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MOLECULAR FEATURES NECESSARY FOR THE UPTAKE OF DIAMINES AND RELATED COMPOUNDS BY THE POLYAMINE RECEPTOR OF RAT LUNG SLICES

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Abstract—The influence of 17 putrescine analogues on the uptake of putrescine and/or paraquat by rat lung slices has been determined. Most of these compounds are competitive inhibitors of putrescine and/or paraquat uptake, but three show no inhibiting activity. Apparent K_i values of the putrescine derivatives increase, and thus the inhibitory effects decrease, with increasing N-methylation. Comparison of *N*-methyl-1,4-diaminobutane ($K_i = 8\ \mu M$) with *N,N'*-bis-methyl-1,4-diaminobutane ($K_i = 25.5\ \mu M$) shows that a single primary amino group is desirable for high inhibiting activity. Dimethylation at one amino function does not greatly decrease inhibitory potential (thus *N,N*-dimethyl-1,4-diaminobutane has $K_i = 11.5\ \mu M$). Increasing the size of *N*-alkyl substituents in putrescine derivatives, decreased their inhibitory action on the uptake of putrescine. Investigation of the effect of conformationally-restricted analogues of putrescine shows that both (*E*) and (*Z*) isomers of 1,4-diaminobut-2-ene are poor inhibitors of putrescine uptake. Analogues of putrescine with bulky substituents on the butyl chain, i.e. the *meso*- and *rac*-isomers of 1,1-dichloro-2,3-diaminomethylcyclopropane, do not inhibit putrescine uptake. Inhibiting putrescine derivatives which contain aziridine groups are competitive inhibitors of putrescine and paraquat uptake. Surprisingly, *N*-(4-aminobutyl)aziridine is the most effective inhibitor of putrescine uptake studied, and is a better inhibitor of paraquat uptake than the endogenous polyamine, putrescine. *N*-(4-Aminobutyl)aziridine binds reversibly to the polyamine transporter and its inhibitory effects do not appear to be due to any cytotoxic activity of the aziridine. The parameter A $(mM)^{-1}$ defined as $1000/K_i$ (where K_i units are μM) was taken as a measure of the affinity of a compound for the polyamine receptor in this paper.

The uptake of polyamines and their analogues by cells is a topic of much current interest [1–3]. Polyamines are ubiquitous, having been found in every living system investigated [4]. Endogenous polyamines appear to be involved in many biological processes including DNA replication [5], protein synthesis [6], cellular differentiation [7], enzyme regulation [8], and membrane stability [9].

Cellular uptake of polyamines has been investigated using many cell types and uptake involves specific polyamine transporters [1, 10]. Little is known of the biochemical basis of polyamine uptake since no polyamine transport proteins have been isolated, although Byers *et al.* [11] have recently obtained clones of Chinese-hamster ovary cells which express human genes for polyamine transport.

Ingestion of paraquat leads to a selective uptake of this herbicide into both rat and human lung tissues [12–15]. The cell types in lung tissue which accumulate polyamines and paraquat (1,1'-dimethyl-4,4-bipyridinium dichloride) are the Clara cells and alveolar epithelial type I and type II cells [15]. The transport system for the accumulation of paraquat in lung slices appears to be identical to that for natural polyamines including putrescine (1,4-diaminobutane), spermidine (*N*-3-aminopropyl-1,4-diaminobutane) and spermine (*N,N'*-bis-3-aminopropyl-1,4-diaminobutane) [12]. The process responsible for the uptake of paraquat into rat and human lung slices is saturable, energy dependent, and competitively inhibited by endogenous polyamines [14, 16, 17]. Previous studies of the inhibiting action of various amines on the uptake of putrescine or paraquat by lung slices have shown that effective inhibitors require one or preferably two nitrogen-containing species, such as amino groups that are protonated at physiological pH, separated by at least four methylene groups [16].

We have investigated the entry of a range of polyamine analogues into rat lung slices, in order to elucidate the structural requirements necessary in molecules for their uptake by this transport system. Also, we hoped to prepare one or more compounds that would attach specifically to the paraquat-binding site of the uptake system, and then covalently bind to an amino acid located at or near the binding site. The intramolecular distance between the charged nitrogen atoms in paraquat and putrescine is 7.02 and 6.6 Å, respectively. It appears that effective inhibitors of the paraquat uptake system should have two cationic nitrogen atoms separated by approximately this distance [14]. We envisaged that the paraquat-binding site in the lung contains two anionic groups, presumably carboxylates, separated by about 7 Å. Therefore, our approach, in the search

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for an affinity label for the paraquat uptake system, was to employ a putrescine analogue containing a group with characteristics similar to those of an amino group, that would react with carboxylates. Both aziridines [18–20] and carbodiimides [21–23] react with carboxylates and have been widely used as affinity labels and enzyme inactivators. Hence, we prepared analogues of putrescine, containing either aziridine or carbodiimide groups.

All the inhibiting compounds that we studied were competitive inhibitors of putrescine uptake. Surprisingly, *N*-(4-aminobutyl)aziridine (**1**) was the most effective inhibitor of both putrescine and paraquat uptake that emerged from these studies.

MATERIALS AND METHODS

Materials. [1,4-^{14}C]Putrescine dihydrochloride (118 mCi/mmol) and [methyl-^{14}C]paraquat dichloride (111 mCi/mmol) were both obtained from Amersham International Ltd (Amersham, U.K.). Putrescine dihydrochloride was obtained from the Sigma Chemical Co. (Poole, U.K.). Paraquat dichloride (99.9%) was obtained from Imperial Chemical Industries PLC (Bracknell, U.K.). Halothane (Fluothane) was supplied by ICI PLC (Macclesfield, U.K.). Soluene-350 (tissue solubilizer) and Dimilume-30 (liquid scintillation fluid) were obtained from Packard Ltd (Poole, U.K.). Optiphase MP (liquid scintillation fluid) was obtained from FSA Laboratory Supplies Ltd (Loughborough, U.K.). The dihydrochloride salts of *N*-methyl-1,4-diaminobutane (**2**), *N*,*N*-dimethyl-1,4-diaminobutane (**3**), *N*,*N*′-dimethyl-1,4-diaminobutane (**4**), *N*-methyl-*N*′,*N*′-dimethyl-1,4-diaminobutane (**5**), *N*-propyl-1,4-diaminobutane (**7**) and *N*-isobutyl-1,4-diaminobutane (**8**) were prepared as described by Golding *et al.* [24]. *N*,*N*,*N*′,*N*′-Tetramethyl-1,4-diaminobutane dihydrochloride (**6**) was prepared from 1,4-diaminobutane using the Eschweiler–Clarke procedure [25]. (*Z*)-1,4-Diaminobut-2-ene (**9**) was prepared from (*Z*)-1,4-dihydroxybut-2-ene as described by Fabiano *et al.* [26]. (*E*)-1,4-Diaminobut-2-ene (**10**) was prepared from (*Z*)-1,4-dihydroxybut-2-ene by acetylation to give (*Z*)-1,4-diacetoxybut-2-ene, which was converted into the (*E*) isomer using tetrakis(triphenylphosphine)palladium [27], followed by removal of the acetate groups and amination of the hydroxy groups [26]. *meso*-1,1-Dichloro-2,3-diaminomethylcyclopropane (**11**) was prepared from 2,3-dimethyl-4,7-dihydro-1,3-dioxepin [28] by reaction of this dioxepin with dichlorocarbene (generated *in situ* from chloroform and 19 M aqueous sodium hydroxide in the presence of the phase transfer catalyst cetyltrimethylammonium bromide), followed by the acid-catalysed hydrolysis of the acetal group to give a diol, which was then aminated [26] to give the required *meso* compound (**11**). *rac*-1,1-Dichloro-2,3-diaminomethylcyclopropane (**12**) was prepared from (*E*)-1,4-dihydroxybut-2-ene by first protecting the alcohol groups as *p*-methoxybenzylethers, followed by reaction with dichlorocarbene. Removal of the *p*-methoxybenzyl protecting groups by oxidation with cerium (IV) ammonium nitrate,* followed by amination [26] of the diol gave the desired *rac* compound (**12**). *N*-(4-Aminobutyl)aziridine (**1**) was prepared essentially using the route described by Piper *et al.* [29]. *N*,*N*′-Bis(2-bromoethyl)-1,4-diaminobutane was prepared by the reaction of 1,4-diaminobutane with ethylene oxide to yield, after fractional distillation, *N*,*N*′-bis(2-hydroxyethyl)1,4-diaminobutane. This diol was reacted with hydrobromic acid to give *N*,*N*′-bis(2-bromomethyl)-1,4-diaminobutane which, at pH 7.4, undergoes ring closure to form *N*,*N*′-bis-aziridinyl-1,4-butane (**13**) [3]. *N*-(4-Aminobutyl)-2,3-*exo*-aziridinonorbornane (**14**) was obtained by reaction of *N*-phenylmethoxycarbonyl-4-azidobutanamine [24] with norbornene, followed by silica-gel catalysed decomposition of the resulting triazoline and removal of the phenylmethoxycarbonyl group to give the desired aziridine (**14**). 1-Propyl-3-(4-dimethylaminobutyl)-carbodiimide (**17**) was obtained by the dehydration of 1-propyl-3-(4-dimethylaminobutyl)urea using *p*-toluenesulphonyl chloride and triethylamine [3]. The urea was prepared by the reaction of *N*,*N*-dimethyl-1,4-diaminobutane with propyl isocyanate. *N*-(2-Bromoethyl)-1,4-diaminobut[2,3-^{3}H]ane was prepared from (*Z*)-1,4-diaminobut-2-ene (**9**) by reaction with ethylene oxide followed by tritiation of the double bond by Amersham International PLC (Amersham, U.K.). To the resulting tritiated alcohol was added unlabelled *N*-(2-hydroxyethyl)-1,4-diaminobutane (**16**). Bromination of this alcohol with hydrobromic acid gave *N*-(2-bromoethyl)-1,4-diaminobut[2,3-^{3}H]ane which was diluted with cold *N*-(2-bromoethyl)-1,4-diaminobutane to give a final activity of 0.343 Ci/mmol.

Rat lung slice preparation. Slices of rat lung were prepared according to the method of Smith *et al.* [17]. Male Alpk:APfSD (Wistar-derived) specific pathogen-free rats (body weight approximately 200 g) were killed by inhalation of halothane (Fluothane) and the lungs removed immediately after cessation of breathing. The lungs were washed with modified Krebs–Ringer phosphate buffer (KRP) containing sodium chloride (130 mM), potassium chloride (5.2 mM), calcium chloride (1.9 mM), magnesium sulphate (1.2 mM), disodium hydrogen phosphate (10 mM) and glucose (11 mM); the pH being adjusted to pH 7.4 by addition of 1 M hydrochloric acid. Lung tissue slices 0.5 mm thick, were obtained using a McIlwain tissue chopper.

Measurement of the effect of compounds on the uptake of putrescine. Flasks were prepared containing 2.0, 5.0, 10 or 50 μM [^{14}C]putrescine (0.3 μM [^{14}C]putrescine dihydrochloride plus the required quantity of unlabelled putrescine dihydrochloride) in KRP buffer (3.0 mL). Other flasks contained the above, but also contained concentrations of 10, 25 or 100 μM of the compound under investigation. These concentrations were obtained by adding the appropriate volume of 10 mM aqueous solution of test compound. For compounds of low water solubility [*N*-(4-aminobutyl)aziridine (**1**), *N*-(4-aminobutyl)-2,3-*exo*-aziridinonorbornane (**14**) and 1-propyl-3-(4-dimethylaminobutyl)-carbodiimide (**17**)] ethanol replaces water in the 10 mM solutions.

* Crilley MML and Golding BT, unpublished work.

The flasks were incubated at 37° and freshly prepared lung slices (20–40 mg) were added to each flask. These slices possess ample excess of polyamine receptor sites for the kinetic studies. The lung slices were incubated in the media at 37°, under air, for 30 min in a shaking water bath at 70 rpm.

Aliquots (0.1 mL) of each of the incubation media were then collected. The slices were removed from the media, washed with KRP and dissolved by warming with Soluene-350. Water (0.9 mL) and then Optiphase MP (10 mL) was added to each of the aliquots of incubation media. Dimilume-30 (10 mL) was added to the solutions containing dissolved slices at room temperature. The disintegrations per minute of each sample were measured by liquid scintillation spectroscopy.

For each compound under assay this procedure was repeated three times using one rat for each procedure (i.e. three rats per compound).

Measurement of the effect of compounds on the uptake of paraquat. The same procedure as that used to measure the effect of compounds on the uptake of putrescine was used but [^{14}C]putrescine was replaced by [^{14}C]paraquat dichloride. A [^{14}C]paraquat dichloride concentration of 5.0, 10, 20, 50 or 100 μM (0.3 μM [^{14}C]paraquat plus the required amount of unlabelled paraquat) was added to the media and the lung slices were incubated at 37° at 70 rpm for 2 hr.

Measurement of the effect of N,N′*-bisaziridinyl-1,4-diaminobutane* (**13**) *on putrescine uptake. N,N-*Bis(2-bromoethyl)-1,4-diaminobutane was incubated at 37° in KRP buffer (10 mL) for 1.5 hr, which resulted in the formation of *N,N′*-bisaziridinyl-1,4-diaminobutane (**13**) [3]. Then quantities of this aziridine (**13**) solution were used in the uptake assays as described previously.

The effect of preincubating lung slices in the presence of N-*(4-aminobutyl)aziridine* (**1**) *on the subsequent uptake of putrescine.* Lung slices, prepared as described previously, were incubated at 37° in a shaking water bath at 70 rpm for 15 min in KRP buffer (3.0 mL) containing ethanol (30 μL), putrescine dihydrochloride (100 μM) and ethanol (30 μL), or aziridine (**1**) (100 μM added as 30 μL of a 10 mM solution in ethanol). The lung slices were then removed, washed with KRP buffer and transferred to flasks containing 2.0, 5.0, 10, 20 or 50 μM [^{14}C]putrescine (0.3 μM [^{14}C]putrescine plus the required amount of unlabelled putrescine) in KRP buffer (3.0 mL). The flasks were incubated at 37° at 70 rpm for 30 min and the samples were then treated in the same way as that used to measure the effect of compounds on the uptake of putrescine.

Calculation of inhibitor constant K_i *and affinity* A. The ratio of the disintegrations per min per 100 mg of lung slice divided by the DPM per 100 μL of media for each incubated flask was calculated. This ratio multiplied by the concentration (μM) of [^{14}C]putrescine or [^{14}C]paraquat in each flask gave v (nmol putrescine/g wet weight lung/30 min or, nmol paraquat/g wet weight lung/2 hr). The v values were used to plot Lineweaver–Burk graphs, i.e. $1/v$ against $1/[S]$, where $[S]$ is the concentration of putrescine or paraquat in μM with the intercept on the x-axis giving $-1/K_m$. The intercept on the $1/[S]$-axis, of a Lineweaver–Burk plot for each concentration $[I]$ (μM) of a competitive inhibitor, gives $-1/K_p$. The K_i of each competitive inhibitor is calculated from the equation:

$$K_i = [I]\left(\frac{K_p}{K_m} - 1\right)^{-1}$$

and, if the units for K_i are μM, then $A = 1000/K_i$ has the units $(\text{mM})^{-1}$.

RESULTS

An aim of this study was to explore the systematic changes of the putrescine structure on the uptake of these compounds into rat lung slices, with the intention of defining the groups important in substrate recognition. We also envisaged that putrescine analogues containing aziridine or carbodiimide groups might covalently bind to the receptor. Hence, the results are presented as studies using putrescine derivatives, conformationally-restricted analogues of putrescine, and aziridine- and carbodiimide-containing derivatives of putrescine.

The K_m (Michaelis–Menten constant) values for the uptake of [^{14}C]putrescine, in the absence of any inhibitor, in eleven uptake studies, varied from 12 to 18 μM. This range in K_m values for putrescine is an indication of the variation that can occur in uptake studies.

The effects of derivatives of putrescine on the uptake of putrescine by rat lung slices

The K_i and A values for the putrescine derivatives studied, when acting as inhibitors against the uptake of [^{14}C]putrescine by rat lung slices are shown in Table 1. Relative inhibitory power is given directly by affinity A. All compounds displayed competitive inhibition of putrescine uptake, as determined by Lineweaver–Burk plots, i.e. V_{max} had the same value when obtained in the presence or absence of all compounds tested. In this section, the best inhibitor of putrescine uptake is *N*-methyl-1,4-diaminobutane (**2**) (Fig. 1), structurally close to the substrate, putrescine and with a K_i of 8. Dimethylation at one of the amino groups appears not to influence greatly the uptake of the compound, i.e. the K_i values of *N*-methyl-1,4-diaminobutane (**2**) and *N,N*-dimethyl-1,4-diaminobutane (**3**) are of similar magnitude. *N,N′*-Disubstituted derivatives of putrescine are much poorer inhibitors than mono-substituted derivatives, i.e. the A value of *N,N-′*-dimethyl-1,4-diaminobutane (**4**) is half that of *N*-methyl-1,4-diaminobutane (**2**). Increasing the number of groups attached to the amino groups of 1,4-diaminobutane, decreases the inhibitory potential of these compounds, i.e. the A value of *N*-methyl-*N′,N′*-dimethyl-1,4-diaminobutane (**5**) is 24 whereas, that for *N,N,N′,N′*-tetramethyl-1,4-diaminobutane (**6**) is 10 $(\text{mM})^{-1}$.

N-Propyl-1,4-diaminobutane (**7**) is a notably better inhibitor of putrescine uptake than *N*-isobutyl-1,4-diaminobutane (**8**). This difference must relate to the greater steric bulk of the isobutyl group compared to the propyl group. *N*-(2-Hydroxyethyl)-1,4-diaminobutane (**16**) is a slightly better inhibitor than the propyl derivative (**7**) and this may be related to

Table 1. Inhibitory effects of putrescine derivatives on the uptake of putrescine into rat lung slices

		Inhibitor constant K_i (μM)	Affinity for receptor A (mM)$^{-1}$
(2)	$H_2N(CH_2)_4NHCH_3 \cdot 2HCl$	8	125
(3)	$H_2N(CH_2)_4N(CH_3)_2 \cdot 2HCl$	11.5	87
(4)	$CH_3NH(CH_2)_4NHCH_3 \cdot 2HCl$	25.5	39
(5)	$CH_3NH(CH_2)_4N(CH_3)_2 \cdot 2HCl$	41.5	24
(6)	$(CH_3)_2N(CH_2)_4N(CH_3)_2 \cdot 2HCl$	100	10
(7)	$H_2N(CH_2)_4NHCH_2CH_2CH_3 \cdot 2HCl$	23	43
(8)	$H_2N(CH_2)_4NHCH_2CH(CH_3)_2 \cdot 2HCl$	51	20
(16)	$H_2N(CH_2)_4NHCH_2CH_2OH$	14	71

Lung slices were incubated at 37° for 30 min in buffer containing 2.0, 5.0, 10, 20 or 50 μM [^{14}C]putrescine (0.3 μM [^{14}C]putrescine plus the required amount of unlabelled putrescine) and the compound under study (0, 10, 25 or 100 μM). The accumulation of ^{14}C-label into the lung slices was measured. This procedure was repeated using three animals for each compound being studied. The mean value from three rats at each compound concentration was calculated. The K_i at each concentration of the compound being studied was determined from Lineweaver–Burk plots and the mean value is listed in the table.

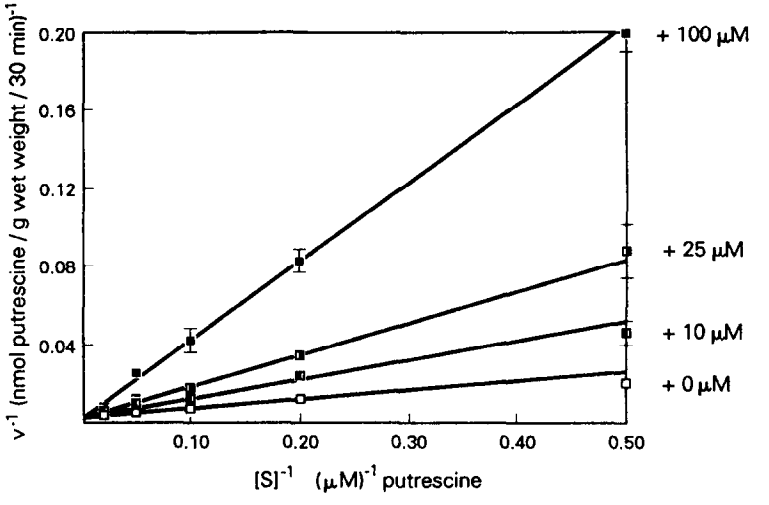


Fig. 1. The effect of *N*-methyl-1,4-diaminobutane dihydrochloride (**2**) on the uptake of [^{14}C]putrescine. Lineweaver–Burk plot of the uptake of [^{14}C]putrescine into rat lung slices after 30 min at 37°, at 70 rpm, in buffer, in the presence of 0 μM (□), 10 μM (◧), 25 μM (◨) and 100 μM (■) of *N*-methyl-1,4-diaminobutane dihydrochloride (**2**). The values shown represent mean values plus or minus the standard error of the mean of three observations. Where the standard error is not shown, it did not extend beyond the point plotted.

the slightly smaller steric bulk of the hydroxyethyl compared to the propyl group.

The effects of conformationally restricted derivatives of putrescine on the uptake of putrescine into rat lung slices

Both the (*Z*) and (*E*) isomers of 1,4-diaminobut-2-ene (**9**) and (**10**), respectively, are poorer competitive inhibitors of putrescine uptake than *N*-methyl- (**2**), *N*,*N*-dimethyl- (**3**), *N*,*N*′-dimethyl- (**4**), *N*-(2-hydroxyethyl)- (**16**) and *N*-propyl-1,4-diaminobutane (**7**) (Table 2). The difference between the K_i values of the (*Z*) and (*E*) isomers of 1,4-diaminobut-2-ene may not be significant and are within the margin of experimental error.

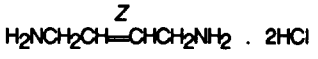
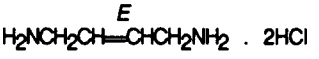
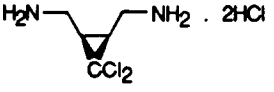
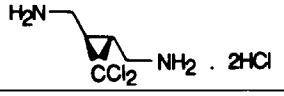
Both *meso*- and *rac*-1,1-dichloro-2,3-diaminomethylcyclopropanes (**11**) and (**12**) respectively, do not inhibit the uptake of putrescine by the receptor.

The effects of some aziridines and a carbodiimide on the uptake of putrescine or paraquat by rat lung slices

All the inhibiting compounds studied were competitive inhibitors of putrescine or paraquat uptake.

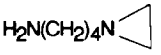
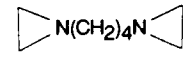
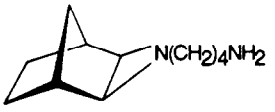
N-(2-Bromoethyl)-1,4-diaminobutane (**15**) (Table 3) appears to be a better competitive inhibitor of putrescine uptake than its hydroxyl precursor (**16**) (Table 1). We have shown, by proton NMR studies [3], that in the buffer conditions used for the uptake

Table 2. Inhibitory effects of conformationally restricted analogues of putrescine on the uptake of putrescine into rat lung slices

		Inhibitor constant K_i (μM)	Affinity for receptor A (mM)$^{-1}$
(9)		40	25
(10)		31	32
(11)		No inhibition	0
(12)		No inhibition	0

See footnote to Table 1.

Table 3. Inhibitory effects of some aziridines and 1-propyl-3-(4-dimethylaminobutyl)carbodiimide (**17**) on the uptake of putrescine or paraquat into rat lung slices

		Inhibition of putrescine uptake		Inhibition of paraquat uptake	
		K_i (μM)	A (mM)$^{-1}$	K_i (μM)	A (mM)$^{-1}$
	$H_2N(CH_2)_4NH_2$. 2HCl			10	100
(15)	$H_2N(CH_2)_4NHCH_2CH_2Br$. 2HBr	9	111		
(1)		7.5	133	5	200
(13)		31.5	32		
(14)		197	5		
(17)	$CH_3(CH_2)_2N{=}C{=}N(CH_2)_4N(CH_3)_2$	No inhibition	0		

For details see footnote to Table 1. In addition, for paraquat uptake studies, lung slices were incubated at 37° at 70 rpm for 2 hr in buffer containing 5.0, 10, 20, 50 or 100 μM of [^{14}C]paraquat (0.3 μM [^{14}C]paraquat plus the required amount of unlabelled paraquat) and the compound under study (0, 10, 25 or 100 μM).

assays, bromide (**15**) forms *N*-(4-aminobutyl)-aziridine (**1**). Hence, the effect measured is due to a mixture of bromide (**15**) and aziridine (**1**).

The most potent inhibitor studied is *N*-(4-aminobutyl)aziridine (**1**) (Fig. 2) and this aziridine appears to be a better competitive inhibitor of paraquat uptake than the endogenous polyamine, putrescine. The modest difference in A values between *N*-(4-aminobutyl)aziridine (**1**) and *N*-methyl-1,4-diaminobutane (**2**) suggests that the inhibitory effects of aziridine (**1**) may not be due to the reactivity of the aziridine ring. *N,N'*-Bisaziridinyl-1,4-butane (**13**) is a much less effective inhibitor than *N*-(4-aminobutyl)aziridine (**1**). This is consistent with the results obtained from the methyl derivatives of putrescine, indicating that effective inhibitors require one primary amino group. However, *N,N'*-bisaziridinyl-1,4-butane (**13**) is a much better inhibitor than *N,N,N',N'*-tetramethyl-1,4-diaminobutane (**6**). The aziridine group has a stronger affinity for the substrate binding site than the *N,N*-dimethyl group.

The final aziridine assayed, *N*-(4-aminobutyl)-2,3-aziridinonorbornane (**14**) is a poor inhibitor of putrescine uptake and has low affinity for the polyamine receptor.

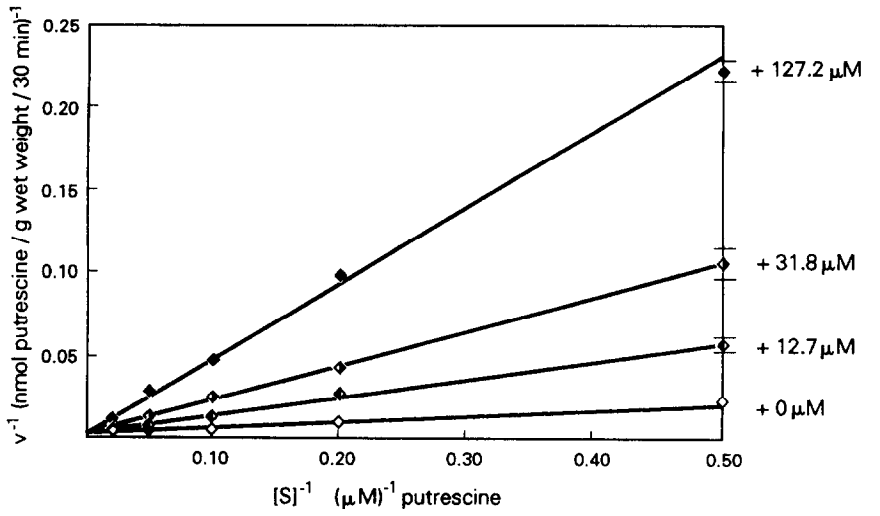


Fig. 2. The effect of *N*-(4-aminobutyl)aziridine (**1**) on the uptake of [^{14}C]putrescine. Lineweaver–Burk plot of the uptake of [^{14}C]putrescine into rat lung slices after 30 min at 37°, at 70 rpm in buffer, in the presence of 0 μM (◇), 10 μM (◆), 25 μM (◆) or 100 μM (◆) of *N*-(4-aminobutyl)aziridine (**1**). The values shown represent mean values plus or minus the standard error of the mean of three observations. Where the standard error is not shown, it did not extend beyond the point plotted.

1-Propyl-3-(4-dimethylaminobutyl)carbodiimide (**17**), with no primary amino group, did not inhibit the uptake of putrescine.

The effect of pre-incubating rat lung slices on the subsequent uptake of putrescine

Rat lung slices were pre-incubated for 15 min in buffer containing either *N*-(4-aminobutyl)aziridine (**1**) (100 μM) in ethanol (30 μL), or putrescine (100 μM) and ethanol (30 μL), or ethanol (30 μL). The lung slices were then removed, washed, and the uptake of putrescine into these lung slices was measured by incubating these lung slices in buffer containing [^{14}C]putrescine (2.0, 5.0, 10, 20 or 50 μM) at 37° with shaking for 30 min. The uptake of labelled putrescine was then determined in the same way as described for the other uptake studies. The K_m value of putrescine uptake in lung slices that had been pre-incubated with 100 μM putrescine was 16 μM, for lung slices pre-incubated with 100 μM *N*-(4-aminobutyl)aziridine was 14 μM and for lung slices pre-incubated with 30 μL ethanol was 11 μM (Fig. 3).

DISCUSSION

Most of the compounds that we assayed reduce the uptake of [^{14}C]putrescine and/or [^{14}C]paraquat into rat lung slices indicating that these compounds inhibit putrescine or paraquat transportation into cells by the polyamine uptake mechanism. The Lineweaver–Burk plots show that all our tested inhibitors of putrescine or paraquat uptake display competitive inhibition. Thus, the inhibition is reversible and none of the inhibiting molecules covalently bind to the polyamine transporter.

Smith *et al.* [17] have shown previously that measurement of carbon-14 is an acceptable method for determining the amount of [^{14}C]putrescine or [^{14}C]paraquat in lung slices. In the present assays, the lung slices were washed with buffer, after incubation with [^{14}C]putrescine or [^{14}C]paraquat. The work of Gordonsmith and coworkers [16] suggests that some of the ^{14}C-labelled compound, measured in our assays, arises from diffusion rather than from receptor-mediated uptake. The amount of ^{14}C-labelled compound that was accumulated in the lung slices because of diffusion is probably a constant factor in all of our determinations. No correction for this factor was deemed to be necessary as we sought only comparable K_i values for the inhibitors.

Assuming that the K_i values of test compounds inversely reflect their affinity for the polyamine receptor in rat lung slices under the experimental conditions used, then $A = 1000/K_i$ will be a measure of this affinity. If K_i is in μM units, A will be in $(\text{mM})^{-1}$ units. The data for *N*-alkyl derivatives of putrescine (Table 1) indicates that the larger the steric size of the alkyl group, the poorer the association of the compound with the substrate-binding site of the receptor. This is illustrated by the sequence: *N*-methyl-1,4-diaminobutane (**2**) (A = 125), *N*-propyl-1,4-diaminobutane (**7**) (A = 43) and *N*-isobutyl-1,4-diaminobutane (**8**) (A = 20). Also, increasing the number of substituents on the amino groups of putrescine decreases the uptake of the compound, as can be seen by the large drop in A values on increasing the number of *N*-methyl groups from *N*-methyl-1,4-diaminobutane (**2**) to *N*,*N*,*N*′,*N*′-tetramethyl-1,4-diaminobutane (**6**). The ratio of A values for *N*-methyl- (**2**) and *N*,*N*-dimethyl-1,4-diaminobutane (**3**) is 1.44, whereas, the ratio for *N*-methyl-1,4-diaminobutane (**2**) and the *N*,*N*′-dimethyl compound (**4**) is 3.2. Therefore, increasing the methyl substituents at one amino group of putrescine has a minor effect on affinity and inhibitory potential, but methylation at both amino groups has a more marked effect.

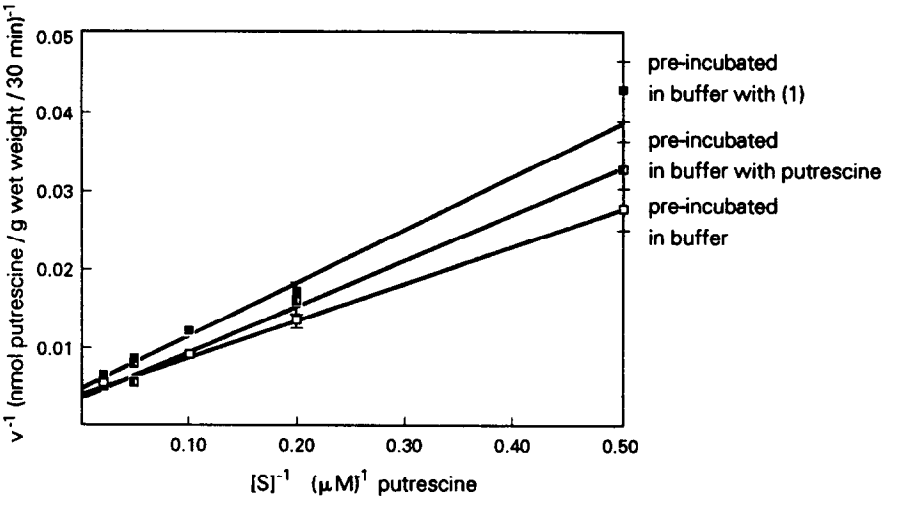


Fig. 3. The effect of pre-incubation of lung slices in buffer containing *N*-(4-aminobutyl)aziridine (**1**) (100 μM) or putrescine (100 μM) on the subsequent uptake of [^{14}C]putrescine. Rat lung slices were incubated at 37°, at 70 rpm for 15 min in buffer containing 30 μL ethanol (□), 100 μM putrescine dihydrochloride and 30 μL ethanol (◧), or 100 μM *N*-(4-aminobutyl)aziridine (**1**) (added as 30 μL of a 10 mM solution in ethanol) (■). The lung slices were then washed with buffer. Lineweaver–Burk plot of the subsequent uptake of [^{14}C]putrescine into the lung slices after 30 min, at 70 rpm in buffer containing [^{14}C]putrescine (2.0, 5.0, 10, 20 or 50 μM). The values shown represent mean values plus or minus the standard error of the mean of three observations. Where the standard error is not shown, it did not extend beyond the point plotted.

We investigated the inhibitory action of some conformationally-restricted analogues of putrescine and showed that both the (*E*) and (*Z*) isomers of 1,4-diaminobut-2-ene [(**10**) and (**9**)], were relatively poor competitive inhibitors of [^{14}C]putrescine uptake (Table 2). 1,4-Diaminobut-2-enes are not only less flexible than putrescine, but as carbon–carbon double bonds are shorter than carbon–carbon single bonds (bond lengths; C=C (ethylene) 1.34 Å [30], C–C (ethane) 1.54 Å [31]) the distance between the amino groups in 1,4-diaminobut-2-enes is less than in putrescine. Either or both of these factors may account for the lower affinity of 1,4-diaminobut-2-ene than that of putrescine for the receptor. Work carried out by Heston *et al.* [32] on the uptake of [^{14}C]putrescine and its derivatives into Dunning R3327 MAT-Lu rat prostate-derived tumour cells supports this result.

Another class of conformationally restricted analogues of putrescine that we investigated were 1,1-dichloro-2,3-diaminomethylcyclopropanes. The distance between the amino groups in both *meso*- (**11**) and *rac*-1,1-dichloro-2,3-diaminomethylcyclopropane (**12**) is similar to the distance between the amino groups of the extended conformation of putrescine. 1,3-Diaminopropane and 1,2-diaminoethane are poor inhibitors of putrescine uptake into rat lung slices [16], indicating that putrescine, in its extended conformation, binds to the polyamine transporter. Neither *meso*- (**11**) nor *rac*-1,1-dichloro-2,3-diaminomethylcyclopropane (**12**) were found to inhibit the uptake of putrescine. This inactivity may relate to conformational rigidity in these molecules in comparison with putrescine or the bulk of the two chlorine substituents in the cyclopropane ring may prevent these molecules fitting into the putrescine-binding receptor site.

In attempts to label covalently the paraquat and polyamine transporter, we investigated the uptake of polyamine analogues, containing an aziridine or a carbodiimide function. The carbodiimide-containing analogue of putrescine, 1-propyl-3-(4-dimethylaminobutyl)carbodiimide (**17**), failed to inhibit the uptake of putrescine, probably because of the absence of a primary amino group. (N.B. the tri-*N*-substituted diimide (**17**) was synthetically accessible, compared to analogues lacking one or more *N*-alkyl substituents.) Compounds containing one primary amino group have been shown to be poor inhibitors of putrescine uptake [16], and the carbodiimide (**17**) has just a single (tertiary) amino group. The presence of the single dimethylamino group confers no affinity for the receptor in this case.

The least effective aziridine studied was *N*-(4-aminobutyl)-2,3-aziridinonorbornane (**14**), indicating that the substrate-binding site cannot tolerate the bulky norbornyl group.

Both *N*-(4-aminobutyl)aziridine (**1**) and *N*,*N*′-bis-aziridinyl-1,4-butane (**13**) are competitive inhibitors of putrescine and/or paraquat uptake. The monoaziridine (**1**) is a better inhibitor than *N*,*N*-dimethyl-1,4-diaminobutane (**3**) and the bisaziridine (**13**) more effective than *N*,*N*,*N*′,*N*′-tetramethyl-1,4-diaminobutane (**6**), indicating that the aziridine group has a stronger affinity for the substrate binding site than the *N*,*N*-dimethylamino group.

A surprising result is that *N*-(4-aminobutyl)aziridine (**1**) appears to be a better substrate

for the uptake system than putrescine, and is the most effective inhibitor we studied.

N-(4-Aminobutyl)aziridine (**1**) might covalently bind to the receptor to hinder, but not totally block, the transport of putrescine and paraquat. This is consistent with the kinetics, but to eliminate this possibility, we pre-incubated lung slices in buffer containing either putrescine (100 μM) or aziridine (**1**) (100 μM) for 15 min. The uptake of [^{14}C]putrescine into these lung slices was then measured. There was very little difference in the subsequent uptake of [^{14}C]putrescine in lung slices that had been pre-incubated with either putrescine or *N*-(4-aminobutyl)aziridine. This indicates that the aziridine binding is readily reversible and confirms that the aziridine does not covalently bind to the receptor.

In order to elucidate further the reasons for the inhibitory action of *N*-(4-aminobutyl)aziridine (**1**) on the uptake of putrescine and paraquat, we prepared *N*-(2-bromoethyl)-1,4-diaminobut[2,3-^{3}H]ane. We have shown that in an aqueous medium at pH 7.4, the conditions used for the uptake studies, *N*-(2-bromoethyl)-1,4-diaminobutane (**15**) ring closes to form *N*-(4-aminobutyl)aziridine (**1**) [3]. The use of *N*-(2-bromoethyl)-1,4-diaminobutane (conveniently as its crystalline dihydrobromide) renders much safer the handling of the aziridine, which is expected to be very toxic [33]. The *N*-(2-bromoethyl)-1,4-diaminobut[2,3-^{3}H]ane was diluted with *N*-(2-bromoethyl)-1,4-diaminobutane (**15**) to give a mixture of specific activity 0.343 Ci/mmol. The resulting mixture was found to have a K_m value of 12.2 ± 3.1 μM and a V_{max} of 203.8 ± 12 nmol (g wet weight lung)$^{-1}$ hr^{-1} (unpublished results, I. Wyatt and R. Moore). The K_m values for the uptake of competitive inhibitors into rat lung slices appears to be equivalent to the K_i values of these inhibitors against putrescine uptake [34] and whereas $A = 1000/K_i$ by definition, A is also given approximately by $1000/K_m$. The K_i value of *N*-(2-bromoethyl)-1,4-diaminobutane (**15**) (9 μM) against putrescine uptake is similar to the K_m value obtained for the tritiated compound. The V_{max} value of the tritiated bromide is significantly less than the V_{max} value of 1000 nmol (g wet weight)$^{-1}$ hr^{-1} reported for putrescine uptake [34].

Autoradiography studies of rat lung slices which have been incubated with tritiated aziridine (**1**) indicate that this compound is selectively accumulated into Clara cells and Type I and Type II epithelial cells (unpublished results, I. Wyatt and A. Soames). These are the cell types that have been shown previously to accumulate putrescine and paraquat in rat lungs [15]. These results confirm that *N*-(4-aminobutyl)aziridine (**1**) is a substrate for the polyamine transporter.

It is known that *N*-(4-aminobutyl)aziridine is cytotoxic [35], and its affinity A might be high not just because it fits the uptake system better than putrescine but, perhaps, the aziridine enters the cell and its subsequent cytotoxic activity causes the uptake system to be impaired. However, we have shown that 15 min exposure to *N*-(4-aminobutyl)aziridine (**1**) does not unduly influence subsequent uptake of putrescine into lung slices and,

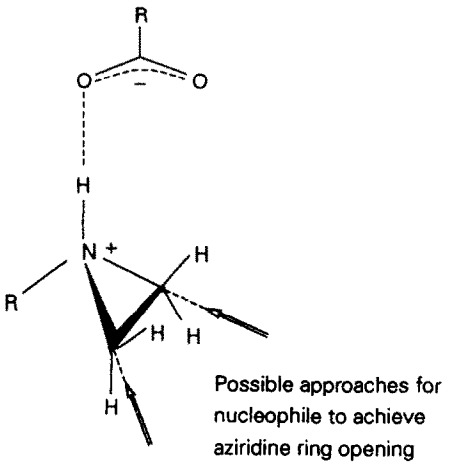


Fig. 4. Hypothesis concerning the interactions between *N*-(4-aminobutyl)aziridine (**1**) and the substrate binding site of the paraquat uptake system.

therefore, the cytotoxic activity of the aziridine (**1**) either does not damage the polyamine uptake system, or damage takes longer than 15 min to become apparent. Since the uptake studies we performed involved lung slices incubated with *N*-(4-aminobutyl)aziridine (**1**) for 30 min, this longer period needs consideration.

N-(4-Aminobutyl)aziridine (**1**) might be protonated to form an aziridinium ion near the substrate-binding site of the polyamine uptake system. Aziridinium ions are reactive towards nucleophilic species [34] and we envisaged that one of the putative carboxylate groups of the substrate-binding site would react with one of the methylene groups of the aziridinium ion, causing ring opening with concomitant formation of a covalent bond. A possible explanation for the lack of covalent binding of *N*-(4-aminobutyl)aziridine to the receptor is shown in Fig. 4. The aziridine (**1**) might fit into the substrate-binding site of the receptor in a particular conformation. Nucleophilic attack to induce the formation of a covalent bond is a S_N2 reaction and has to come from a specific direction [36]. If the substrate-binding site of the receptor does not contain any suitable nucleophiles in positions which can result in ring opening, a covalent bond will not form.

Our results for *N*-(4-aminobutyl)aziridine (**1**) and *N*,*N*′-bisaziridinyl-1,4-butane (**13**) using lung slices are in general agreement with the results published by Heston *et al.* [35] using PC-3 human prostatic carcinoma cells. They showed that the K_i for *N*-(4-aminobutyl)aziridine (**1**) against putrescine uptake was 1.0 μM, and for *N*,*N*′-bisaziridinyl-1,4-butane (**13**) was 430 μM. Both compounds displayed competitive inhibition, whereas, the apparent K_m for putrescine was 3.3 μM. They obtained K_i values for *N*,*N*-diethyl-1,4-diaminobutane and *N*,*N*′-diethyl-1,4-diaminobutane of 2.0 and 135 μM, respectively. The conclusion reached was in agreement with our observations that di-*N*-alkyl substitution of both nitrogens of putrescine produces

compounds that are much less effective competitive inhibitors of putrescine uptake than substitutions on just one nitrogen.

Porter *et al.* [37] have suggested that as cellular uptake of polyamine molecules is carrier mediated, polyamine analogues, containing antineoplastic groups, might be useful vehicles for delivering an antineoplastic function into tumour cells. Also, certain polyamine analogues, utilizing the polyamine transport system, might accumulate in cells and interfere with polyamine metabolism and function. In consequence they might act as antiproliferatives [37]. The sequence of affinities for the polyamine receptors of rat lung and the molecular structure-affinity relations developed in this paper help in the formulation of molecular structures of potential therapeutic value.

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