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Cytotoxic and non-cytotoxic *N*-alkyl derivatives of putrescine: effect on polyamine uptake and growth of prostatic cancer cells *in vitro*

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All mammalian cells have an active transport system for polyamines such as putrescine, spermidine, and spermine [1]. The chemotherapeutic agent methylglyoxal bis guanylhydrazone (MGBG) utilizes this transport system to enter the cell [2]. The ability of the cell to synthesize polyamines is inactivated irreversibly by alpha-difluoromethylornithine (DFMO) [3]. The resultant decrease in intracellular polyamines in DFMO-treated cells is associated with an increase in the uptake of exogenous polyamines via the polyamine transport system [1]. Treatment with DFMO and then MGBG increases MGBG uptake and cell death [4]. MGBG has many actions but probably acts as a cytotoxin because of its damaging effects on mitochondria [5]. We are synthesizing analogs of putrescine which are potential alkylating agents and may differ in their cytotoxic activity profiles from MGBG [6].

Materials and methods

Cells. PC-3 human prostatic carcinoma cells were obtained from the American Type Tissue Culture Collection, Rockville, MD.

Chemicals. N^1,N^1-Diethyl putrescine and N^1,N^4-diethyl putrescine were purchased from Alpha Products, Danvers, MA. Aziridinyl putrescine (AZP; 1-aziridinyl-4-aminobutane) and diaziridinyl putrescine (DAZP; 1,4 diaziridinylbutane) were synthesized according to published procedures [7, 8]. DFMO was the gift of Merrell Dow Pharmaceuticals Inc., Cincinnati, OH, through the courtesy of Dr. Peter McCann and Dr. W. J. Hudack. [^{3}H]Putrescine (28 Ci/mmol) and [^{3}H]thymidine (5 mCi/mmol) were purchased from Amersham, Arlington, IL. All other chemicals were obtained from Sigma, St. Louis, MO. All tissue culture media and additives were obtained from the media preparation facility, Memorial Sloan-Kettering Cancer Center.

[^{3}H]*Putrescine uptake.* [^{3}H]Putrescine (28 Ci/mmol) was diluted with unlabeled putrescine and incubated with or without competing putrescine analogue for 20 min at 37° with 5×10^5 cells in RPMI 1640, 10% fetal calf serum (FCS), 100 μM aminoguanidine. Following incubation with [^{3}H]putrescine, the cells were rinsed four times with Hanks' balanced salt solution (HBSS), the [^{3}H]putrescine was solubilized with 0.1 N NaOH, and the putrescine uptake was determined by scintillation counting. Michaelis constants were determined graphically from Lineweaver–Burk plots as described by Dixon and Webb [9].

[^{3}H]*Thymidine incorporation.* PC-3 cells (10^4) were plated in 12-well CoStar plates, and the next day the medium was replaced with RPMI 1640, 10% FCS, 100 μM aminoguanidine with or without 1 mM DFMO. Forty-eight hours later the medium was replaced with or without medium containing the putrescine analogues for 1 hr. The cells were rinsed twice with HBSS, and the incubation was continued with fresh RPMI 1640, 10% FCS, 100 μM aminoguanidine. The next day 0.1 μCi [^{3}H]thymidine (5 mCi/mmol) was added to each well. After 3 hr of exposure to the [^{3}H]thymidine, the cells were rinsed twice with HBSS and twice with ice-cold 5% trichloroacetic acid (TCA). The DNA was solubilized with 1 ml of 0.1 N NaOH, and a 0.2-ml aliquot was taken for scintillation counting.

Effect on cell growth. PC-3 cells 3×10^4 were plated in 62 mm petri dishes. The following day the medium was replaced with RPMI 1640, 10% FCS, 100 μM aminoguanidine with or without 1 mM DFMO. Two days later the cells were rinsed with HBSS, and the medium was exchanged for RPMI 1640, 10% FCS, 100 μM aminoguanidine with or without the putrescine analogues and incubated for 1 hr at 37°. The cells were rinsed twice with HBSS, fresh RPMI 1640, 10% FCS, 100 μM aminoguanidine, and the incubation was continued for 5 more days. The cells were lifted from the plate by trypsinization and counted with the aid of a hemocytometer. The completeness of the cell recovery was verified by inspection of the petri dish using an inverted microscope.

Results and discussion

The structural modifications of putrescine utilized in this study are depicted in Fig. 1.

Aminoguanidine (AMG) was included in these experiments because it inhibits the diamine oxidase of FCS without affecting polyamine transport, [^{3}H]thymidine incorporation, or growth of the PC-3 cells.

Figure 2a shows Lineweaver–Burk plots of putrescine uptake with and without the presence of 10 μM AZP or 1 mM DAZP. The inhibition patterns are typical of competitive inhibition. The calculated V_{max} was 280 pm per 20 min per 5×10^5 PC-3 cells. The apparent K_m for putrescine was 3.3 μM, and the K_I values for AZP and DAZP were 1 and 430 μM respectively. Figure 2b illustrates experiments with N^1,N^1-diethyl putrescine and N^1,N^4-diethyl putrescine which also resulted in competitive inhibition with apparent K_I values of 2 and 135 μM respectively. It was apparent that *N*-alkyl substitutions of just one putrescine nitrogen resulted in compounds that were competitive inhibitors of putrescine transport, and their K_I values for inhibition were the equivalent of the K_m observed for putrescine uptake. Di-*N*-alkyl substitution of both nitrogens of putrescine produced compounds that were competitive inhibitors of putrescine uptake but were at least 50-fold less inhibitory than substitutions of just one nitrogen.

In initial toxicity studies we examined the activities of these putrescine analogues for their effects on [^{3}H]thymidine incorporation into PC-3 prostatic carcinoma cells, with and without DFMO pretreatment (Table 1). Neither of the non-aziridinyl *N*-alkyl modified putrescine analogues inhibited [^{3}H]thymidine incorporation into the AMG-pretreated cells, nor did putrescine. However, at the stated concentration, both AZP and DAZP inhibited [^{3}H]thymidine incorporation into the AMG-pretreatment cells. After 48 hr of DFMO/AMG pretreatment, the rate of [^{3}H]thymidine incorporation was decreased by 69%. This is in keeping with our observation that by 48 hr these DFMO/AMG-treated cells were depleted by over 90% of their content of putrescine and spermidine and with the report that cells, whose polyamine is depleted to this extent, exhibit an increased number of cells in G_1 and a decreased number in the S phase of the cell cycle [10]. A 1-hr exposure to putrescine significantly increased the rate of [^{3}H]thymidine incorporation consistent with a natural polyamine reversing the polyamine-depleted state. However, neither N^1,N^1 nor N^1,N^4-diethyl putrescine reversed the DFMO-induced decrease in [^{3}H]thymidine incorporation. Either these diethyl analogues of putrescine were not entering the PC-3 cell or they were unable to substitute for the natural polyamines in nucleic acid synthesis. While AZP

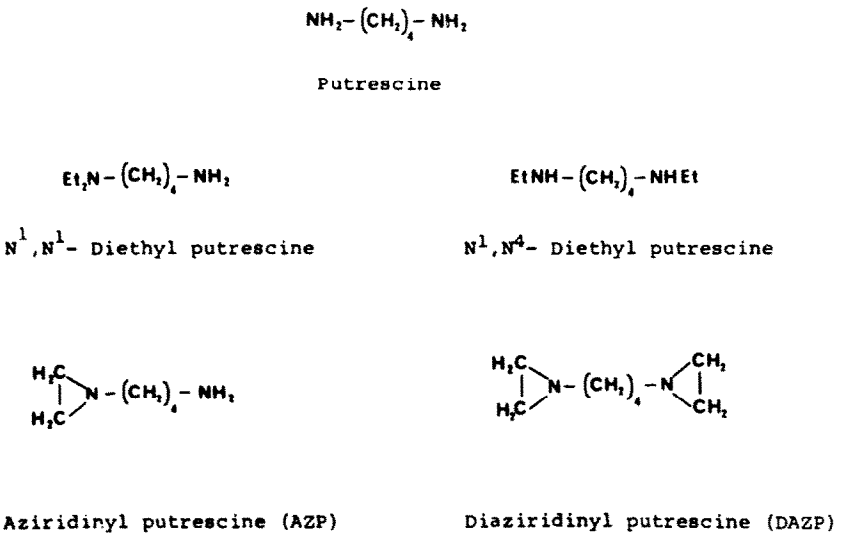

Fig. 1. Structures of the putrescine analogues utilized in this report.

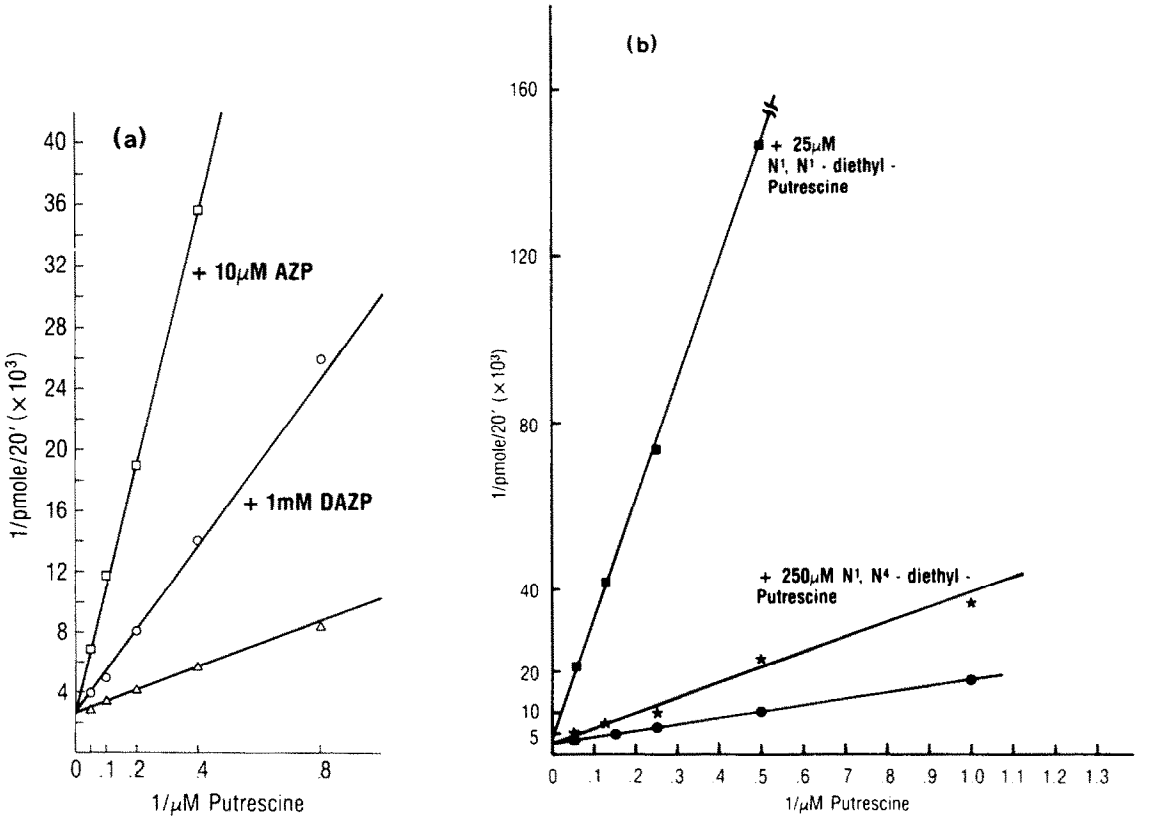

Fig. 2. Lineweaver–Burke plot of [^{14}C]putrescine uptake in (a) the presence and absence of 10 μM aziridinyl putrescine (AZP) or 1 mM diaziridinyl putrescine (DAZP), and (b) in the presence and absence of 25 μM N^1,N^1-diethyl putrescine or 250 μM N^1,N^4-diethyl putrescine.

Table 1. Effects of putrescine derivatives on [^{3}H]thymidine incorporation into PC-3 human prostatic carcinoma cells

Conditions	[^{3}H]Thymidine incorporation (cpm)	
	Pretreatment*	
	AMG (% of control)	DFMO/AMG (% of control)
No additions (control)	11,100 ± 616 (100%)	3,460 ± 183 (100%)
+500 μM N^1,N^4-Diethyl putrescine	10,200 ± 567 (92%)	3,480 ± 249 (100%)
+500 μM N^1,N^1-Diethyl putrescine	11,700 ± 920 (105%)	3,060 ± 149 (89%)
+50 μM Putrescine	11,100 ± 1,440 (100%)	7,520 ± 206† (217%)
+100 μM DAZP	5,860 ± 352† (53%)	2,810 ± 55† (81%)
+10 μM AZP	5,640 ± 614† (51%)	1,450 ± 24† (42%)

* Cells were plated in 12-well CoStar plates in RPMI 1640, 10% FCS. The following day the medium was replaced with RPMI 1640, 10% FCS, 100 μM AMG with or without 1 mM DFMO. Forty-eight hours later, the medium was replaced with fresh RPMI 1640, 10% FCS, 100 μM AMG with or without the designated putrescine analogues and incubated for 1 hr, following which the medium was removed, the cells were washed twice, and fresh RPMI 1640, 10% FCS, 100 μM AMG medium was added. Twenty-four hours later, 0.1 μCi of [^{3}H]thymidine (5 Ci/mmol) was added to each well, and the incubation was continued for an additional 3 hr. The medium was then aspirated, and the cells were washed twice with HBSS and then twice with ice-cold 5% TCA. The counts were solubilized with 0.1 N NaOH, and an aliquot was taken for scintillation counting. Values represent the mean ± SE of triplicates, and values in parentheses represent the mean value as a percentage of the respective pretreated control.

† Significantly different from their own controls ($P < 0.05$) by Student's *t*-test.

Table 2. Effects of 1-hr exposure to putrescine derivatives on the growth of PC-3 human prostatic carcinoma cells

Conditions	Cell number ($\times 10^{-4}$)	
	Pretreatment*	
	AMG (% of control)	DFMO/AMG (% of control)
No additions (control)	282 ± 17.6 (100%)	141 ± 10 (100%)
500 μM N^1,N^1-Diethyl putrescine	282 ± 26 (100%)	127 ± 33 (90%)
500 μM N^1,N^4-Diethyl putrescine	297 ± 35 (105%)	139 ± 27 (99%)
500 μM DAZP	63 ± 10† (22%)	39 ± 7† (28%)
250 μM DAZP	193 ± 11† (68%)	87 ± 5† (62%)
125 μM DAZP	232 ± 45 (82%)	114 ± 14 (81%)
50 μM DAZP	302 ± 12 (107%)	152 ± 14 (108%)
No additions	476 ± 12 (100%)	496 ± 8 (100%)
25 μM AZP	176 ± 27† (37%)	4 ± 1† (0.8%)
10 μM AZP	344 ± 18† (72%)	12 ± 3† (2.4%)
5 μM AZP	422 ± 30 (85%)	112 ± 20† (23%)
2.5 μM AZP	493 ± 54 (104%)	304 ± 21† (61%)
No additions	268 ± 28 (100%)	140 ± 8 (100%)
1000 μM Putrescine	190 ± 12† (71%)	100 ± 6† (71%)
25 μM AZP	22 ± 2† (8%)	1 ± 0.3† (0.7%)
AZP + Put	112 ± 14† (42%)	44 ± 6† (31%)
500 μM DAZP	60 ± 10† (22%)	45 ± 7† (32%)
DAZP + Put	68 ± 12† (25%)	52 ± 12† (37%)

* Cells were plated in 62-mm Corning petri dishes in RPMI 1640, 10% FCS. The following day the medium was aspirated and replaced with RPMI 1640, 10% FCS, 100 μM AMG with or without DFMO 1 mM (DFMO/AMG). Forty-eight hours later, the medium was replaced with or without the designated putrescine analogues and incubated for 1 hr at 37°. The medium was removed, the cells were rinsed twice with HBSS, and the incubation was continued for 5 more days. The medium was aspirated, the cells were lifted from the plate by trypsinization, and the total number of cells was determined by microscopic examination with the aid of a hemocytometer. Numbers equal cell number $\times 10^4$ ± SD from triplicate assays, and values in parentheses represent the mean values as a percent of the respective pretreated control.

† Significantly different from their own controls ($P < 0.05$) by Student's *t*-test.

decreased [^{3}H]thymidine incorporation in the DFMO/AMG-pretreated cells to the same extent as in AMG-pretreated cells (58 vs 49%), DAZP did not suppress [^{3}H]thymidine incorporation to the same degree in the DFMO/AMG- as in the AMG-pretreated cells (19 vs 47% respectively).

The effects of these alkyl substitutions on the growth of the PC-3 prostatic carcinoma cells are listed in Table 2. Again, the nonalkylating *N*-alkyl derivatives did not exhibit growth inhibitory activity when the PC-3 cells were exposed to a 500 μM concentration of either N^1,N^1- or N^1,N^4-diethyl putrescine for 1 hr, whether or not the cells were pretreated with DFMO. The dialkylating putrescine derivative DAZP was cytotoxic in a dose–response fashion, with 500 μM DAZP suppressing growth by 78 and 72% in AMG- and DFMO/AMG-pretreated cells respectively. DFMO pretreatment did not alter the percentage DAZP cell growth inhibition relative to the DFMO control when compared with the AMG-treated group.

AZP activity, on the other hand, was increased by DFMO pretreatment. This is consistent with AZP behaving like a polyamine and having its uptake into PC-3 cells increased by intracellular polyamine depletion by DFMO pretreatment.* If AZP were to use the polyamine transport system for its uptake into the cell to exhibit its subsequent activity, putrescine co-administration should reverse its cytotoxic activity, and putrescine did. The activity of DAZP was not reversed by co-incubation with putrescine. Usually dialkylating agents are more effective inhibitors of growth than are monoalkylating agents; however, DAZP appeared to be a much poorer compound than AZP for the polyamine transport system, and AZP was more effective as a cytotoxin at these concentrations than DAZP.

A number of structurally modified spermidine analogues have been synthesized, and the position and form of substitution on the nitrogens of the spermidine molecule have been shown to be critical for uptake and cellular polyamine-related functions [11]. This report demonstrates that the nature of *N*-alkyl modification of putrescine is also important with regard to the design of cytotoxic agents.

In summary, aziridinyl or diethyl alkyl modification of one nitrogen of putrescine did not decrease the activity of the molecule as an inhibitor of putrescine uptake. Diaziridinyl or N^1,N^4-diethyl modification of both nitrogens reduced the putrescine uptake inhibitory activity of the molecule over 50-fold relative to substitutions of just one nitrogen. Consistent with AZP behaving like putrescine and DAZP not acting like putrescine was the observation that intracellular polyamine depletion increased AZP activity. The activity of DAZP was not increased. Also, the cytotoxic activity of AZP could be reduced by coincubation with putrescine; the growth inhibitory activity of DAZP could not.

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‡*Urologic Oncology Research Laboratory and* §*Laboratory of Organic Chemistry*, *Memorial Sloan-Kettering Cancer Center*, *New York, NY 10021, U.S.A.*
WARREN D. W. HESTON†‡, KYOICHI A. WATANABE§, KRZYSZTOF W. PANKIEWICZ§

Department of Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.
DOUGLAS F. COVEY

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† Address all correspondence to: Warren D. W. Heston, PhD., Urologic Oncology Research Laboratory, Box 334, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021, U.S.A.