Inhibition of putrescine uptake by polypyridinium quaternary salts in B16 melanoma cells treated with difluoromethylornithine

Rodney F. MINCHIN,*† Roger L. MARTIN,* Lindsay A. SUMMERS† and Kenneth F. ILETT*

*Drug Metabolism and Toxicology Section, Department of Pharmacology, University of Western Australia, WA, and †Department of Chemistry, University of Newcastle, Newcastle, NSW, Australia

Several bipyridinium, tetrapyridinium and hexapyridinium quaternary salts have been found to be potent inhibitors of putrescine uptake into B16 melanoma cells which had previously been treated with difluoromethylornithine. In general, the potency of inhibitors increased as the number of quaternary centres increased. A relationship between the distance apart of the positively charged nitrogen atoms and the potency of the salts as inhibitors of uptake has been established by comparison with a number of diaminoalkanes. It was found that an inter-nitrogen distance of 0.6–0.7 nm or 1.0–1.1 nm was optimal for high activity. This finding is significant in determining structural features of the polyamine transport system.

INTRODUCTION

During normal exponential growth, B16 melanoma cells have limited capacity to accumulate extracellular polyamines. However, inhibition of polyamine synthesis *de novo* with difluoromethylornithine (DFMO) results in a substantial increase in the uptake of putrescine, spermidine and spermine [1]. Similar observations have been reported by other investigators for a variety of cell lines [2–6].

The enhanced uptake of polyamines is accompanied by an increase in the accumulation of the anti-cancer agent methylglyoxal bis(guanyldiazide) (MGBG). It has been proposed that the polyamine uptake system might provide a means of targeting tumour cells with cytotoxic agents that share the uptake system [7,8]. Using various derivatives of spermidine, Porter and associates have shown that both the charge on the terminal nitrogens and the distance between the charged centres are important determinants for transport [7,8]. An understanding of the structural requirements for the polyamine transporter could well assist in the design of new anti-cancer drugs.

Studies of the polyamine transport system in normal lung tissue have shown that diquaternary salts of 4,4'-bipyridine may be accumulated by this transporter [9]. These compounds are similar in structure to putrescine in that they contain two nitrogen atoms separated by approx. 0.7 nm. The bipyridinium salts are generally rigid planar molecules, whereas the endogenous polyamines exhibit considerable flexibility around their aliphatic backbone. We now report the results of an investigation of a number of bipyridinium, tetrapyridinium and hexapyridinium quaternary salts as potential substrates for the putrescine transport system. Interaction of these compounds with putrescine uptake in B16 melanoma cells and their ability to inhibit DNA synthesis in both untreated and DFMO-treated cells has been determined.

MATERIALS AND METHODS

Chemicals

[¹⁴C]Putrescine (101 mCi/mmol) [³H]thymidine (120 Ci/mmol) and [³H]sucrose (15 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Difluoromethylornithine (DFMO) was generously supplied by Dr. P. McCann, Merrell Dow Pharmaceuticals, Cincinnati, OH, U.S.A. Diaminoalkanes, paraquat (compound 2) and Benzyl Viologen (compound 3) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other inhibitors were synthesized by methods previously published (compounds 4–9 [10–15], compounds 10–14 [16] and compounds 15–16 [11]). All other chemicals were of analytical grade.

Putrescine uptake

B16 melanoma cells were plated at a density of 1.75×10^4 cells/cm² in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 µg of gentamicin/ml and 2.5 µg of amphotericin B/ml. The doubling time of the B16 cells is 18 h. All experiments were undertaken while the cells were in log phase growth. The cells were grown for 24 h in 5% CO₂ in air. DFMO (1 mM) was added and the cells grown for a further 24 h. Putrescine uptake was then determined by washing the cells with phosphate-buffered saline (Delbecco's Ca²⁺-free and Mg²⁺-free), incubating in serum-free medium for 60 min followed by the addition of [¹⁴C]putrescine and [³H]sucrose (extracellular marker). The final putrescine concentration was adjusted by the addition of unlabelled putrescine. After 30 min, 0.2 ml of medium was removed for quantification of ¹⁴C (CM) and ³H (HM) by liquid scintillation spectroscopy. Dual-isotope analysis was performed with a Packard TriCarb 1500 liquid scintillation analyser and counting efficiencies were determined by the external standard ratio method. The remainder of the medium in each well was removed and 0.5 ml of 1 M-NaOH was

Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanyldiazide).

† To whom correspondence should be addressed.

added to the cells for 30 min at 60 °C for digestion. Each well was neutralized by 0.5 ml of 1 M-acetic acid; a 0.5 ml aliquot of the digest was used to quantify cellular ^{14}C (CT) and ^3H (HT), while 0.1 ml was used to determine DNA content [17]. Intracellular putrescine (CI) was determined as:

$$\text{CI} = \frac{\text{CT} - (\text{HT} \times \text{CM}/\text{HM})}{\text{Putrescine sp. radioactivity}}$$

Uptake of putrescine was determined over a concentration range of 0–120 μM and preliminary studies established linearity of uptake under these conditions. Approx. 10–20% of the accumulated putrescine was converted to spermidine during the 30 min uptake period, as determined by h.p.l.c. [18]. However, since no measurable efflux occurred over the duration of the experiment, accumulated radiolabel was assumed to represent putrescine.

The various inhibitor studies were undertaken, as described above, by the simultaneous addition of inhibitor and putrescine to the cells. In initial studies, the effect of 1, 10 and 100 μM concentrations of inhibitor on the uptake of 1 μM -putrescine was measured to determine approximate potencies. A concentration of inhibitor capable of increasing the apparent K_m by 2–3-fold was then chosen to examine putrescine uptake kinetics in detail.

[^3H]Thymidine incorporation

B16 cells were exposed to each inhibitor at concentrations ranging from 0–100 μM for 24 h. Following treatment, the cells were washed and 0.5 ml of [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$) in complete medium was added to each well. The cells were incubated for 80 min at 37 °C following which they were washed twice with phosphate-buffered saline. The cells were then incubated with methanol/acetic acid (3:1, v/v) for 30 min followed by three washes with 5% trichloroacetic acid containing 1 mM unlabelled thymidine. Cells were digested in 500 μl of 1 M-NaOH at 60 °C for 30 min, neutralized with 500 μl of 1 M-acetic acid and ^3H content determined by liquid scintillation spectroscopy. Preliminary studies showed that none of the compounds studied inhibited thymidine uptake at concentrations up to 100 μM .

Data analysis

The velocities of uptake-concentration curves were fitted to the general single saturable transport model where

$$\text{velocity of uptake} = \frac{V_m \times [\text{putrescine}]}{(K_m + [\text{putrescine}])}$$

and to a model comprising saturable transport plus a non-saturable component. Model parameters were optimized by non-linear least-squares regression with data weighted with the reciprocal of the observed velocity of uptake squared. Convergence was confirmed by initiating the iterative process from at least two independent sets of initial parameter values. Models were compared using the method of Boxenbaum *et al.* [19].

The K_i for each bipyridinium compound was calculated as:

$$K_i = \frac{[I] \times K_m}{(K_a - K_m)}$$

where $[I]$ = inhibitor concentration, K_m = Michaelis-Menten constant in the absence of inhibitor and K_a = apparent Michaelis-Menten constant in the presence of inhibitor.

Molecular structures and intra-molecular distances were determined using the molecular graphics program of Henkel & Clarke [20].

RESULTS

Bipyridinium diquaternary salts

The structures of the bipyridinium compounds examined as inhibitors of putrescine transport are shown in Fig. 1(a). In the absence of inhibitor, putrescine kinetics were described by a K_m of $4.49 \pm 0.39 \mu\text{M}$ and a V_{max} of $135 \pm 11 \text{ pmol}/30 \text{ min per } \mu\text{g}$ of DNA. The inter-day variability in V_{max} (101 – $192 \text{ pmol}/30 \text{ min per } \mu\text{g}$ of DNA) necessitated that each compound be compared with its own control. K_m values, however, were highly reproducible.

Compound 1, the parent 4,4'-bipyridine, did not affect putrescine uptake. Several of the diquaternary salts inhibited putrescine transport. The salts 2, 3, 5, 6, 7 and 8 increased the apparent K_m for putrescine uptake without altering the V_{max} . Estimated K_i values ranged from $3.1 \pm 0.7 \mu\text{M}$ to $131 \pm 49 \mu\text{M}$ (Table 1). Compound 4, which contains one methoxyl quaternizing group, was inactive. This compound is reported to be unstable [10] and its lack of activity may be related to that fact. Compound 9, a 1-(4-pyridyl) pyridinium salt, did not inhibit putrescine transport at concentrations of up to 100 μM .

Tetrapyridinium and hexapyridinium salts

The structures of these compounds are shown in Figs. 1(b) and 1(c). All of the tetrapyridinium compounds exhibited classical competitive inhibition and their K_i values are shown in Table 1.

Compound 14 is not quaternized on the end nitrogens, and consequently contains only two quaternary groups. The potency of this compound ($K_i = 9.12 \mu\text{M}$) was similar to that of the bipyridinium salts. Compounds 10–13 all have four quaternary nitrogens and inhibited putrescine transport with K_i values ranging from 0.24 to 1.57 μM . The most potent of the tetrapyridinium salts were compounds 10 and 11, with two methylene groups between the bipyridine rings. Increasing the aliphatic bridge to three or four carbons significantly decreased activity, although these compounds were more potent than the bipyridinium salts.

Both hexapyridinium compounds (15 and 16) were competitive inhibitors of putrescine transport in B16 cells. Compound 16 was the most potent inhibitor tested ($K_i = 150 \pm 20 \text{ nM}$). Absence of the quaternary groups on the end nitrogens (compound 15) significantly decreased activity ($362 \pm 70 \text{ nM}$) although this compound was as potent an inhibitor as the tetrapyridinium salts.

Diaminoalkane analogues

The ability of the series $\text{NH}_2-(\text{CH}_2)_n-\text{NH}_2$ (where $n = 2$ –12) to inhibit putrescine uptake was also assessed. Only 1,2-diaminoethane failed to inhibit putrescine transport. The other analogues were competitive inhibitors with K_i values ranging from $0.63 \pm 0.09 \mu\text{M}$ for 1,7-diaminoheptane to $559 \pm 109 \mu\text{M}$ for 1,3-diaminopropane. A graph of K_i versus n is shown in Fig. 2 (K_i for

Fig. 1. Structures of the (a) bipyridinium, (b) tetrapyridinium and (c) hexapyridinium inhibitors

Table 1. Inhibition constants for the inhibitors of putrescine uptake into B16 melanoma cells

Data are expressed as means $\pm$ S.E.M. NI, no measurable inhibition.

Compound	K_i (μ M)
1	NI
2	54.4 ± 10.6
3	7.6 ± 0.9
4	NI
5	3.1 ± 0.7
6	9.8 ± 2.9
7	77.4 ± 13.4
8	131 ± 49
9	NI
10	0.24 ± 0.05
11	0.31 ± 0.03
12	1.57 ± 0.44
13	0.82 ± 0.09
14	9.12 ± 3.00
15	0.36 ± 0.07
16	0.15 ± 0.02

putrescine = K_m). The curve shows two minima at $n = 4$ and $n = 7$ corresponding to the inter-nitrogen distances of putrescine and the N^1N^8 bridge of spermidine.

Incorporation of thymidine

The ability of the bipyridinium, tetrapyridinium and hexapyridinium compounds to inhibit thymidine incor-

Fig. 2. Inhibition of putrescine uptake by diaminoalkanes

The values along the abscissa show the number of methylene groups separating the two amines. Data are presented as means.

poration was assessed in control and DFMO-treated cells. Table 2 lists the estimated ED_{50} (concentration at which 50% inhibition occurred) for each compound. There was no correlation between the ability of each analogue to inhibit polyamine transport and its ability to inhibit thymidine incorporation. In addition, treatment

Table 2. ED₅₀ values for the inhibition of thymidine incorporation into B16 melanoma cells.

Data are expressed as means $\pm$ S.E.M. NT, no evidence of inhibition of thymidine incorporation.

Compound	ED ₅₀ (μ M)	
	– DFMO	+ DFMO
2	40.4 $\pm$ 3.3	42.7 $\pm$ 8.1
3	2.79 $\pm$ 0.19	4.70 $\pm$ 1.12
4	NT	NT
5	5.87 $\pm$ 0.82	6.82 $\pm$ 1.14
6	NT	NT
7	8.83 $\pm$ 0.63	9.07 $\pm$ 1.06
8	NT	NT
9	NT	NT
10	3.99 $\pm$ 0.15	6.14 $\pm$ 0.79
11	0.54 $\pm$ 0.08	0.54 $\pm$ 0.07
12	25.1 $\pm$ 3.12	36.2 $\pm$ 1.13
13	25.3 $\pm$ 25.1	25.1 $\pm$ 5.9
14	NT	NT
15	NT	NT
16	NT	NT

of the cells with DFMO, which markedly increased polyamine transport, did not alter the ED₅₀ for any of the compounds investigated. Putrescine uptake in the DFMO-treated cells was increased by 36-fold (results not shown).

DISCUSSION

In the present study, we have shown that the transport of putrescine into DFMO-treated B16 melanoma cells was inhibited in a competitive manner by a number of bi-, tetra- and hexapyridinium salts. This study has shown that both the number of positive charges and the distance separating these charges influence the potency of the inhibitors of polyamine uptake. As the number of quaternary nitrogens increased from none (compound 1) to two (compound 2), to four (compound 10) and to six (compound 16), potency increased. This suggests that a greater number of ionic interactions between the inhibitor and the recognition site of the transporter occurs with increasing number of quaternary nitrogens. Consequently, it is implied that the recognition site comprises several negatively charged domains to which the substrates bind.

Because the bipyridinium and tetrapyrindinium salts are relatively rigid molecules, they are useful compounds for investigating possible structural features of the transporter. Extension of the normal distance of 0.7 nm separating the 4,4'-bipyridine quaternary nitrogens was achieved by addition of various groups between the pyridine rings, as seen with compounds 6, 7 and 8. Compound 6 was an effective inhibitor of uptake with the inter-nitrogen distance of 1.0 nm. Similarly, the addition of a 1,3,4-thiadiazole moiety between the pyridine rings (compound 7) extended the inter-nitrogen distance to 1.17 nm without complete loss of activity. However, when the distance separating the quaternary nitrogens was decreased to 0.42 nm in a 1-(4-pyridinium)

salt (compound 9), the compound was inactive. Additional studies with a series of diaminoalkane derivatives showed two peaks of activity corresponding to inter-nitrogen distances of 0.6–0.7 nm and 1.0–1.1 nm. These data support observations by Porter *et al.* [7], who showed that the distance separating the charged nitrogens contributed to activity, with the optimal distance being similar to that separating the N¹ and N⁸ atoms of spermidine [8].

We also examined the effects of DFMO treatment on the ability of each salt to inhibit thymidine incorporation. These experiments were undertaken as an indirect method for determining if the compounds investigated in the present study could act as substrates for the polyamine transporter. DFMO increased putrescine uptake, confirming induction of the transporter. However, the toxicities of all compounds tested were unaffected by DFMO, strongly suggesting that the polypyridinium compounds are not transported by this system. To support this conclusion, we have previously reported that compound 2 (paraquat) is not a substrate for the polyamine transport system in B16 cells [21].

It has been suggested that the polyamine transporter may provide a useful means of directing cytotoxic agents to tumour cells *in vivo* [8]. Recently, Maddox and associates [22] showed an increase in the cellular content of MGBG following DFMO therapy in patients with haematological malignancies. MGBG is known to be transported by the polyamine uptake system [6]. It is evident that the development of cytotoxic agents that are substrates for this transporter may provide a new means of treating cancer. However, the design of such drugs is dependent on an understanding of the polyamine transporter and its regulation. In this study we have examined a number of polypyridinium salts as potential substrates for the polyamine uptake system and, while many showed a high affinity for the transporter, there was no evidence to suggest that these compounds are substrates for uptake. The potent inhibition of putrescine uptake by compounds such as 10, 11, 15 and 16 indicates that the tetrapyrindinium and hexapyridinium compounds are useful probes for characterizing this transport system.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

1. Minchin, R. F., Martin, R. L., Demasi, E. & Ilett, K. F. (1987) Clin. Exp. Pharmacol. Physiol. Suppl. **11**, 118
2. Alhonen-Hongisto, L., Levin, L. A. & Marton, L. J. (1985) Cancer Res. **45**, 509–514
3. Alhonen-Hongisto, L., Seppanen, P. & Janne, J. (1980) Biochem. J. **192**, 941–945
4. Maddox, A. M., Orenco, I. & Haddox, M. K. (1987) Chemotherapy (Basel) **33**, 110–122
5. Porter, C. W., Ganis, B., Vinson, T., Marton, L. J., Kramer, D. L. & Bergeron, R. J. (1986) Cancer Res. **46**, 6279–6285
6. Rinehart, C. A. & Chen, K. Y. (1984) J. Biol. Chem. **259**, 4750–4756
7. Porter, C. W., Bergeron, R. J. & Stolowich, N. J. (1982) Cancer Res. **42**, 4072–4078

8. Porter, C. W., Miller, J. & Bergeron, R. J. (1984) *Cancer Res.* **44**, 126–128
9. Smith, L. L. (1985) *Biochem. Soc. Trans.* **13**, 332–334
10. Fielden, R. & Summers, L. A. (1974) *J. Heterocycl. Chem.* **11**, 299–301
11. McGeachie, J. & Summers, L. A. (1986) *Z. Naturforsch.* **41B**, 1255–1259
12. Dickeson, J. E. & Summers, L. A. (1969) *J. Chem. Soc. C* 1643–1645
13. Kennedy, D. A. & Summers, L. A. (1981) *J. Heterocycl. Chem.* **18**, 409–410
14. Rockley, J. E. & Summers, L. A. (1981) *Aust. J. Chem.* **34**, 2683–2686
15. Black, A. L. & Summers, L. A. (1970) *Aust. J. Chem.* **23**, 1495–1497
16. Attalla, M. I., McAlpine, N. S. & Summers, L. A. (1984) *Z. Naturforsch.* **39B**, 74–78
17. Labarca, C. & Paigan, K. (1980) *Anal. Biochem.* **102**, 344–352
18. Flores, E. H. & Galson, A. W. (1982) *Plant Physiol.* **69**, 701–706
19. Boxenbaum, H. G., Riegelman, S. & Elashoff, R. M. (1974) *J. Pharmacokinet. Biopharm.* **2**, 123–147
20. Henkel, J. G. & Clarke, F. H. (1986) *Molecular Graphics on the IBM PC Microcomputer*, Academic Press, New York
21. Minchin, R. F., Demasi, E., Martin, R. L. & Ilett, K. F. (1987) *Clin. Exp. Pharmacol. Physiol. Suppl.* **11**, 139
22. Maddox, A., Freireich, E. J., Keating, M. J. & Haddox, M. K. (1988) *Cancer Res.* **48**, 1367–1373

Received 29 December 1988/9 March 1989; accepted 7 April 1989