Inhibition of IMP-specific cytosolic 5'-nucleotidase and adenosine formation in rat polymorphonuclear leucocytes by 5'-deoxy-5'-isobutylthio derivatives of adenosine and inosine

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1. The partially purified IMP-specific cytosolic 5'-nucleotidases from rat liver, polymorphonuclear leucocytes and heart were inhibited by 50 % by 2–6 mM-5'-deoxy-5'-isobutylthioadenosine (IBTA) or 7–10 mM-5'-deoxy-5'-isobutylthioinosine (IBTI). IBTA and IBTI inhibited the rat liver and polymorphonuclear-leucocyte enzymes non-competitively. IBTA, but not IBTI, also inhibited the ecto-5'-nucleotidase of polymorphonuclear leucocytes. IBTI was, by contrast, a more potent inhibitor than IBTA of the AMP-specific soluble 5'-nucleotidase from pigeon heart. 2. During 2-deoxyglucose-induced ATP-catabolism in rat polymorphonuclear leucocytes, adenosine formation was inhibited by approx. 80 % by 3 mM-IBTA and by approx. 70 % by 7 mM-IBTI. 3. The results show that 5'-modified nucleosides are inhibitors of cytosolic 5'-nucleotidases and that they penetrate to inhibit their target enzymes in intact cells. Such inhibitors may be useful to clarify the mechanisms of adenosine formation and to prevent mononucleotide hydrolysis during ATP breakdown.

INTRODUCTION

Adenosine formation in response to cellular ATP catabolism may function as a signal which tends to prevent further depletion of ATP (Newby, 1984). The molecular mechanism of adenosine formation is, however, controversial. It may be formed either from extracellular ATP, ADP or AMP via a cascade of ecto-enzymes (Pearson, 1985) or from cytosolic AMP by the action of AMP-specific (Truong *et al.*, 1988; Newby, 1988) or IMP-specific (Van den Berghe *et al.*, 1977; Itoh, 1981a,b; Itoh & Oka, 1985; Itoh *et al.*, 1986) 5'-nucleotidases (EC 3.1.3.5). Inhibitors of the ecto-5'-nucleotidase have proved useful in testing its involvement in adenosine formation (see Newby *et al.*, 1987), but inhibitors of the cytosolic 5'-nucleotidases have not been described. Such inhibitors might have a further use in preventing loss of purine nucleotides from cells undergoing ATP breakdown, which is of relevance to myocardial protection and to the preservation of organs for transplantation.

We showed in a previous study (Worku & Newby, 1982) that during hydrolysis of [³H]IMP by the cytosolic 5'-nucleotidase, inosine apparently inhibited non-competitively the formation of P_i, but did not inhibit formation of [³H]inosine. This breach of stoichiometry was explained by the demonstration that the enzyme catalysed exchange of the nucleoside moiety via an enzyme-phosphate intermediate (Worku & Newby, 1982). Thus incubation in the presence of radiolabelled IMP and unlabelled inosine led to the stoichiometric formation of unlabelled IMP and radiolabelled inosine,

and to the suppression of P_i release. A prediction from that study is that other nucleosides with a free 5'-hydroxy group should also participate in nucleoside exchange (depending on their affinity for the phosphoenzyme intermediate) and thereby should apparently inhibit formation of P_i, but not inosine. Hydrolysis of the enzyme-phosphate intermediate was furthermore shown to require prior release of nucleoside (Worku & Newby, 1982), thus predicting that nucleoside analogues with a modified 5'-position should act as non-competitive inhibitors of both P_i and inosine formation.

Following up those predictions, we show here that 5'-deoxy-5'-isobutylthioadenosine (IBTA) and 5'-deoxy-5'-isobutylthioinosine (IBTI) are non-competitive inhibitors of the IMP-specific cytosolic 5'-nucleotidases from rat liver and polymorphonuclear leucocytes. These nucleoside analogues also inhibited adenosine and hypoxanthine formation during 2-deoxyglucose-induced ATP catabolism in polymorphonuclear leucocytes, showing that they penetrate intact cells to inhibit the cytosolic 5'-nucleotidase *in situ*.

MATERIALS AND METHODS

Preparation and incubation of intact polymorphonuclear leucocytes

Polymorphonuclear leucocytes were prepared and counted for cell density as described previously (Newby, 1980), except that the Krebs-Ringer bicarbonate buffer used (Cohen, 1957) was modified by replacing half of the bicarbonate with 13.5 mM-Hepes/NaOH (pH 7.4). Incubations were performed at 37 °C as described previously

Abbreviations used: IBTA, 5'-deoxy-5'-isobutylthioadenosine; IBTI, 5'-deoxy-5'-isobutylthioinosine; 2'-deoxycoformycin, *R*-3-(deoxy-β-D-pentofuranosyl)-3,6,7,8-tetrahydro[4,5-*d*]diazapin-8-ol; 5-iodotubercidin, 4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine.

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(Worku & Newby, 1983). Briefly, suspensions of cells (0.2 ml , containing 4.7×10^7 – $10.5 \times 10^7/\text{ml}$) were preincubated for 30 min at 37°C with inhibitors of adenosine deaminase (2'-deoxycoformycin, $30\text{ }\mu\text{M}$), adenosine kinase (5-iodotubercidin, $10\text{ }\mu\text{M}$) and xanthine oxidase (allopurinol, $10\text{ }\mu\text{M}$) to prevent further degradation of adenosine and hypoxanthine (Newby *et al.*, 1983), with, in addition, IBTA (3 mM) or IBTI (7 mM) when indicated. ATP catabolism was induced with 30 mM -2-deoxyglucose so as to produce a maximum rate. Incubations were terminated 2–10 min after addition of 2-deoxyglucose with 0.8 ml of 5% (w/v) trichloroacetic acid, and extracts were then deproteinized, neutralized and filtered as described previously (Newby, 1980). Alternatively, 10 min after addition of 2-deoxyglucose, 0.8 ml of buffer was added and the cell suspensions were then centrifuged at 1000 g for 2 min. Supernatants were then removed, and the activity of lactate dehydrogenase in sonicated cells and supernatants was measured as described by Newby (1980) so as to quantify cell intactness.

The activity of ecto-5'-nucleotidase was measured in 0.5 ml portions of polymorphonuclear-leucocyte suspensions (1.0×10^7 – 1.8×10^7 cells/ml) as described by Newby *et al.* (1975). Incubations contained (final concentrations) 10.3 mM -sodium β -glycerophosphate, pH 7.4, 1.5 mM -adenosine, 0.2 mM -AMP, 2–3 kBq of $[2\text{-}^3\text{H}]\text{AMP}$, with or without 3 mM -IBTA or 5 mM -IBTI. Incubations were performed at 37°C for 15 min, after which 0.3 ml of 0.15 M - ZnSO_4 and 0.2 ml of 0.15 M - $\text{Ba}(\text{OH})_2$ were added. The resulting suspensions were centrifuged at 15000 g for 2 min, after which a 0.7 ml portion of the supernatant was removed and radioactivity was measured.

Determination of metabolite concentrations

The concentration of adenosine was measured by h.p.l.c. as described previously (Newby & Holmquist, 1981), except that the concentration of methanol in the buffer system was 15% (v/v). The reversed-phase chromatography of IMP, ATP, ADP, AMP, hypoxanthine and inosine was performed on a 25 cm -long Apex1 $5\text{ }\mu\text{m}$ -Octadecyl-silica column (Jones Chromatography, Hengoed, Mid Glamorgan, U.K.) eluted at 2 ml/min at 21°C with 50 mM -ammonium phosphate, pH 6.7. The respective elution times were 2.3, 2.8, 3.2, 4.0, 4.7 and 18 min. For quantification, the peak heights of unknowns were compared with those of standards, except for inosine, where the peak areas were used. ATP concentration was also measured in some experiments by the luciferin-luciferase method (Spielman *et al.*, 1981).

Partial purification of cytosolic 5'-nucleotidases

The IMP-specific cytosolic 5'-nucleotidase was purified from rat heart or liver homogenates by two-step phosphocellulose chromatography as described by Itoh (1981). Only the first phosphocellulose-chromatography step was used for extraction of the rat polymorphonuclear-leucocyte enzyme. The specific activities of enzyme preparations used were 6.1, 0.3 and 0.7 units/mg of protein respectively for liver, heart and polymorphonuclear leucocytes. Bovine serum albumin (fraction V; final concn. 1 mg/ml) was added to liver and heart enzyme preparations, and they were frozen in small portions for several weeks without detectable loss of activity. Enzyme preparations from polymorphonuclear leucocytes were used for experiments immediately. The AMP-specific pigeon heart soluble 5'-nucleotidase was

purified by the procedure described by Truong *et al.* (1988), and the preparation, of specific activity about 10 units/mg of protein, was used immediately for measurements.

Protein was measured as described by Bradford (1976), with bovine serum albumin as standard.

Measurement of cytosolic 5'-nucleotidase and AMP deaminase activity

The soluble 5'-nucleotidases isolated either from polymorphonuclear leucocytes or from rat heart or liver were assayed as described by Newby (1988). The purified pigeon heart soluble 5'-nucleotidase was assayed by a modification of the method of Newby (1988). The assay contained 100 mM -Tes/NaOH, pH 6.5, 10 mM - MgCl_2 , 30 mM -NaCl, 10 mM -AMP (plus 2–3 kBq of $[2\text{-}^3\text{H}]\text{AMP}$), 0.25 mM -ATP and $5\text{ }\mu\text{g}$ of enzyme.

AMP deaminase isolated from rat heart or polymorphonuclear leucocytes was assayed by the modified procedure of Skladanowski *et al.* (1979). Briefly, $50\text{ }\mu\text{l}$ ($0.5\text{ }\mu\text{g}$) of rat heart enzyme (sp. activity 14.5 units/mg of protein) or $10\text{ }\mu\text{l}$ ($0.8\text{ }\mu\text{g}$) of polymorphonuclear-leucocyte enzyme (sp. activity 1.8 units/mg of protein) obtained as by-products during soluble 5'-nucleotidase isolation was added to the assay medium, which in the final volume (0.1 ml) contained 0.1 M -potassium succinate buffer, pH 6.5, 0.1 M -KCl, 10 mM -AMP and 1 mM -ATP. Reaction was terminated after 20 min incubation at 37°C for the heart enzyme or after 30 min for the polymorphonuclear-leucocyte enzyme, and ammonia liberated from AMP was measured as described by Chaney & Marbach (1962).

One unit of enzyme activity for either 5'-nucleotidase or AMP deaminase was defined as the amount needed to convert $1\text{ }\mu\text{mol}$ of substrate/min at 37°C .

Statistical methods

The concentration of compounds giving 50% inhibition of 5'-nucleotidase were computed from second- or third-order polynomial fit to the experimental points.

Values are expressed throughout as means $\pm$ S.E.M. Values were compared by using Student's *t* test with unpaired data unless otherwise stated.

Chemicals

IBTA was obtained from Sigma (Poole, Dorset, U.K.). IBTI was prepared by selective modification of the 5'-position of inosine (Cusack, 1985). The procedure involved synthesis of 5'-deoxy-5'-toluene-*p*-sulphonyl-inosine (Hampton *et al.*, 1968) and 5'-deoxy-5'-chloro-inosine (Hanessian *et al.*, 1972) as the intermediate precursors. IBTI was obtained by the reaction of 5'-deoxy-5'-chloro-inosine with isobutylmercaptan (Vuilborgne *et al.*, 1978). The product was purified by preparative h.p.l.c. on a 30 cm -long C_{18} - μ Bondapak column (Waters, Harrow, Middx., U.K.) eluted at 2 ml/min with 20% (v/v) acetonitrile in 20 mM -ammonium acetate (pH 4.2). Peaks corresponding to IBTI were pooled from multiple runs and freeze-dried. Other nucleosides were obtained from either Sigma or P-L Biochemicals (Northampton, U.K.).

RESULTS

Inhibitors of IMP-specific cytosolic 5'-nucleotidase

The hydrolysis of 3 mM -IMP by the purified rat liver

IMP-specific 5'-nucleotidase was assessed by measurement of both products, P_i and inosine. Production of P_i , but not that of inosine, was apparently inhibited by nucleosides with a free 5'-hydroxy group. The percentage inhibitions (means of four observations) obtained by compounds at 5 mM final concentration were: inosine, 49; 1-methylinosine, 7; 7-methylinosine, 4; 8-brominosine, 15; 2'-deoxyinosine, 22; guanosine, 8; 6-mercaptopurine riboside, 53; 6-methylthiopurine riboside, 14; 6-methoxypurine riboside, 4; 6-chloropurine riboside, 25; adenosine (10 mM), 7; 2-chloroadenosine (10 mM), 20. Production of both P_i and inosine was inhibited by 5'-substituted nucleosides. The percentage inhibitions obtained with compounds at 5 mM final concentration were: 5'-deoxy-5'-amininosine, 0; 5'-deoxy-5'-chlorinosine, 11; 5'-deoxy-5'-brominosine,

17; 5'-deoxy-5'-isobutylthioinosine (IBTI), 40; 5'-deoxy-5'-aminoadenosine (10 mM), 2; 5'-deoxy-5'-methylthioadenosine (10 mM), 11 and 5'-deoxy-5'-isobutylthioadenosine (IBTA), 79. The most promising compounds for further study were IBTA and IBTI.

From a series of experiments with IMP as substrate, the kinetics of the inhibition of the rat liver enzyme by 0.5–3.0 mM-IBTA or 1–7 mM-IBTI were non-competitive (Figs. 1a and 1b); similar results were also obtained for the effect of 0.5–3.0 mM-IBTA on the enzyme from rat polymorphonuclear leucocytes (Fig. 2). IBTA (3 mM) and IBTI (7 mM) inhibited the polymorphonuclear-leucocyte enzyme to a similar degree when 0.1 mM- or 10 mM-IMP was used as substrate (Table 1), confirming non-competitive inhibition. Similar results were also obtained with 0.1 mM- or 10 mM-AMP as substrate (Table 1).

IBTA was found to be approximately twice as potent as IBTI as an inhibitor of the IMP-specific cytosolic 5'-nucleotidases from rat polymorphonuclear leucocytes (Table 1), rat liver (Table 2), and also rat heart (Table 2). The concentration of IBTA giving 50% inhibition of the rat liver enzyme was 2–4 mM (Table 2). The inhibitory potency of IBTA against IMP hydrolysis was slightly less when ATP was present (Table 2). With AMP as substrate

Fig. 1. Inhibition of rat liver IMP-specific cytosolic 5'-nucleotidase by IBTA (a) and IBTI (b)

Dixon (1953) plots relating the velocities of reaction, measured as the amount of [3 H]inosine liberated from [3 H]IMP during 30 min incubation at 0.05 mM- ($\square$), 0.1 mM- ($\blacksquare$), 0.3 mM- ($\circ$) and 0.5 mM- ($\bullet$) IMP in the presence of 5 mM-ATP. Values represent the means of triplicate observations ($\pm$ S.E.M.) and are representative of three experiments.

Fig. 2. Inhibition of rat polymorphonuclear-leucocyte IMP-specific 5'-nucleotidase by IBTA

Dixon (1953) plot relating the velocities of reaction, measured as the amount of [3 H]inosine liberated from [3 H]IMP during 30 min incubation at 0.1 mM- ($\blacksquare$), 0.3 mM- ($\circ$), 0.5 mM- ($\bullet$) and 1.0 mM- ($\triangle$) IMP in the presence of 5 mM-ATP. Values represent the means of triplicate observations ($\pm$ S.E.M.).

Table 1. Inhibition of the soluble cytoplasmic IMP-specific 5'-nucleotidase from polymorphonuclear leucocytes

The enzyme was assayed with 5 mM-ATP present. Reactions were started by adding 3.9 munits of enzyme (5 µg of protein) and conducted for 30 min. Values are means ± S.E.M. of three observations. Inhibition by IBTA or IBTI was significant ($P < 0.01$) under all conditions.

Substrate	Activity without inhibitors (munits/ml)	Activity (% of control) with:	
		3 mM-IBTA	7 mM-IBTI
0.1 mM-IMP	15.1 ± 0.6	59 ± 2	56 ± 3
10.0 mM-IMP	194 ± 2	46 ± 5	46 ± 5
0.1 mM-AMP	4.2 ± 0.1	44 ± 9	63 ± 1
10.0 mM-AMP	130 ± 13	49 ± 1	41 ± 3

Table 2. Concentrations of IBTA and IBTI giving 50% inhibition of purified cytoplasmic 5'-nucleotidases

The enzymes were assayed with IMP (10 mM) or AMP (10 mM) as substrate, with or without 5 mM-ATP when indicated. The inhibitor concentrations varied from 0.5 to 3.0 mM for IBTA and from 1.0 to 7.0 mM for IBTI. Reactions were started by adding 11.2 munits of liver enzyme (1.8 µg of protein) and conducted for 10 min, or with 1.5 munits of heart enzyme (5.3 µg of protein) and conducted for 40 min. Values are means ± S.E.M. ($n = 4$; * $P < 0.05$ versus absence of ATP; † $P < 0.01$ versus IMP in the presence of ATP; ‡ $P < 0.05$ versus rat liver; § $P < 0.05$ versus corresponding value for IBTA).

Enzyme source	Substrate	Activator	IBTA concn. (mM)	IBTI concn. (mM)
Rat liver	IMP	None	2.7 ± 0.2	7.1 ± 1.0§
	IMP	ATP	3.5 ± 0.2*	8.7 ± 1.2§
	AMP	ATP	2.1 ± 0.2†	6.4 ± 1.1§
Rat heart	IMP	None	3.7 ± 0.3‡	8.1 ± 0.8§
	IMP	ATP	6.0 ± 0.7*‡	10.2 ± 1.5§
	AMP	ATP	4.2 ± 0.5‡	6.9 ± 0.9§

in the presence of ATP, IBTA was more potent than with IMP plus ATP (Table 2). The same trends were seen with IBTI and with the action of IBTA and IBTI on the IMP-specific cytosolic 5'-nucleotidase from rat heart, but differences with IBTI were not statistically significant (Table 2). IBTA was less potent against the heart enzyme than the liver enzyme, whereas IBTI appeared equipotent (Table 2). Comparison of the more limited data in Table 1 with the data in Table 2 indicates that the potency of IBTA and IBTI for the enzyme from polymorphonuclear leucocytes was similar to that for the liver enzyme.

Inhibition of other 5'-nucleotidases and AMP deaminase

The ecto-5'-nucleotidase of polymorphonuclear leucocytes was significantly inhibited by 3 mM-IBTA ($25 \pm 8\%$, $n = 6$, $P < 0.02$), but was not significantly inhibited by 5 mM-IBTI ($8 \pm 8\%$, $n = 6$). With 10 mM-AMP as substrate, the AMP-specific soluble 5'-nucleotidase from pigeon heart was significantly inhibited by 3 mM-IBTA by $19 \pm 3\%$ ($n = 4$, $P < 0.02$) and by 7 mM-IBTI by

$55 \pm 4\%$ ($n = 4$, $P < 0.01$). The degree of inhibition by IBTI was similar with 1, 2.5, 5 and 10 mM-AMP as substrate.

The AMP deaminase from rat heart was not inhibited by either 2.5 mM-IBTA (108 and 110% of control activity) or 7 mM-IBTI (109 and 90% of control activity). The enzyme from polymorphonuclear leucocytes was also not inhibited by 3 mM-IBTA ($100 \pm 1\%$ of control activity, $n = 3$) and only slightly inhibited by 10 mM-IBTI ($91 \pm 3\%$ of control activity, $n = 3$).

Adenosine formation by intact polymorphonuclear leucocytes

IBTA and IBTI did not significantly affect cell viability, as the ratio of lactate dehydrogenase activity released in supernatants of 2-deoxyglucose-poisoned cells to total activity in homogenates was $1.4 \pm 0.7\%$ ($n = 5$), $1.3 \pm 0.1\%$ ($n = 3$) and $2.1 \pm 0.3\%$ ($n = 3$) for control, 3 mM-IBTA- and 7 mM-IBTI-preincubated cells respectively.

The changes in concentrations of ATP, AMP, IMP and adenosine at 2 min intervals after the cells were poisoned with 30 mM-2-deoxyglucose are presented in Fig. 3. The concentrations of metabolites in control cells before and after incubation with 2-deoxyglucose are similar to those previously reported (Newby, 1980; Newby & Holmquist, 1981; Worku & Newby, 1983). The initial rapid fall in ATP concentration ($28.3 \pm 3.3\%/min$) was significantly slower ($13.7 \pm 4.6\%/min$) after preincubation with IBTA, but was unaltered ($28.6 \pm 1\%/min$) after preincubation with IBTI. In the presence of IBTI, the extent of the fall in ATP concentration appeared greater, but this was not statistically significant. IBTI, but not IBTA, caused an apparent increase in the concentration of AMP in cells before incubation with 2-deoxyglucose (Fig. 3b). The rate of increase in AMP concentration during the first 2 min of incubation (Fig. 3b), however, was the same in control cells (291 ± 53 pmol/min per 10^7 cells, $n = 5$) and in cells preincubated with IBTI (328 ± 87 pmol/min per 10^7 cells, $n = 3$); it was non-significantly decreased by IBTA (145 ± 83 pmol/min per 10^7 cells, $n = 3$). IBTA, but not IBTI, significantly decreased the increase in IMP concentration over the first 2 min (Fig. 3c). Both IBTA and IBTI delayed and inhibited the decrease in IMP concentration seen between 2 and 10 min of incubation (Fig. 3c). As a result, both IBTA and IBTI increased the total concentration of purine mononucleotides remaining at 10 min. Despite some variability in the absolute rate of adenosine formation seen in control cells, a strong inhibitory effect of both IBTA and IBTI was visible in each experiment (Fig. 3d). When changes in adenosine concentration were normalized for each batch of cells (Fig. 4), it was apparent that IBTA totally inhibited adenosine formation in the first 2 min and decreased subsequent formation by approx. 80%. IBTI decreased adenosine formation by approx. 70%.

In agreement with our previous work (Newby & Holmquist, 1981; Worku & Newby, 1983), the concentrations of inosine and, in particular, hypoxanthine rose to account for ATP broken down (Figs. 3e and 3f). Although IBTA delayed the rise in inosine concentration (Fig. 3e), neither IBTA nor IBTI affected the maximum rate of inosine formation. Both IBTA and IBTI, however, significantly decreased the maximum rate of hypoxanthine formation (Fig. 3f) by $35 \pm 5\%$ ($P < 0.001$) and $51 \pm 6\%$ ($P < 0.001$) respectively.

Fig. 3. Metabolite concentrations in 2-deoxyglucose-treated rat polymorphonuclear leucocytes

Polymorphonuclear leucocytes (4.7×10^7 – 10.5×10^7 cells/ml) were preincubated without inhibitor ($\circ$), with 3 mM-IBTA ($\bullet$) or with 7 mM-IBTI ($\square$). 2-Deoxyglucose was then added, and incubations were continued for the times shown. The concentrations of ATP (a), AMP (b), IMP (c) adenosine (d), inosine (e) and hypoxanthine (f) were then measured as described in the Materials and methods section. The values are means ($\pm$ S.E.M.) for five experiments without inhibitors, three with IBTA and three with IBTI. ** $P < 0.01$, * $P < 0.05$ (paired t test) versus no inhibitors.

Fig. 4. Inhibition of adenosine formation by IBTA and IBTI

The data in Fig. 3(d) were normalized by relating the change in adenosine concentration without inhibitor ($\circ$), with IBTA ($\bullet$) or with IBTI ($\square$) to the change seen between 0 and 10 min of incubation in cells without inhibitor. The values are means $\pm$ S.E.M.; * $P < 0.01$ versus no inhibitor.

DISCUSSION

The IMP-specific cytosolic 5'-nucleotidase of rat liver, heart and polymorphonuclear leucocytes were all shown to catalyse exchange of the nucleoside moiety between IMP and inosine (Worku & Newby, 1982). Other nucleosides with a free hydroxy group should also participate in this exchange reaction, as demonstrated with the antiviral agent acyclovir by Keller *et al.* (1985) and with the cytotoxic nucleoside tiazafurin by Fridland *et al.* (1986). Nucleoside exchange can therefore also account for the apparent inhibition of production of P_i but not of inosine, observed here with a series of nucleoside analogues. The IMP-specific cytosolic 5'-nucleotidases appear to have an ordered Uni-Bi mechanism in which inosine must dissociate from the enzyme before hydrolysis of the putative enzyme-phosphate intermediate (Worku & Newby, 1982). Thus nucleoside analogues with a modified 5'-position should inhibit the enzyme non-competitively by substituting for inosine at the active site of the phosphoenzyme. In the present study, several 5'-substituted nucleosides were shown to be inhibitors of the rat liver enzyme, and both IBTA and IBTI were shown to be non-competitive inhibitors. The inhibitory potency of nucleoside analogues appeared to depend on the hydrophobicity of the 5'-substituent and on the structure of the purine base. The greater potency of IBTA than IBTI, although IMP is a better substrate than AMP, is surprising. Interestingly, the AMP-specific 5'-nucleotidase of pigeon heart was conversely more potently inhibited by IBTI. These data might be explained by differences in binding specificity of the enzyme-phosphate intermediates of these enzymes compared with their ground states. The different inhibitory potencies of IBTA and IBTI with IMP and AMP as substrates for the IMP-specific 5'-

nucleotidase suggest the existence of different conformational states of the protein for the two substrates. Consistent with this, AMP appears to protect the enzyme from heat-inactivation, whereas IMP does not (Itoh, 1982). Different potencies against heart and liver enzymes may also suggest the existence of different isoforms, as suggested by immunological data of Itoh & Oka (1985).

Inhibition by IBTA and IBTI of ecto-5'-nucleotidase and by IBTI of the AMP-specific cytosolic 5'-nucleotidase will limit, but not preclude, their usefulness. Failure to inhibit adenosine formation with specific inhibitors of the ecto-5'-nucleotidase, as previously shown for polymorphonuclear leucocytes (Newby & Holmquist, 1981), taken together with inhibition by IBTA still makes a conclusive case for the involvement of cytosolic 5'-nucleotidases. To interpret the effect of IBTI, it will be necessary to define the type of 5'-nucleotidase present in each cell type. In polymorphonuclear leucocytes fractionated as described by Truong *et al.* (1988), we found no evidence for AMP-specific cytosolic 5'-nucleotidase (results not shown); thus the only known target for IBTA and IBTI was the IMP-specific enzyme. Inhibition of both cytosolic 5'-nucleotidases might, on the other hand, be an advantage when using IBTI to prevent the hydrolysis of nucleotide monophosphates in cells during ATP breakdown.

The ability of IBTA and IBTI to penetrate intact cells and hence inhibit their target enzymes *in situ* was tested with polymorphonuclear leucocytes, since there is strong evidence in this cell type that both adenosine and hypoxanthine formation during ATP breakdown involves the cytosolic IMP-specific 5'-nucleotidase (Worku & Newby, 1983). Both IBTA and IBTI inhibited the formation of adenosine and hypoxanthine by 2-deoxyglucose-poisoned polymorphonuclear leucocytes. IBTA, but not IBTI, also slowed ATP degradation and tended, presumably as a consequence, to decrease the rate of rise in AMP and hence IMP concentration. The inhibitory action of IBTA may not therefore be purely at the level of 5'-nucleotidase. The basis for the apparent increase in AMP concentration in cells preincubated with IBTI is not clear. IBTI did not delay the rise in AMP or IMP concentration, and had no effect on AMP deaminase activity measured in cell-free preparations. Its inhibitory effect on adenosine and hypoxanthine formation is consistent with action solely at the level of cytosolic 5'-nucleotidase. Both IBTA and IBTI increased the nucleoside monophosphate concentration remaining after treatment for 10 min with 2-deoxyglucose, suggesting that they might be useful to bolster nucleoside monophosphate concentrations in other cells undergoing ATP breakdown.

This work establishes the feasibility of designing nucleoside inhibitors of cytosolic 5'-nucleotidases and demonstrates that they are effective in intact cells. Effort should now be directed to improving the potency and specificity of the inhibitors.

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