methyl-^{3}H]-dTMP (40Ci/mmol), [U-^{14}C]-CDP (438 mCi/mmol) and ACS scintillant were obtained from Amersham (Amersham, Bucks, U.K.). HPLC columns were purchased from Jones Chromatography (Glamorgan, U.K.), acetonitrile from Romil Chemicals (Loughborough, Leics, U.K.) and trioctylamine in 1,1,2-trichlorotrifluoroethane from Aldrich Chemicals (Gillingham, Dorset, U.K.). All other chemicals were supplied by BDH Chemicals Ltd (Poole, Dorset, U.K.), AR grades being used where available. Polygram Cel 300 PEI plates were purchased from Camlab (Cambridge, U.K.). The synthesis of Ap_3T, Ap_4T, Ap_5T, Ap_6T, Ap_2cpT, Ap_3cpT, Ap_4cpT and Ap_5cpT is described elsewhere.*

* L. C. Davies, J. A. Stock, R. M. Orr, S. E. Barrie and K. R. Harrap, *J. Med. Chem.*, submitted.

Methods

dTMP kinase. Twenty grams of blast cells removed from a patient with acute myeloid leukaemia (M_4 subtype classification) were homogenised, using a Potter Elvehjem homogeniser, in 200 ml of 10 mM Tris/HCl buffer pH 7.5 containing 2 mM dithioerythritol and 40 μM dTMP to stabilise the enzyme. Following centrifugation at 40,000 g at 4° for 1 hr, the supernatant was dialysed against the same buffer for 18 hr and aliquots stored in liquid nitrogen. Enzyme activity remained stable under these conditions for at least one year. dTMP kinase activity was assayed by a modification of the method described by Lee and Cheng [19]. The reaction mixture contained ATP (1.5 mM), $MgCl_2$ (4 mM), [methyl-^{3}H]-dTMP (24 μM, 1.25 μCi), dithioerythritol (5 mM), nucleoside 5'-diphosphate kinase (1.25 U), creatine phosphokinase (0.27 U), phosphocreatine (3 mM), bovine serum albumin (0.12%), 12.5 μl of enzyme, Tris/HCl buffer pH 7.5 (50 mM) and variable concentrations of inhibitor, in a final volume of 125 μl. Following incubation at 37° for 10 min, the reaction was terminated by boiling for 3 min and assay tubes centrifuged for 5 min in a Beckman microfuge. Ten microlitre aliquots of supernatant were applied to strips of PEI coated plastic (0.5 in. × 0.75 in.) which were dried and then washed 3 times with 4 M formic acid/1 mM ammonium formate solution for 20 min and finally, 3 times with ethanol for 30 sec. These were counted for radioactivity in 10 ml of ACS scintillant using an Intertechnique beta spectrometer. In experiments to determine the K_m values, when dTMP was the variable substrate, the enzyme preparation was passed down a Dowex-1-borate column [20] to remove stabilising dTMP immediately prior to assay.

CDP reductase. A crude extract was prepared from 10 g of L1210 cells harvested from the ascitic fluid of BDF_1 mice and CDP reductase activity

measured as described by Cory and Mansell [21, 22]. The precipitate collected after adjustment of the crude extract to pH 5.2 with 1 M acetic acid, was dissolved in 0.05 M Tris/HCl buffer, pH 8.0. This fraction was used as enzyme source for testing the inhibitory activity of compounds in a CDP reductase assay with [U-^{14}C]-CDP (50 μM, 0.1 μCi) as substrate. Enzyme activity remained stable for at least 6 months in liquid nitrogen. The 20–40% ammonium sulphate fraction [21] was used for determination of the K_m value for CDP. Protein concentrations were measured by the method of Lowry *et al.* [23] using bovine serum albumin as a standard.

Statistics. Experimental data were fitted to a non-linear least squares regression program based on the algorithm described by Jennrich and Sampson [24]. IC_{50} values were expressed as the concentration of inhibitor required to reduce the control enzyme rate by 50% under the conditions of the assay. For determination of IC_{50} values, the equation $i = a + b \ln (c)$ where i is response and c is inhibitor concentration, equivalent to a semi-log plot, was fitted to the data using an error weighting of $1/(y + \hat{y})^2$ [25, 26].

HPLC. Optimal isocratic conditions for the separation of relevant nucleotides were defined on a 20 × 2 mm column [27]. Samples of reaction mixtures were precipitated with 0.5 M perchloric acid. After standing on ice for 20 min the samples were centrifuged and the supernatants neutralised with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane [28]. The analytical separations were performed on a 100 × 4.6 mm Apex NH_2 column running in 65% 0.2 M ammonium phosphate pH 6.15/35% acetonitrile at a flow rate of 1 ml/min. Figure 2 illustrates the chromatogram for 20 μl of an extract of a CDP reductase reaction mixture incubated for 20 min at 37° in the presence of 90 μM Ap_5T. Under these conditions the capacity factors for the nucleotides of interest were: Ap_5T, 2.82; ADP, 2.88; CDP, 3.30; TTP, 4.52; ATP, 5.84; CTP, 6.76.

RESULTS

dTMP kinase

The reaction rate was linear for at least 25 min in the assay used and the control enzyme rate was routinely 1 nmole of dTTP produced per hour per mg of protein. The K_m value for ATP was calculated to be 266 μM (SE ± 49 μM) (Fig. 3) for dTMP kinase from acute myeloid leukaemia. This is in agreement with others who reported the K_m values to be 250 μM for ATP and 40 μM for dTMP using dTMP kinase derived from acute myeloid leukaemia and chronic myeloid leukaemia [4, 19]. However, we determined the K_m value for dTMP to be 5.4 μM (SE ± 1.2 μM) for our enzyme. This discrepancy may be due to enzyme source, as Bone *et al.* [4] did not report the leukaemic subtype classification of the cells used for their enzyme preparation. Neither Ap_5A nor dTTP inhibited the enzyme reaction significantly (Table 1). The inhibitory potencies of the Ap_nT dinucleotides increased with extension of the chain length of phosphoryl groups. Ap_5T and Ap_6T were the best inhibitors with IC_{50} values of 7.9 μM and 5.4 μM respectively (Table 1). The introduction of an alpha, beta-methylene group did not reduce the inhibitory activity of either compound.

CDP reductase

Enzyme activity related to protein concentration did not yield a linear relationship at low protein concentrations. This phenomenon has been described previously for CDP reductase activity measurements [5]. However, activity was related to the enzyme amount between 100 μg and 500 μg of protein per assay tube. Under the assay conditions, the reaction rate was linear for 20 min. The control enzyme rate was 0.9 nmoles of dCDP produced per hour per mg of protein for the 20–40% ammonium sulphate fraction. The K_m value for CDP was calculated to be 7.8 μM (SE ± 1.8 μM) using this enzyme preparation from L1210 cells (Fig. 4). A

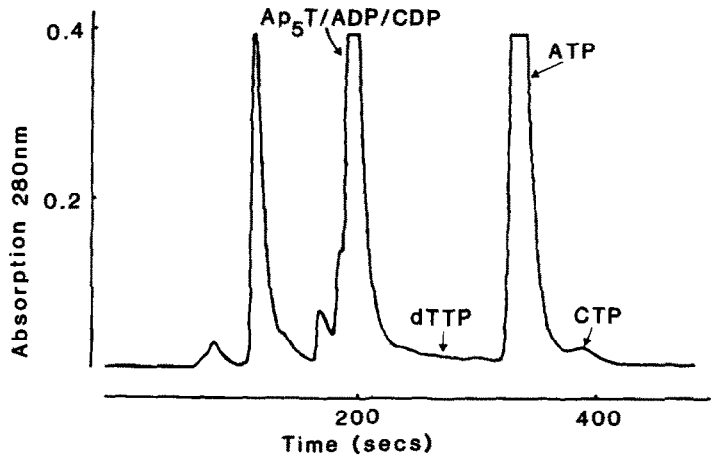

Fig. 2. HPLC nucleotide profile of a CDP reductase reaction mixture incubated for 20 min at 37° in presence of 90 μM Ap_5T.

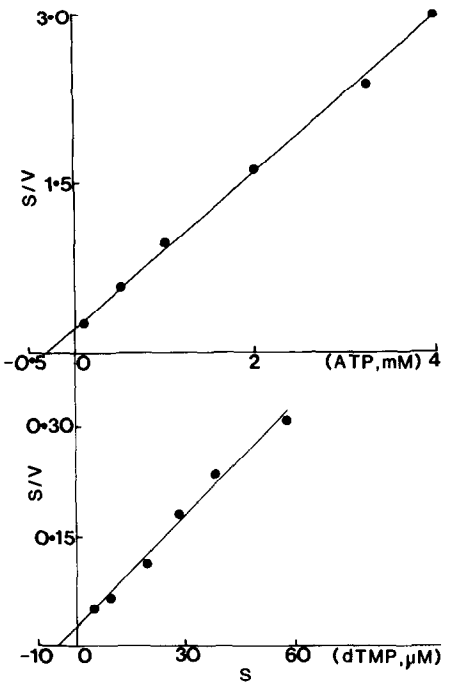

Fig. 3. S/V against S plot for the determination of K_m values for dTMP and ATP using human leukaemic dTMP kinase, where S = substrate concentration (dTMP, μM, or ATP, mM) and V = initial velocity, nmoles of dTTP produced per hour per mg protein. Each point represents the mean of duplicate determinations.

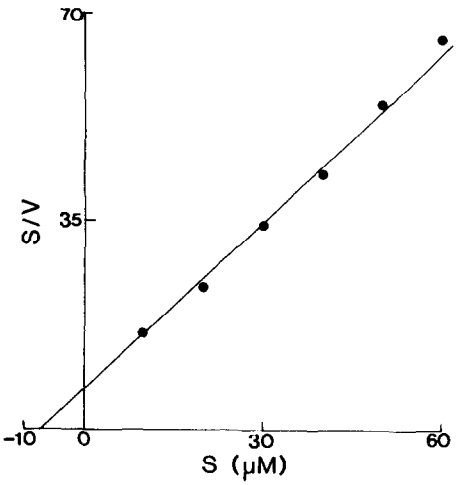

Fig. 4. S/V against S plot for the determination of the K_m value for CDP using L1210 CDP reductase, where S = substrate concentration (CDP, μM) and V = initial velocity, nmoles of dCDP produced per hour per mg protein. Each point represents the mean of triplicate determinations.

wide range of K_m values for CDP has been reported for CDP reductase extracted from different sources, ranging from 3.6 μM for the Novikoff tumour [29] to 370 μM for normal and transformed human fibroblasts [30]. Of the dinucleotides tested, Ap_5T and Ap_6T were the most potent inhibitors (Table 1). The introduction of an α,β-methylene group decreased the inhibitory potency approximately 10-fold in both cases. Ap_5A did not inhibit the enzyme. Since dTTP was a substantial negative effector (Table 1), it was important to determine whether these compounds could be degraded to release dTTP in the assay system. However, less than 3 μM dTTP (the limits of detection of dTTP in the HPLC assay described in the Methods section) was produced in a CDP reductase incubation mixture which contained 90 μM Ap_5T (Fig. 2).

Table 1. Inhibition of human thymidylate kinase and L1210 CDP reductase by dinucleotides of adenosine and thymidine and their phosphonate analogues

Compound	IC_{50} (μM)* dTMP kinase	CDP reductase
Ap_3T	1230 (± 180)†	>500
Ap_2cpT	730 (± 70)	455
Ap_4T	290 (± 40)	76.7 (± 4.3)
Ap_3cpT	340 (± 60)	296 (± 9.0)
Ap_5T	7.9 (± 1.9)	14.4 (± 2.5)
Ap_4cpT	5.8 (± 1.01)	128 (± 9.6)
Ap_6T	5.4 (± 1.36)	20.3 (± 5.8)
Ap_5cpT	4.0 (± 0.36)	231 (± 8.9)
Ap_5A	>1000	>500
dTTP	690 (± 80)	19.3 (± 0.15)

* IC_{50} values as defined in Methods.

† Figures in parenthesis are standard errors of the IC_{50} values.

No cytotoxicity against L1210 cells *in vitro* was observed when these inhibitors were added at a final concentration of 1 mM (data not shown). This was presumably due to the inability of these compounds to penetrate cells.

DISCUSSION

In the Ap_nT series of compounds there was a direct relationship between the degree of inhibition and the number of phosphoryl groups, with a chain length of 5 and 6 phosphorus atoms being optimal for inhibition of both enzymes. The thymidine moiety was essential for inhibition since Ap_5A was without activity. With thymidylate kinase, for which these compounds may act as bisubstrate analogues, maximum inhibition was achieved with compounds containing one or two phosphoryl groups more than the two natural substrates combined. This confirms an earlier observation by Bone *et al.* [4] for dTMP kinase and that of Lienhard and Secemski [3] using Ap_5A as an inhibitor of adenylate kinase. The introduction of an alpha,beta-methylene group in the series of novel compounds Ap_ncpT, still conferred inhibitory potency against dTMP kinase.

The inhibition of L1210 CDP reductase by Ap_5T and Ap_6T may be due to bridging the proposed nucleotide effector site, which binds dTTP, and an activity site on the M1 subunit, which binds ATP. This suggestion is speculative, since although the thymidine moiety was essential for inhibition, it is not known whether the adenosine moiety is an important

structural requirement in these compounds, and awaits competitive binding studies and further elucidation of the enzyme structure. However, Ap_3T showed negligible reductase inhibition compared with dTTP. Therefore, it is possible that this compound binds preferentially to the ATP binding site and does not have the structural requirements to reach the nucleotide effector site. As compounds in the Ap_ncpT series showed markedly reduced activity, this indicates that for CDP reductase there is a binding requirement for a phosphoryl group at the thymidine end of the molecule.

Weak inhibition of ribonucleotide reductases by polynucleotides and tRNA has been documented [31, 32]. Also, Lewis *et al.* [33] isolated an unusual dinucleotide from glutamine-deprived Chinese hamster ovary cells which they suggested might be an important regulator of ribonucleotide reductase, although the structure of this dinucleotide has not been fully elucidated. These observations, together with the known physiological properties of Ap_4A [34] suggest that naturally occurring dinucleotides may play key regulatory roles *in vivo*. Our studies have shown that compounds such as Ap_5T and Ap_6T act not only as potential bisubstrate analogues for dTMP kinase, but are also inhibitors of the CDP reductase reaction, where ATP is not involved in the direct transfer of a phosphoryl group.

Acknowledgements—We are grateful to Miss V. L. Shepherd (Royal Marsden Hospital, Sutton, Surrey, U.K.) for supplying the leukaemic blast cells used in this study and also to Dr L. I. Hart for useful discussions in the preparation of this manuscript.

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