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STUDIES ON SEVERAL PYRROLO[2,3-*d*]PYRIMIDINE ANALOGUES OF ADENOSINE WHICH LACK SIGNIFICANT AGONIST ACTIVITY AT A1 AND A2 RECEPTORS BUT HAVE POTENT PHARMACOLOGICAL ACTIVITY *IN VIVO*

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Abstract—5′-Deoxy-5-iodotubercidin was previously reported [11] to cause potent muscle relaxation and hypothermia when injected i.p. into mice. In normotensive rats, i.v. injection reduced blood pressure and heart rate. 5-Iodotubercidin possessed the same *in vivo* activities whereas tubercidin was pharmacologically almost inactive. None of these compounds interacted significantly with Al adenosine receptors, as determined by their ability to displace ^{3}H-N^6-phenylisopropyladenosine or ^{3}H-5′-*N*-ethylcarboxamidoadenosine bound to rat brain membranes. Furthermore these compounds were much weaker than adenosine as agonists of adenosine-stimulated adenylate cyclase in guinea-pig brain slices (A2 receptors). A previous report showed that 5′-deoxy-5-iodotubercidin and 5-iodotubercidin were very potent inhibitors of adenosine kinase from rat or guinea-pig brain and were potent inhibitors of ^{3}H-adenosine uptake‖ into brain slices; relative to the halogenated derivatives, tubercidin was quite weak as an inhibitor of adenosine kinase and of adenosine uptake. We therefore propose that a significant part of the *in vivo* activity of the two halogenated tubercidin analogues may not be due to a direct agonist action at A1 and/or A2 adenosine sites (as proposed for a number of other metabolically-stable analogues of adenosine) but may result from an inhibition of reuptake of endogenously-released adenosine; the increased extracellular levels of adenosine resulting from this action could then interact directly with membrane receptors. Consistent with this, low concentrations of 5′-deoxy-5-iodotubercidin were shown to significantly potentiate the effects of exogenous adenosine on blood pressure and heart rate in anaesthetized rats and on adenosine-stimulated cAMP generation in guinea-pig brain slices.

None of these compounds interacted with central benzodiazepine receptors.

The cardiovascular and behavioural effects of 5′-deoxy-5-iodotubercidin and 5-iodotubercidin were blocked by theophylline; results from the cardiovascular studies suggest there may be different adenosine receptors in heart and blood vessels.

Cardiovascular responses to peripherally administered adenosine were first described over 50 years ago [1] and since then numerous other studies have reported on both the cardiovascular and central actions of adenosine.

Recent studies have reported on the sedative, anticonvulsant, muscle-relaxant and hypothermic actions of adenosine or metabolically-stable analogues, following oral, parenteral or intracerebral administration (e.g. [2–9]).

There appear to be at least two types of adenosine recognition sites on the external surface of many different cells (A1, mediating inhibition, and A2, stimulation of adenylate cyclase) as well as an internal P site, located on the catalytic subunit of adenylate cyclase [10]. Current research is aimed at determining which of these receptor site subtypes are involved in the different actions of adenosine.

We have reported that 5′-deoxy-5-iodotubercidin, a novel analogue of adenosine isolated from a marine red alga *Hypnea valentiae*, potently inhibited adenosine kinase and adenosine uptake in brain *in vitro* and had potent muscle-relaxant/hypothermic action *in vivo* [11]. In order to ascertain the possible biochemical mechanism(s) for the pharmacological action of this compound, we have extended these *in vivo* and *in vitro* investigations using two closely related compounds, 5-iodotubercidin and tubercidin (Fig. 1).

MATERIALS AND METHODS

In vivo *testing in mice*. For each experiment, groups of five male Füllinsdorf mice (20–30 g) were acclimatized at 23° in white plastic boxes with sawdust bedding for approx 1 hr prior to intraperitoneal administration of saline or the compound under test. Volumes administered did not exceed 0.2 ml/10 g body wt. Rectal temperature was measured using a YSI thermistor probe (Model No. 423) and Telethermometer (Yellow Springs Instrument Co., OH). All measurements were compared with a vehicle-treated control group. Muscle relaxation was

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‖ *Uptake* refers to the cellular accumulation of a nucleoside permeant and its metabolic products whereas *transport* refers only to the transporter-mediated passage of nucleosides across the plasma membrane.

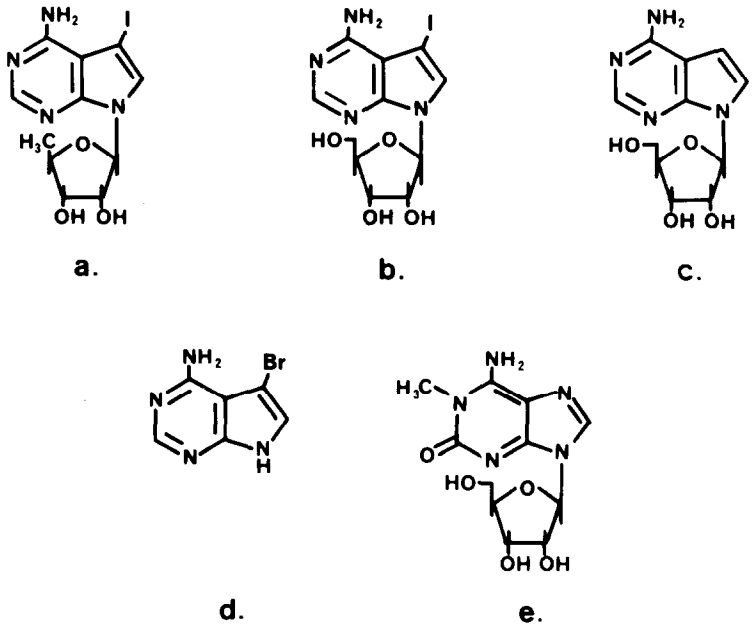

Fig. 1. Structures of (a) the natural product 5′-deoxy-5-iodotubercidin, isolated from the red alga *Hypnea valentiae*, (b) the related synthetic compound 5-iodotubercidin, (c) tubercidin, (d) the natural product 4-amino-5-bromopyrrolo[2,3-*d*]pyrimidine, isolated from a sponge of the genus *Echinodictyum* and (e) the natural product 1-methylisoguanosine, isolated from the sponge *Tedania digitata*.

assessed subjectively as previously detailed [2], using an arbitrary scale (four increments from 0 to 2) to assess animals which ranged from normal to those which exhibited pronounced general flaccidity and much-reduced spontaneous locomotor activity.

Cardiovascular testing in rats. Female Sprague–Dawley rats were anaesthetized with 100 mg/kg i.p. of Inactin (5-sec-butyl-5-ethyl-2-thiobarbitone sodium; Byk Gulden, West Germany). The trachea was intubated to facilitate breathing, the carotid artery catheterized for blood pressure measurement and the jugular vein for drug administration. Recording was done using a Neotrace Pen Recorder (Neomedix, Australia); heart rate was derived electronically from the blood pressure pulse. Animals were left for 30 min after surgery to stabilize before experiments commenced. Other details are given in the legends to Figs 4 and 5.

Receptor-binding assays. Washed synaptosomal membranes from whole rat brain (Sprague–Dawley) were prepared as previously described and frozen for 1–14 days [12, 13]. On the day of assay membranes were thawed, washed once by centrifugation and resuspension in ice-cold distilled water, then resuspended in 50 mM Tris–HCl buffer, pH 7.1. For purine binding assays, tissue was pretreated (10 min at 37°) with adenosine deaminase (0.4 μg/mg protein) to remove endogenous adenosine. ^{3}H-PIA and ^{3}H-NECA binding assays were performed as described earlier [13]; incubation periods were 20 min, in a final volume of 1 ml 50 mM Tris–HCl buffer pH 7.1, containing 1 mM $MgCl_2$. ^{3}H-Diazepam binding was performed essentially as previously described [14]; incubation periods were 30 min in a final volume of 2 ml 50 mM Tris–HCl buffer, pH 7.45 at 0–4°. At the end of the incubation period bound and free radioligand were separated by filtration on Whatman GF/B filters, followed by rapid washing of the tube and filter with 7.5 ml (PIA and NECA) or 12 ml (diazepam) of buffer.

Adenosine deaminase. The assay procedure was as previously described [15]; assays, in a final volume of 0.5 ml of 50 mM sodium phosphate buffer, pH 6.5, contained an excess of purified adenosine deaminase (from calf intestinal mucosa) and 500 μM of adenosine or the analogue being tested. After 50 min incubation at 37°, assays were stopped and released ammonia determined by a colorimetric procedure.

Adenosine kinase. Adenosine kinase activity was assayed in supernatants (20,000 *g* for 1 hr) or brain extracts prepared by homogenizing brain tissue in 50 mM sodium acetate buffer, pH 5.6, using a glass–glass homogenizer. Activity was assayed essentially according to the procedure of De Jong [16] as previously detailed by the present authors [17], using small discs of DEAE ion-exchange paper. 2′-Deoxycoformycin (10 nM) was added to inhibit adenosine deaminase.

cAMP Generation in guinea-pig brain slices. Guinea-pigs (Himalayan white) were killed by cervical dislocation and decapitation and 250 μm cerebral cortical "prisms" prepared using a McIlwain tissue chopper. Slices were washed extensively by decantation and preincubated for 60–70 min at 37° in Krebs bicarbonate buffer, with buffer changes every 10 min. Aliquots of the slices (approx. 2 mg protein) were then incubated with the appropriate compound for 15 min in a final volume of 1 ml. The procedures for extraction and assay of cAMP were essentially as previously reported [18].

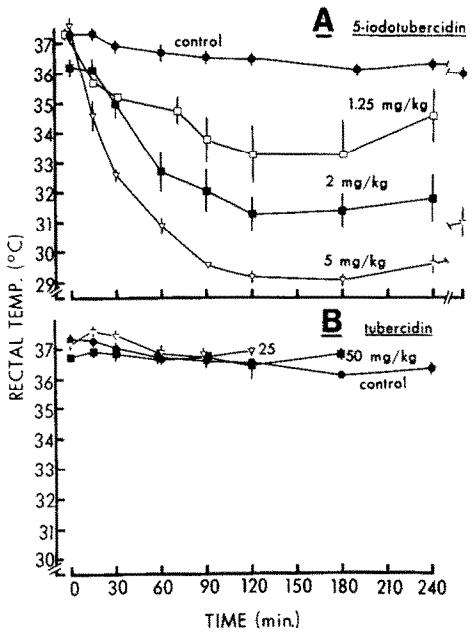

Fig. 2. Hypothermic effects *in vivo* of 5-iodotubercidin and tubercidin. The rectal temperatures of male Füllinsdorf mice (five per dosage group) were recorded at various times after administration of compounds by the intraperitoneal route. Saline control (●). (A) The effect of 5-iodotubercidin at 1.25 mg/kg (□), 2.0 mg/kg (■) and 5 mg/kg (▽). (B) The effect of tubercidin at 25 mg/kg (▽) and 50 mg/kg (■). Results for 5-iodotubercidin may be compared with similar results for 5′-deoxy-5-iodotubercidin [11] and 1-methylisoguanosine [2].

Materials. 5′-Deoxy-5-iodotubercidin was purified from a methanol extract of the red alga *Hypnea valentiae* collected at Quobba lagoon, Western Australia [19]. 5-Iodotubercidin was a kind gift from Professor Leroy B. Townsend, University of Michigan, while tubercidin was supplied by F. Hoffmann-La Roche & Co. (Basle). 2-^{3}H-Adenosine (10–20 Ci/mmole) and methyl-^{3}H-diazepam (60 Ci/mmole) were from New England Nuclear (Boston, MA) while 8(*n*)-^{3}H-5′-ethylcarboxamidoadenosine (20–40 Ci/mmole), 2,8-^{3}H-N^6-*R*-2-phenylisopropyladenosine (20–40 Ci/mmole) and cAMP assay kits were from Amersham International (Amersham, U.K.). Adenosine deaminase was supplied by Boehringer Mannheim (F.R.G.).

RESULTS

Pharmacological effects of 5′-deoxy-5-iodotubercidin and related compounds in mice

As previously reported, the marine natural product 5′-deoxy-5-iodotubercidin had a potent muscle-relaxant/hypothermic action when given i.p. to Füllinsdorf mice [11]. The related synthetic compound 5-iodotubercidin had the same pharmacological action (Fig. 2a and Table 1). Limited supplies of 5-iodotubercidin precluded an accurate estimation of the ED50 for the muscle-relaxant or hypothermic action, but it appeared to be approximately equipotent with 5′-deoxy-5-iodotubercidin which had an ED50 of 1.7 mg/kg for muscle-relaxation (1.0–2.8, 95% confidence limits). 5′-Deoxy-5-iodotubercidin might be somewhat shorter acting than 5-iodotubercidin (Fig. 2a, cf. Fig. 3 of [11]).

Table 1. Muscle-relaxant effects of intraperitoneal administration of tubercidin, 5-iodotubercidin and 1-methylisoguanosine in mice

Treatment	Muscle-relaxation score (out of ten) at various times (min)									
	−30	0	15	30	60	90	120	180	240	360
Saline control	0	0	0	0	0	0	0	0	0	0
Theophylline, 10 mg/kg	0	0	0	0	0	0	0	0	0	—
Tubercidin, 10 mg/kg	—	0	0	0	0	0	0	0	—	—
25 mg/kg	—	0	0	0	0	0	0	—	—	—
50 mg/kg	—	0	0	0	0	0	0	0	0	—
5-ITu, 0.5 mg/kg	—	0	0	0	0	0	0	—	—	—
1.25 mg/kg	—	0	0	1	1.5	2.5	2	2.5	2	—
5.0 mg/kg	—	0	9.5	10	10	10	9.5	6	6	3.5
5-ITu, 2.0 mg/kg	0	0	0.5	3.5	6	6	8	4.5	1	—
+ Theo. (−30′)	0	0	0	0	0	2	4.5	4	3.5	—
5 ITu, 2.0 mg/kg	0	0	0	4	6.5	8	9	8.5	7	0.5
+ Theo. (−30′ and 90′)	0	0	0	0	1	1.5	0	0	0	—
1-MeiG, 10 mg/kg	—	0	5	6	5.5	2.5	0	0	—	—

Male Füllinsdorf mice (five/group) were treated with the compounds as stated and muscle-relaxation scored as indicated in Materials and Methods. Values given are a muscle relaxation score out of 10 (with a maximum of two being scored for each animal). Data also indicate the effects of pretreatment with theophylline (10 mg/kg i.p.), given either at 30 min prior to dosing, or at 30 min prior to *and* 90 min after dosing with 5-iodotubercidin. Results for muscle-relaxation correlate very closely with the degree of hypothermia, as given in Figs 2 and 3 (see also [1]).

5-ITu = 5-iodotubercidin, 1-MeiG = methylisoguanosine, Theo. = theophylline. A dash indicates that a measurement was not recorded.

A comparison of areas under the muscle-relaxation vs time (to 4 hr) curves showed that theophylline did not significantly antagonize the effects of 5-ITu after the single dose ($P = 0.17$), but theophylline dosing at −30 and 90 min had a significant effect ($P < 0.0001$) (Student's *t*-test for unpaired experiments).

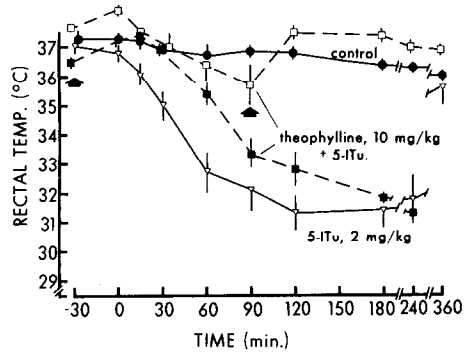

Fig. 3. The effect of pretreatment with theophylline on the *in vivo* hypothermic responses to 5-iodotubercidin. Saline control (●). Intraperitoneal 5-iodotubercidin at 2.0 mg/kg (▽). Pretreatment with intraperitoneal theophylline (10 mg/kg), 30 min prior to 2.0 mg/kg 5-iodotubercidin (■) or at 30 min prior to *and* 90 min after 2.0 mg/kg 5-iodotubercidin (□) (black arrows indicate times of theophylline treatment).

It was also noted that pretreatment of the mice with theophylline (10 mg/kg i.p.) 30 min prior to 5-iodotubercidin administration resulted in a partial inhibition of both the hypothermic and muscle-relaxant responses (Fig. 3 and Table 1). A second treatment with theophylline at 90 min maintained almost a complete inhibition of hypothermic and muscle-relaxant responses; these doses of theophylline alone had little effect on muscle tone or body temperature, although there was slightly increased spontaneous activity for 60–90 min after dosing.

In marked contrast to the effects of the halogenated compounds, tubercidin tested at doses as high as 50 mg/kg produced no muscle-relaxation or hypothermia whatsoever (Fig. 2b and Table 1).

Results obtained for the hypothermic and muscle-relaxant activity (Table 1) of the marine natural product purine, 1-methylisoguanosine, in the current series of experiments were the same as those previously published by the present authors [1].

Cardiovascular effects in rats

The effect of i.v. administration of tubercidin and the two halogenated analogues are given in Fig. 4. 5-Iodotubercidin and 5-deoxy-5-iodotubercidin at doses as low as 100 μg/kg caused approximately equipotent reductions in heart rate and blood pressure. Tubercidin at much higher doses had negligible effects on these parameters until doses of 10 mg/kg were reached and effects were transient (less than 1 min).

It is interesting to note that whereas i.v. theophylline almost completely reversed the effects of the iodo-compounds on heart rate, it only partially reversed their effects on blood pressure.

In another study, the effect of a low dose of 5′-deoxy-5-iodotubercidin (0.1 mg/kg i.v.) which had no statistically-significant effects alone on HR or BP was tested on responses to adenosine given at four different i.v. doses (Fig. 5). As illustrated, the dose–response (DR) curves for both BP and HR were shifted significantly to the left (2.1 for BP, 1.69 for HR). It appeared that the experiment underestimated the extent of potentiation because the dITu action wore off fairly quickly over the approx. 10 min it took to finish the DR curve.

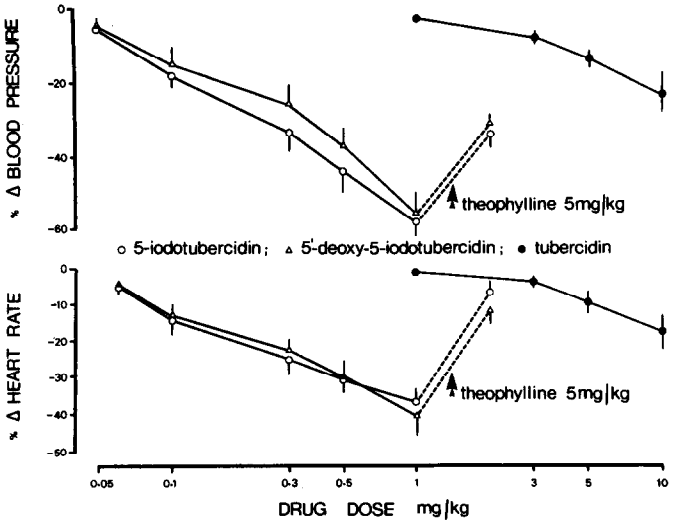

Fig. 4. The effect of i.v. tubercidin (●), 5-iodotubercidin (○) and 5′-deoxy-5-iodotubercidin (△) on heart rate and blood pressure in anaesthetized rats (see Materials and Methods). Data are means ± SEM for five rats in each drug group (each rat received only one compound) and are given as the percentage change in the parameter. Except for tubercidin, compounds were given cumulatively—as the effect of one dose reached its peak, the next was given. Tubercidin had only transient effects and the next dose was administered after the effects of the previous dose had worn off. Theophylline (5 mg/kg) was given after the peak effect of the last dose of 5-iodotubercidin and 5′-deoxy-5-iodotubercidin. Initial mean blood pressures and heart rates (±SEM) in the three groups were; tubercidin (123.4 ± 6.5 mm Hg, 320 ± 23 beats/min), 5-iodotubercidin (115.2 ± 4.7, 279 ± 20) and 5′-deoxy-5-iodotubercidin (124 ± 5.0, 300 ± 14).

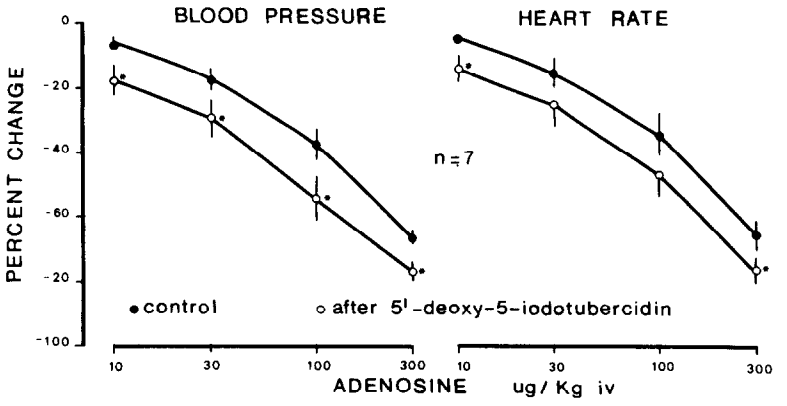

Fig. 5. The effect of i.v. 5′-deoxy-5-iodotubercidin on blood pressure and heart rate responses to i.v. adenosine in anaesthetized rats (see Materials and Methods). Data illustrate control responses (●) to adenosine and responses after 0.1 mg/kg 5′-deoxy-iodotubercidin (○) and are means ± SEM for seven rats. Each adenosine dose was given when the effects of the previous dose wore off. Initial mean blood pressure and heart rate was 119 ± 5 mm Hg and 310 ± 15 beats/min, and after 5′-deoxy-iodotubercidin, 108 ± 5 and 289 ± 17; these reductions of less than 10% were not statistically significant. Asterisks indicate significant differences ($P < 0.05$) from control.

Inhibition of adenosine kinase

5′-Deoxy-5-iodotubercidin and 5-iodotubercidin were very potent inhibitors of adenosine kinase from rat and guinea-pig brain [11]. However, tubercidin was inactive even when tested (rat brain extracts) at high doses (Table 2). Divekar and Hakala [20] reported that tubercidin was an inhibitor of adenosine kinase from sarcoma 180 cells; however it was quite weak in that its K_i for inhibition was approximately 30-fold larger than the K_m for adenosine as substrate.

Adenosine deaminase

In the presence of an excess of intestinal mucosa adenosine deaminase, adenosine was rapidly and completely degraded whereas the three tubercidin compounds were untouched (in agreement with the findings of others).

Interaction with ^{3}H-PIA and ^{3}H-NECA binding sites

The potency of 5′-deoxy-5-iodotubercidin in displacing ^{3}H-PIA and ^{3}H-NECA from rat brain membranes was compared with related structures (5-iodotubercidin and tubercidin) and with other known A1 and A2 receptor ligands including 1-methylisoguanosine, a purine with *in vivo* muscle-relaxant and hypothermic activity [14, 15] (Table 3). 4-Amino-5-bromopyrrolo[2,3-*d*]pyrimidine was also tested; this compound, closely related to 5′-deoxy-5-iodotubercidin but lacking the 5′-deoxy sugar (Fig. 1), had some CNS depressant activity in that it was observed to potentiate barbiturate-induced sleeping times and to have anti-amphetamine activity [11]. However its predominant *in vivo* activity was as a bronchodilator [11].

Compared with the purines, the four pyrrolo[2,3-*d*]pyrimidines tested were only very weak in displacing ^{3}H-PIA and ^{3}H-NECA.

While NECA is significantly more potent than R-PIA at A2 receptors (and vice versa for A1 receptors; [13]), it appears that under the conditions used for ligand receptor binding, ^{3}H-NECA may predominantly bind to A1 sites (e.g. [10]). Therefore the weak activity of compounds vs ^{3}H-NECA binding cannot be taken as an indication of lack of agonist activity at A2 sites. For this reason we tested the compounds in an adenosine-stimulated adenylate cyclase system.

Table 2. Inhibition of rat and guinea-pig brain adenosine kinase activity

Compound	Concentration (μM)	Inhibition (%) Rat	Guinea-pig
5′-Deoxy-5-iodotubercidin*	10	98 ± 1 (4)	91 ± 3 (2)
5-Iodotubercidin*	10	67 ± 4 (2)	83 ± 1 (2)
Tubercidin	100	5 ± 1 (4)	n.t.

Per-cent inhibitions are expressed as means ± SEM (or range) for the number of separate experiments in brackets; in each experiment determinations were performed in triplicate.

* Data published by the present authors [11].

Kinetic constants for the inhibition of rat and guinea-pig brain adenosine kinase by 5′-deoxy-5-iodotubercidin have been published [11].

n.t. = not tested.

Table 3. Effects of tubercidin analogues on the binding of ^{3}H-PIA and ^{3}H-NECA to rat brain membranes

Compound	K_i (nM) vs binding ^{3}H-PIA	^{3}H-NECA
5′-Deoxy-5-iodotubercidin	29,800 ± 2,300 (4)	22,400 ± 5,000 (3)
5-Iodotubercidin	28,500 ± 5,600 (3)	13,400 ± 5,500 (2)
Tubercidin	44,100 ± 10,100 (2)	67,100 ± 21,100 (3)
4-Amino-5-bromopyrrolo[2,3-*d*]pyrimidine	>100,000 (3)	>100,000 (2)

In exactly equivalent experiments (see Table 1 of [13]), K_i values of between 1–30 nM (vs both ligands), were measured for L-N^6-phenylisopropyladenosine, N^6-cyclohexyladenosine, 5′-*N*-ethylcarboxamidoadenosine and 2-chloroadenosine; for 1-methylisoguanosine values were around 200 nM.

K_i values were calculated using the Cheng–Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ where [L] = concentration of the radioligand used (5.6 nM for ^{3}H-PIA and 3.4 nM for ^{3}H-NECA) and K_d = equilibrium dissociation constant for the ligand. Four to five inhibitor concentrations were used in each experiment and IC_{50} values calculated using log–logit analysis; values are means ± SEM or range for the number of experiments in brackets. Kinetic parameters (mean ± SEM) of purine ligand binding were: ^{3}H-PIA; $K_d = 3.31 \pm 0.24$ nM, $B_{max} = 458 \pm 19$ fmole/mg protein ($N = 5$): ^{3}H-NECA; $K_d = 9.54 \pm 0.72$ nM, $B_{max} = 517 \pm 17$ fmole/mg protein ($N = 3$).

Table 4. Effects of adenosine and tubercidin analogues on cyclic AMP levels in guinea-pig brain slices

Sample	Concentration (μM)	cAMP level/unit protein (normalized)
Control	—	15.5 ± 1.6 (20)
Adenosine	100	100 ± 8.5 (20)
Tubercidin	2.5	12.4 ± 1.1 (4)
	100	14.3 ± 3.5 (4)
	250	10.5 ± 2.2 (8)
5-ITu	2.5	24.7 ± 2.3 (4)
	100	31.4 ± 2.0 (8)
	250	44.3 ± 2.9 (14)
dITu	2.5	28.1 ± 3.8 (4)
	100	34.2 ± 3.1 (8)
	250	49.4 ± 3.1 (14)
Adenosine	100	
+ tubercidin	250	43.7 ± 6.1 (4)

Data are means ± SEM for the number of determinations given in brackets. Cyclic AMP levels (measured as pmoles per mg of protein) were normalized (taking the level in the presence of 100 μM adenosine as 100) to reduce interexperiment variation. Absolute levels in the presence of 100 μM adenosine were 103.5 ± 15.4 pmole/mg protein (20 determinations).

5-ITu = 5-iodotubercidin; dITu = 5′-deoxy-5-iodotubercidin.

Table 5. Potentiation of the effects of adenosine on cyclic AMP levels in guinea-pig brain slices by 5′-deoxy-5-iodotubercidin

Sample	Concentration (μM)	cAMP level/unit protein (normalized)
Control	—	15.5 ± 0.8 (8)
Adenosine	20	26.4 ± 2.0 (8)
dITu	2.5	34.0 ± 2.2 (8)
Adenosine	20	
+ dITu	2.5	64.3 ± 4.6 (7)

Cyclic AMP levels (measured as pmoles per mg of protein) were normalized to reduce interexperiment variation; absolute control levels were 21.5 ± 4.1 pmole/mg protein (eight determinations) in these experiments.

dITu = 5′-deoxy-5-iodotubercidin.

Adenosine-stimulated cAMP generation in guinea-pig brain slices

Table 4 shows the effects of adenosine, tubercidin, 5-iodotubercidin and 5'-deoxy-5-iodotubercidin on cAMP levels in guinea-pig brain slices. At a concentration of 250 μM of the halogenated tubercidin analogues, the stimulated level of cAMP found in slices was less than half that found after 100 μM adenosine. Tubercidin did not produce any increase in cAMP levels.

It should be noted that in our assay system known adenosine uptake inhibitors produce a small increase (2–3-fold) in cAMP over basal levels (e.g. [11, 18]) by acting to inhibit reuptake of endogenously-released adenosine. Therefore it appears that 5'-deoxy-5-iodotubercidin and 5-iodotubercidin are quite weak as A2 site agonists, the cAMP increase being largely due to their ability to inhibit adenosine uptake.

This possibility was further tested by examining the combined effect of a low concentration of 5'-deoxy-5-iodotubercidin (2.5 μM) and of adenosine (20 μM) (Table 5). While both compounds had some effects alone in stimulating cAMP levels, the combined effect was more than additive. Such a result supports the suggestion that a significant part of the action of dITu could be due to an inhibition of adenosine uptake (endogenous and exogenous adenosine). In contrast to dITu, tubercidin (which is only a very weak adenosine uptake inhibitor; [11]) actually antagonized the cAMP-stimulatory effects of adenosine (Table 4); such an effect has previously been reported for human fibroblasts [21].

Interaction with ^{3}H-diazepam binding sites

None of the pyrrolo[2,3-*d*]pyrimidines was able to significantly displace ^{3}H-diazepam from CNS benzodiazepine receptors. 5'-Deoxy-5-iodotubercidin was tested at 10, 50, 125, 250 and 500 μM, with a maximum inhibition of 16% at the highest concentration. 5-Iodotubercidin was inactive at the highest dose tested (25 μM) while tubercidin had an IC_{50} of 172 μM (113–258; 95% confidence limits). 4-Amino-5-bromopyrrolo[2,3-*d*]pyrimidine had an IC_{50} of 553 μM (384–796).

DISCUSSION

The potent central and circulatory actions of metabolically-stable analogues of adenosine such as *R*-phenylisopropyladenosine, 5'-*N*-ethylcarboxamidoadenosine, 2-chloroadenosine, cyclohexyladenosine and 1-methylisoguanosine are thought to be due to direct interaction with membrane-bound adenosine receptors. These receptors have been classified into A1 (or Ri) and A2 (or Ra) sites, although there is some evidence that there may be further subtypes of these receptors (e.g. [22]). Current evidence suggests that receptors of the A1 subtype may mediate the electrophysiological depressant actions of adenosine in the CNS [23], although information currently available is insufficient to determine clearly whether either A1 or A2 sites (or both) mediate the *in vivo* sedative and behavioural effects of certain metabolically-stable adenosine analogues (e.g. [10, 21]).

It is clear from the results presented here that the direct interaction of 5'-deoxy-5-iodotubercidin and 5-iodotubercidin with A1 and A2 receptors is quite weak and it is therefore unlikely that such a mechanism could fully explain the pharmacological activity *in vivo* of these compounds.

We suggest that a possible explanation for a significant part of the *in vivo* activity of 5'-deoxy-5-iodotubercidin and 5-iodotubercidin is their ability to potently inhibit adenosine uptake (where "uptake" is the net result of membrane "transport" and subsequent rapid intracellular metabolism). We previously reported that both the halogenated pyrrolo[2,3-*d*]pyrimidines were very potent inhibitors of radioactive adenosine uptake in rat and guinea-pig brain slices whereas tubercidin was relatively weak [11]; IC_{50}s for 5'-deoxy-5-iodotubercidin, 5-iodotubercidin, tubercidin and unlabelled adenosine in guinea-pig cortex were 0.133, 0.071, 65.8 and 14.1 μM respectively).

Normal release of endogenous adenosine in excitable tissue may be related to general regulation of excitability [24] and any compound which can act to potently inhibit its reuptake from the extracellular space might be expected to have an effect similar to exogenous adenosine. Thus, rather than having a predominant direct agonist action at A1 and/or A2 adenosine sites, 5'-deoxy-5-iodotubercidin and 5-iodotubercidin may cause increased extracellular levels of endogenous adenosine which itself could then interact with extracellular receptors. The lack of *in vivo* pharmacological activity of tubercidin may be explained as being due to its inability to inhibit adenosine uptake to any significant extent at concentrations attained *in vivo*, despite its very close structural similarity to 5'-deoxy-5-iodotubercidin and 5-iodotubercidin. Our observation that the adenosine receptor antagonist theophylline can act to block the *in vivo* pharmacological effects of 5-iodotubercidin (and of 5'-deoxy-5-iodotubercidin) is consistent with the above proposal.

In further support of this suggestion, low concentrations of 5'-deoxy-5-iodotubercidin were shown to significantly potentiate the effects of exogenous adenosine on blood pressure and heart rate in anaesthetized rats and on adenosine-stimulated cAMP generation in guinea-pig brain slices.

This proposal fits in with the general hypothesis [25] that the CNS excitatory properties of the xanthines such as caffeine and theophylline result from an antagonism of the depressant effects of endogenously-released adenosine acting at extracellular receptors.

Also consistent with the above results is the observation that 4-amino-5-bromopyrrolopyrimidine has some CNS *sedative* activity *in vivo*; while this compound has bronchodilator activity like theophylline (and a not dissimilar structure), it differs biochemically from theophylline in that it lacks adenosine antagonist activity and is a reasonably potent inhibitor of adenosine uptake into CNS tissue [11].

The biochemical profile of 5'-deoxy-5-iodotubercidin and 5-iodotubercidin contrasts sharply with that of 1-methylisoguanosine, despite the fact that they all have similar *in vivo* activity. The latter compound is not an inhibitor of adenosine

kinase and does not inhibit adenosine uptake. However, it can quite potently interact with A1 adenosine receptors [13] and is a good agonist of adenosine-stimulated adenylate cyclase in CNS tissue (A2 receptors; [15]). Thus it may be supposed that 1-methylisoguanosine, unlike 5-iodotubercidin and 5'-deoxy-5-iodotubercidin, has its pharmacological activity through a direct interaction with adenosine receptors ([14, 15]; also electrophysiological studies in rat brain, J. W. Hall and L. P. Davies, unpublished results).

The experimental data also rule out the possibility that an interaction of these compounds with benzodiazepine receptors could contribute to any muscle-relaxant effects.

An additional mechanism to that proposed above is that these halogenated pyrrolopyrimidine compounds, by inhibiting adenosine kinase, may alter intracellular adenosine levels with a consequent effect on the "intracellular" or P-site adenosine receptor which has been suggested to be associated with virtually all adenylate cyclases, perhaps directly associated with the catalytic subunit [26]; however, this is not a likely mechanism since theophylline is not an antagonist at this site [25].

A number of studies have suggested that in many tissues adenosine kinase is intimately involved in adenosine uptake by rapidly phosphorylating and trapping adenosine in the cell once its has been transported across the membrane by the nucleoside carrier (e.g. [27, 28]). It appears likely that the uptake inhibition by 5'-deoxy-5-iodotubercidin and 5-iodotubercidin results primarily from an inhibition of adenosine kinase since Wu *et al.* [29] have shown that 5-iodotubercidin was essentially inactive in inhibiting rapid *transport* in rat brain synaptosomes.

With respect to the cardiovascular effects of these tubercidin analogues, the coronary vasoactivity of some other adenosine analogues appears to be due at least in part to inhibition of adenosine transport and consequent accumulation of endogenous adenosine [22]. The observation that theophylline almost completely reversed the effects of the iodo-compounds on heart rate but only partially reversed their effects on blood pressure is noteworthy, and may support the idea that there are separate receptors for adenosine in cardiac and vascular smooth muscle tissue [30, 31].

In the current experiments there was a correlation between the cardiovascular and the muscle-relaxant/hypothermic activities of the investigated compounds. In previous studies on the effects of peripherally-administered adenosine agonists on CNS function, some controversy has arisen as to whether such effects are peripherally or centrally mediated [10, 32, 33]. The sedative and hypnogenic actions of adenosine could possibly be ascribed to a reduction in blood pressure and in this regard a correlation has been reported for the effects of adenosine analogues on neuronal cell firing and their hypotensive actions. However, Katims *et al.* [33] reported that the cardiovascular effects of *R*-PIA were not seen until doses some ten-fold higher than those causing reduced locomotor activity were attained. It is possible that adenosine may produce its behavioural effects by a combination of both central and peripheral actions (perhaps involving alterations in cerebral blood flow). However, it may be noted that while adenosine itself only poorly crosses the blood brain barrier (and is rapidly metabolized), the metabolically-stable halogenated tubercidin analogues are more lipophilic and thus more likely to enter the brain.

In summary, it appears that while these tubercidin analogues have only weak agonist activity at membrane-bound adenosine receptors, quite a good correlation exists between their ability to inhibit adenosine uptake (and adenosine kinase) and their *in vivo* activity. It is relevant to note that "standard" adenosine uptake inhibitors can potentiate the transient hypothermic and sedative effects of adenosine given i.p. [5, 34].

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