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SHORT COMMUNICATIONS

The interaction of inhibitors with adenosine metabolising enzymes in intact isolated cells

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Inhibitors of adenosine deaminase are of potential clinical value, owing to their ability to increase the potency of cytotoxic nucleoside analogues which are inactivated by deamination [1–5]. Inhibitors of adenosine kinase have recently been used [6] to test the requirement of phosphorylation in the cytotoxic action of other nucleoside analogues. In addition, it has been suggested [7, 8] that these inhibitors may be useful in the study of the pathways of adenosine metabolism *per se* and hence shed light on the factors controlling the concentration of this important regulatory metabolite [9]. These uses of the inhibitors all demand their action not only on purified enzymes but on the enzymes *in situ* in the cell [10]. This study was undertaken to determine specificity and relative potency of four widely used inhibitors of adenosine deaminase or of adenosine kinase on the enzymes both in disrupted and intact rat polymorphonuclear leucocytes. This cell type is suitable for such a study because of its availability in high purity and large quantities and its low mechanical fragility. The latter property avoids complications due to extracellular pools of enzyme arising by cell breakage.

Bovine serum albumin (fraction V) with adenosine deaminase contamination of <0.05 mU/mg protein (batch No. AC 2864) was a gift of Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. 2′ deoxycoformycin was generously given by Dr. H. W. Dion, Parke-Davis, Detroit, MI, EHNA by Burroughs Wellcome, Research Triangle NC, and 5-iodotubercidin by Dr. Leroy B. Townsend, University of Michigan, College of Pharmacy, Ann Arbor, MI, 5′-amino-5′deoxyadenosine was synthesised according to Stout *et al.*, [11] and purified by reverse phase chromatography in aqueous $(NH_4)_2HPO_4$ pH 6.0 as described previously [8]. [2-^{3}H] adenosine (20–25 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. [2-^{3}H] inosine (0.8 Ci/mmol) was prepared from [2-^{3}H] adenosine. 80 μCi was added to 2.5 ml of 40 μM adenosine in 10 mM NH_4HCO_3 ph 7.9. The mixture was incubated with 0.1 U of purified calf intestinal adenosine deaminase (Type 1, Sigma, Poole, U.K.) at room temperature until the A_{265} reached a minimum (approx. 5 min). The enzyme was removed by passing the mixture over a 2 ml bed of DEAE-cellulose (DE–52, Whatman Ltd., Springfield Mill, Maidstone, U.K.) equilibrated with 10 mM NH_4HCO_3. After eluting the column with 10 ml of the same buffer all the eluate fractions were pooled and lyophylised. The residue was resuspended in 1 ml of 140 mM NaCl, 15 mM Na_2HPO_4 pH 7.0 and stored in liquid N_2. Radiochemical purity of 96 per cent inosine was determined for the product by t.l.c. on silica gel as described below.

Rat polymorphonuclear leucocytes were harvested and purified as described previously [8]. Rat hepatocytes were isolated by collagenase perfusion of the liver *in situ* by the method of Berry and Friend [12] as modified by Westwood *et al.*, [13]. Rat spleen lymphocytes were purified according to Stanley *et al.*, [14]. In each case, cells were homogenised by sonication for 5 sec at 0° at 20 μm tip amplitude in a model PG 100 sonicator (MSE, Crawley, U.K.).

All cells were resuspended in Krebs–Ringer bicarbonate buffer (1.3 mM Ca^{2+}, pH 7.4, [15]) containing 0.2 per cent (w/v) bovine serum albumin. Polymorphonuclear leucocytes, spleen lymphocytes and hepatocytes were used at concentrations of 1–2 × 10^7, 1–2 × 10^7 and 0.2–0.6 × 10^7 cells/ml respectively as judged by counting in a haemocytometer. Polymorphonuclear leucocytes, were washed by centrifugation at 15,000 × *g* for 5 sec in an Eppendorf model 5412 centrifuge (Anderman, E. Molesey, U.K.). Hepatocytes were centrifuged at 4000 × *g* for 30 sec in an MSE super-minor centrifuge (MSE, Crawley, U.K.). Inhibitors were added from aqueous solutions at 50 or 100 times the required final concentration.

Incorporation of [2-^{3}H] adenosine or [2-^{3}H] inosine into nucleotides and their metabolism was determined by mixing 25 μl of cell suspension with 25 μl of substrate (20 μM, 0.8 Ci/mmol) diluted in Krebs–Ringer bicarbonate buffer. Reactions were linear for up to 10 min at 37° and with up to 2.5 × 10^5 cells per incubation. Reactions were stopped with 50 μl of a solution containing 1 per cent sodium dodecyl sulphate, 3 mM AMP, 3 mM adenosine or 1 per cent sodium dodecyl sulphate, 3 mM IMP, 3 mM inosine as appropriate. The reaction mixture was analysed in one of two ways.

(1) 10 μl were dried on a disc, 23 mm dia., of DEAE-paper [16] (DE81, Whatman Ltd., Springfield Mill, Maidstone, U.K.). The discs were then incubated for 5 min at room temperature in 5 ml of 1 mM Na HCO_3 before being washed with 2 × 5 ml of the same solution. This procedure removed nucleosides and hypoxanthine as judged by u.v. spectroscopy of the eluates. Nucleotides were eluted in 1 ml of 50 mM HCl in a scintillation vial. 8 ml of scintillant (Liquid Scintillation cocktail T, Hopkin & Williams, Chadwell Heath, Essex, U.K.) was added and radioactivity determined.

(2) Incorporation of nucleoside into nucleotides and nucleoside metabolism were simultaneously assessed by chromatography of the reaction mixtures on Silica gel [17]. The Rf was for AMP or IMP 0–0.06; for inosine, 0.30–0.34; for hypoxanthine, 0.43–0.47 and for adenosine 0.52–0.58. Spots located under ultraviolet light were cut out and eluted with 2 ml of 50 μM HCl, 16 ml of scintillant added and radioactivity determined.

Adenosine deaminase was determined spectrophotometrically as described previously [18, 8]. Adenosine kinase was determined by ECTEOLA-cellulose t.l.c. as described [8] or by separating the reaction mixture on DE-81 filter discs as described above. Nucleoside phosphorylase was determined as previously [8].

The inhibition of purified adenosine deaminase by dCF and EHNA [19] and of adenosine kinase by ITU [20] and 5′-amino-5′-deoxyadenosine [21] have all been previously studied. Therefore only sufficient experiments were per-

Abbreviations used: dCF, 2′deoxycofomycin, R-3-(2-deoxy - β - D - erythropentofuranosyl) - 3, 6, 7, 8 - tetrahydro[4,5-d] [1,3] diazapin-8-ol; EHNA, erythro-9-(2-hydroxy-3nonyl)adenine; ITU, 5-iodotubercidin, 4-amino-5-iodo-7-(β-D-ribofuranosyl) pyrrolo [2,3-d] pyrimidine.

formed to extend these studies to rat polymorphonuclear leucocytes and to determine the potency of inhibition under the standard assay conditions used. The specificity of inhibitors was tested up to the maximal effective concentration on the enzyme *in situ* (see below).

When preincubated with polymorphonuclear leukocyte homogenates for 15 min at 21°, dCF inhibited adenosine deaminase by 50 per cent at $1–3 \times 10^{-9}$ M (range for 5 experiments). EHNA caused a similar inhibition at $3–5 \times 10^{-8}$ M (3 experiments). ITU at 10^{-5} caused no significant inhibition of adenosine deaminase, neither did 5′-amino-5′deoxyadenose at 5×10^{-5} M. Sonicate adenosine kinase was inhibited 50 per cent by $4–6 \times 10^{-8}$ M 5′-amino-5′deoxyadenosine (4 experiments). ITU inhibited to a similar degree at $3–6 \times 10^{-7}$ M (3 experiments). Neither EHNA nor dCF significantly inhibited adenosine kinase at 10^{-4} M consistent with the previous report [8].

Exogenous adenosine added to intact cells can be metabolised by a number of routes [22]. It may be directly incorporated into nucleotides by adenosine kinase or indirectly by prior conversion to inosine and hypoxanthine. Hypoxanthine is converted to IMP by hypoxanthine phosphoribosyl transferase. In addition, hypoxanthine may be converted to uric acid by xanthine oxidase. If [2-^{3}H] adenosine is used as substrate, formation of uric acid results in release of the label as ^3H_2O and hence nucleotides are the only negatively charged radioactive products of adenosine metabolism. These were detected by ion-exchange or silica gel chromatography as described. The rate of nucleotide formation was the sum of the rates of the direct and indirect pathways. On the other hand adenosine metabolism, measured as the rate of disappearance of labelled substrate was the sum of the activities of adenosine kinase and adenosine deaminase. Either adenosine metabolism or its incorporation could have been limited by transport depending on the relative rates of the three processes. Since the object of this study was not to investigate the fate of adenosine *per se* but merely to devise an experimental model against which to judge the effects of inhibitors, only a single adenosine concentration of 10 μM was studied. This was a compromise between the need for high specific radioactivity for sensitivity and that for a high substrate concentration to maintain linear initial rates. By comparing the rates of adenosine incorporation and metabolism with the activities of adenosine kinase and adenosine deaminase determined at the same substrate concentration in sonicated cells, the contribution of nucleoside transport could be determined. The use of inosine as a cold trap and a comparison of the effect of inhibitors on the incorporation of [2-^{3}H] adenosine and [2-^{3}H] inosine allowed the contribution of the direct pathway and hence the activity of adenosine kinase *in situ* to be assessed.

Table 1 shows that the initial rate of incorporation of 10 μM adenosine into nucleotides in intact polymorphonuclear leucocytes was similar to the activity of adenosine kinase in an homogenate of the same cells. This suggested that incorporation into nucleotides was not limited by nucleoside transport. Adenosine metabolism (the sum of adenosine kinase and adenosine deaminase) was 7 times faster indicating that nucleoside metabolism was largely due to catabolism through adenosine deaminase at this substrate concentration. However, when cells were homogenised, 5 times as much adenosine deaminase activity (determined at 10 μM adenosine) was revealed (Table 1). Nucleoside metabolism was, therefore, limited by transport and indeed its rate gives a measure of the rate of nucleoside transport. That this rate was 7 times the rate of incorpoation of adenosine into nucleotides confirms that nucleoside transport was not limiting for this process. Similar conclusions were drawn for inosine metabolism and its incorporation into nucleotides (Table 1).

As shown in Table 2, 10^{-4} M unlabelled inosine reduced by 79 per cent the incorporation of 10 μM [2-^{3}H] inosine into nucleotides. In contrast unlabelled inosine reduced the incorporation of adenosine by only 33 per cent. It was therefore concluded that direct phosphorylation accounted for at least 60–70 per cent of the incorporation of adenosine into nucleotides.

Adenosine incorporation was blocked 66 per cent by 50 μM 5′-amino-5′-deoxyadenosine (Table 2) and 58 per cent by 10^{-6} M 5-iodotubercidin. The effect of these agents plus 10^{-4} M inosine was additive (data not shown). In contrast, neither 5′-amino-5′-deoxyadenosine nor ITU blocked the incorporation of [^{3}H] inosine into nucleotides. Hence it was concluded that both inhibitors blocked adenosine kinase *in situ*. The concentration of ITU needed was approximately the same as needed to completely block the homogenate enzyme. However the potency of 5′-amino-

Table 1. Comparison of the rates of adenosine and inosine metabolism in intact cells and sonicates

Process	Reaction velocity at 10 μM substrate pmol/10^7 cells per min
Incorporation of adenosine into nucleotides	182 ± 13 (12)
Sonicate adenosine kinase	155 ± 20 (5)
Adenosine metabolism by intact cells	1030 ± 90 (8)
Sonicate adenosine deaminase	6900 ± 600 (4)
Incorporation of inosine into nucleotides	100 ± 20 (3)
Inosine metabolism by intact cells	700 ± 50 (3)
Sonicate nucleoside phosphorylase	5600 ± 400 (2)

Polymorphonuclear leucocytes ($1–2 \times 10^7$ per ml) were incubated at 37° with 10 μM [2-^{3}H] adenosine or [2-^{3}H] inosine in Krebs–Ringer bicarbonate buffer and nucleoside metabolism determined as described. Portions of the same cell suspensions were washed 3 times by resuspension in 140 mM NaCl, 15 mM Na_2HPO_4, pH 7.0 and finally resuspended and recounted. Cells were then sonicated and enzyme activities determined with 10 μM adenosine or inosine as substrate as described. Values are mean ±S.E.M. of the number of observations in parenthesis. For less than 4 observations the mean error is given.

Table 2. Comparison of the effects of inhibitors on adenosine and inosine metabolism by polymorphonuclear leucocytes

Compound	Concentration (M)	Per cent control			
		Adenosine incorporation into nucleotides	Adenosine metabolism	Inosine incorporation into nucleotides	Inosine metabolism
Inosine	10^{-5}	82 ± 3 (4)	62 ± 12 (2)	47 ± 1 (3)	51 ± 9 (2)
	10^{-4}	67 ± 3 (5)	35 ± 4 (4)	21 ± 3 (3)	<10 (2)
5′-amino-5′-deoxyadenosine	5×10^{-8}	99 ± 8 (5)	100 ± 10 (2)	–	–
	5×10^{-7}	88 ± 10 (4)	100 ± 10 (2)	–	–
	5×10^{-6}	55 ± 3 (8)	101 ± 4 (4)	96 ± 8 (3)	103 ± 3 (3)
	5×10^{-5}	34 ± 3 (9)	92 ± 5 (5)	96 ± 3 (4)	80 ± 7 (3)
5′-iodotubercidin	10^{-8}	64 ± 8 (3)	100 ± 3 (3)	–	–
	10^{-7}	56 ± 8 (2)	96 ± 8 (2)	–	–
	10^{-6}	42 ± 3 (5)	92 ± 3 (5)	95 ± 3 (4)	97 ± 1 (3)
	10^{-5}	46 ± 4 (4)	82 ± 7 (3)	93 ± 2 (3)	40 ± 10 (3)

Polymorphonuclear leucocytes ($1–2 \times 10^7$/ml) were incubated in quadruplicate at 37° with 10 μM [2-^{3}H] adenosine or [2-^{3}H] inosine for 5 min before estimation of nucleoside metabolism or incorporation into nucleotides. Inhibitors were added either simultaneously with substrate or 30 min previously (except inosine). Since no significant differences were observed, data from either type of experiment have been pooled. Percentages were obtained by comparing the rates of the various processes in cells incubated either with or without inhibitors. Data are the means ±S.E.M. of values pooled from different experiments as indicated in parenthesis. Where less than four similar experiments were performed the average difference from the mean is given.

5'deoxyadenosine was approximately 50 times lower on adenosine kinase *in situ*. This discrepancy was not caused by metabolism of the drug since 101 ± 4 per cent, as judged by high pressure liquid chromatography [8], remained unmodified after a 90 min incubation with cells. Neither was it caused by slow permeation into the cells (legend to Table 2). Hence the altered potency most likely reflects differences between the conditions of assay of adenosine kinase in intact and homogenised cells. Intracellular pH may be particularly important, due to the observation that the unprotonated drug is the true inhibitor [21].

Table 2 also records that neither ITU nor 5'-amino-5'-deoxyadenosine had any significant effect on adenosine metabolism by intact cells. This indicated that they had no detectable effect on adenosine transport nor on adenosine deaminse *in situ*. The ability of ITU and to a lesser extent 5'-amino-5'-dexoyadenosine to inhibit inosine metabolism may suggest that the inhibitors permeate the cell by the inosine transport protein.

Both dCF and EHNA blocked the metabolism of adenosine by intact polymorphonuclear leucocytes (Table 3). However, the inhibitory effect of dCF was half maximal on intact cells at approximately 300 times the concentration needed to inhibit homogenate adenosine deaminase. In the case of EHNA the difference in potency was small. In addition, when concentrations of dCF or EHNA were raised to completely block adenosine deaminase *in situ*, the incorporation of adenosine into nucleotides was also profoundly inhibited. Since dCF has been shown to inhibit adenosine transport [10, 23, 24] the simplest explanation of this phenomenon was that the blockers also inhibited nucleoside transport to such an extent that it became limiting for adenosine incorporation. This conclusion was strengthened in the case of dCF by the experiment described in Table 4. Cells preincubated with dCF which showed a decreased rate of incorporation of adenosine into nucleotides showed an unaltered rate of adenosine kinase when homogenised. This experiment also showed that the effect of dCF on adenosine incorporation was only slowly reversed by washing. This was confirmed in eight separate experiments. In contrast, neither 30 μM dCF nor 10 μM EHNA had a pronounced effect on inosine incorporation or metabolism. This is consistent with previous data with dCF [8].

The possibility that dCF and EHNA interfere with nucleoside transport obviously invalidates the use of adenosine degradation by intact cells to measure their effect on adenosine deaminase activity. Therefore an alternative approach was sought to quantify the effect of inhibitors. The inhibition by dCF was only very slowly reversible [19]. Sonicates pretreated with 10^{-8} M dCF showed that no detectable recovery of adenosine deaminase activity when dialysed against 2 × 250 vol. of 50 mM phosphate buffer pH 7.0 for 4 hr at 21°. Intact cells were therefore preincubated with dCF for 30 min at 37°, washed until the washings failed to inhibit a fresh batch of homogenate adenosine deaminase and then sonicated. The residual adenosine deaminase activity was determined spectrophotometrically.

The concentration of dCF necessary to inhibit *in situ* adenosine deaminase by this experimental approach was 2–3 μM (range of 5 experiments). Complete inhibition was obtained by 10 μM dCF demonstrating the sigmoid nature of the binding isotherm [19]. That the low potency of dCF was caused by slow penetration, was confirmed when intact cells were preincubated with dCF for different times before washing. The time taken for 50 per cent inhibition of the adenosine deaminase revealed by sonication was for 30 μM dCF, 4 min; for 10 μM dCF, 10 min; and for 3 μM dCF 30 min. Thus the rate of inhibition was roughly proportional to the concentration. This rate could be reduced by 10 μM dipyridamole (data not shown) a known inhibitor of adenosine transport.

Studies were also performed on rat spleen lymphocytes and rat hepatocytes isolated by digestion of the liver with collagenase. The homogenate adenosine deaminase of all three cell types was inhibited 50 per cent by preincubation for 15 min at 21° with 0.3–3 × 10^{-9} M dCF (range of 9 experiments). However, when intact cells were preincubated for 30 min at 37° the enzyme *in situ* was 50 per cent inhibited by 2–3 μM for polymorphonuclear leucocytes, 0.3–1 μM for spleen lymphocytes and 0.03 μM for hepatocytes. In no case was the lowered potency due to drug metabolism since supernatants from cells incubated with dCF for 30 min showed, when appropriately diluted, the same capacity to inhibit homogenate adenosine deaminase as unincubated dCF.

Both 5'-iodotubercidin and 5'-amino-5'deoxy adenosine have been shown to be useful inhibitors of adenosine kinase *in situ*. They had no effect on adenosine transport or deamination. dCF has been shown to inhibit adenosine deaminase *in situ* only at concentrations much higher than those which inhibit the homogenate enzyme. Even at these concentrations it did not inhibit adenosine kinase. How-

Table 3. Effect of EHNA and dCF on adenosine metabolism

Compound	Concentration (M)	Per cent control activity: Adenosine degradation	Per cent control activity: Adenosine incorporation into nucleotides
EHNA	2×10^{-8}	85 ± 5	90 ± 6
	2×10^{-7}	55 ± 5	67 ± 2
	2×10^{-6}	26 ± 6	41 ± 5
	2×10^{-5}	11 ± 1	21 ± 1
dCF	10^{7}	93 ± 4	89 ± 6
	10^{6}	54 ± 5	58 ± 3
	10^{5}	0 ± 1	14 ± 1
	10^{4}	6 ± 2	14 ± 1

Polymorphonuclear leucocytes (1.58 ± 0.05 × 10^{7}/ml) were incubated in quadruplicate at 37° for 4 min with 10 μM [2-^{3}H] adenosine. Adenosine degradation and incorporation into nucleotides was then determined as described. Blockers were added 30 min previously as indicated and the values shown calculated by comparing the rates of the two processes in cells with or without inhibitors. Values are the mean ± S.E.M. of the four observations. The results are those of one representative of 5 similar experiments with EHNA and 8 with dCF.

Table 4. The effect of dCF on the incorporation of adenosine into nucleotides and on sonicate adenosine from the same cells

Conditions	Adenosine incorporation into cellular nucleotides (pmol/10^7 cells per min)	Sonicate adenosine kinase (pmol 10^7/cells per min)
Control	350 ± 10	170 ± 8
+ 30 μM dCF	25 ± 1	165 ± 10
+ 30 μM dCF and washed	42 ± 2	150 ± 2
Control sonicate + 30 μM dCF	-	143 ± 9

Polymorphonuclear leucocytes ($1.54 \pm 0.06 \times 10^7$/ml) were incubated for 30 min at 37° with or without 30 μM dCF in 140 mM NaCl, 15 mM Na_2HPO_4 pH 7.0, 0.2 per cent bovine serum albumin. A portion of the inhibited cells was subsequently washed 3 times with the same buffer and resuspended to the original volume. Portions of inhibited, control and inhibited washed cells were incubated for 5 min at 37° with 10 μM [2-^{3}H] adenosine and the incorporation of adenosine into nucleotides determined. A second portion of each batch of cells was sonicated and sonicate adenosine kinase determined. 30 μM dCF was added to a portion of control sonicate before assaying for adenosine kinase. The incorporation of [2-^{3}H] adenosine into a sample of the same cells resuspended in Krebs–Ringer bicarbonate buffer was 280 ± 10 pmol/10^7 cells per min. Values are means ± S.E.M. of quadruplicate observations. The data are from one representative of six similar experiments.

ever, it did block adenosine incorporation into nucleotides, probably via an effect on nucleoside transport [10, 23, 24]. This effect could not readily be reversed by washing (Table 4). The reduced potency of dCF on intact cells was due to slow penetration into the cells. The potency of the drug would be increased by longer exposures, a factor which may be important in its pharmacodynamics. Differences in the potency of inactivation in different cell types may determine preferential sites of action [7, 23]. EHNA also inhibited incorporation into nucleotides as well as adenosine degradation. It may, therefore, also inhibit nucleoside transport, cf. [10]. Quantitation of the effect of EHNA on adenosine deaminase *in situ* may eventually be possible using endogenously generated adenosine.

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