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1,5-Bis(4-amidinophenoxy)pentane (pentamidine) is a potent inhibitor of [^{3}H]idazoxan binding to imidazoline I_2 binding sites

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Abstract

The aromatic diamidine 1,5-bis(4-amidinophenoxy)pentane (pentamidine) is used for treatment and prophylaxis of *Pneumocystis carinii* pneumonia in patients with Acquired Immune Deficiency Syndrome. Clinical use of pentamidine has been restricted by significant toxicity, that includes hypotension, and hypoglycemia. Although clinical toxicity is well described, the mechanisms are still poorly understood. Competitive binding analyses using [^{3}H]idazoxan as the radioligand, and cirazoline to define non-specific binding, demonstrate that pentamidine binds to an imidazoline I_2 binding site on rat liver membranes with a K_i of 1.4 ± 0.22 nM. The K_i indicates that pentamidine inhibits radioligand binding at imidazoline I_2 sites with an affinity approximating the most potent known ligands and may be related to pentamidine toxicity. Moreover, pentamidine analogs inhibit radioligand binding with a range of affinities that vary according to their structure. Two candidate drugs, Compounds 5 and 6, are more active than pentamidine in the corticosteroid-suppressed rat model of *P. carinii* pneumonia, yet have different affinities for the imidazoline I_2 site (K_i 5 = 50.1 ± 1.06 nM and K_i 6 = ~ 3500 nM). Affinity for this site does not correlate with antimicrobial activity ($r = 0.60$; $p = 0.09$) or the calculated log of the octanol:water partition coefficient (ClogP) ($r = -0.38$; $p = 0.22$).

Keywords: Imidazoline I_2 receptor; Pentamidine; Hypoglycemia; *Pneumocystis carinii* pneumonia

1. Introduction

The aromatic diamidine pentamidine was first synthesized in 1939 as an alternative to the hypoglycemic agent synthalin, for use against bloodstream forms of African trypanosomes, which are solely dependent on glucose metabolism for survival (Schoenbach and Greenspan, 1948; Seed and Hall, 1992; Yorke et al., 1929). Pentamidine has been found to be active against a number of organisms including *Cryptosporidium parvum* (Blagburn et al., 1991), *Giardia lamblia* (Bell et al., 1991), and *Toxoplasma gondii* (Lindsay et al., 1991). It has been used clinically to treat African trypanosomiasis (Apted, 1980), antimony resistant leishmaniasis (Bryceson et al., 1985) and, beginning in 1958, *Pneumocystis carinii* pneumonia (Ivady and Paldy, 1958), the most prevalent opportunistic infection in patients with Acquired Immune Deficiency Syndrome. Despite its broad range of antimicrobial activity, pentamidine therapy has been limited due to a number of debilitating side effects, including hypotension, abnormal liver function, nephrotoxicity (Waltzer et al., 1974) and hypoglycemia (Murdoch and Keystone, 1983) with possible progression to insulin-dependent diabetes mellitus (Bouchard et al., 1982; Coyle et al., 1996; Liegl et al., 1994). In a recent five-year study of 106 patients, 24% developed hypoglycemia following intravenous pentamidine therapy (O'Brien et al., 1997). Although clinical toxicity has been well described, mechanisms of toxicity are still poorly understood and attempted explanations remain speculative (Assan et al., 1995; Bouchard et al., 1982; O'Brien et al., 1997).

Our laboratory has synthesized a large number of novel dicationic compounds as possible alternatives to pentamidine, striving for improved antimicrobial activity and reduced host toxicity. The compounds are tested against a variety of microorganisms, including *P. carinii*, as well as their ability to bind DNA, which is the proposed mechanism of action for the antimicrobial activity (Tidwell and

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Bell, 1993). The novel analogs of pentamidine have cationic groups as an integral part of their structure and most of the cationic groups are either amidine or imidazoline moieties. Two cation groups are necessary for binding to the organism DNA and for antimicrobial activity (Cory et al., 1992).

In the early 1980s while studying the different hypotensive effects of imidazoline compounds vs. catecholamines in the nucleus reticularis lateralis, Bousquet et al. (1984) proposed the existence of a class of imidazoline binding sites distinct from adrenergic receptors. Many subsequent studies have supported the presence of these binding sites in a variety of tissues (Coupry et al., 1987; Ernsberger et al., 1987; Meeley et al., 1986; Parini et al., 1989), including the existence of a putative endogenous ligand, (Atlas and Burstein, 1984; Li et al., 1994), and purification of a mitochondrial imidazoline binding site (Limon et al., 1992). Imidazoline binding sites do not bind the catecholamines (Coupry et al., 1987; Ernsberger et al., 1987). They are classified into two groups, I_1 and I_2, according to their affinity for clonidine and idazoxan, respectively, and are located on the plasma and outer mitochondrial membranes. The imidazoline I_2 sites are further sub-classified into I_{2A} and I_{2B} depending on their sensitivity to the compound amiloride. (For a recent review, see Bousquet, 1997). Specific physiological functions of imidazoline binding sites remain somewhat speculative. Imidazoline I_1 sites are known to play a role in hypotension, and studies have shown that insulin release is affected via blockade of a ATP-sensitive K^+ (K_{ATP}) channel by an imidazoline binding site atypical from I_1 and I_2 sites (Brown et al., 1993; Chan et al., 1993; Morgan et al., 1995).

The purpose of the current study was to determine whether pentamidine and the direct analogs of pentamidine synthesized in our laboratory as candidate antimicrobial drugs have an affinity for the imidazoline binding site. Using [^{3}H]idazoxan as the radioligand, competitive binding analyses were performed using a variety of analogs in order to develop structure–affinity relationships. The results show that pentamidine displays an affinity for the imidazoline I_2 binding site approximating that of the most potent known ligands.

2. Materials and methods

2.1. Membrane preparation

Membranes were prepared as previously described (Zonnenchein et al., 1990). Briefly, male Sprague–Dawley rats (Hilltop, Scottsdale, PA, USA) weighing approximately 250–300 g were decapitated, their livers rapidly removed and immediately placed into 0.25-M cold sucrose solution. The livers were weighed and minced with scissors before being homogenized at 4°C in solution A (0.25 M sucrose containing 0.1-mM phenylmethyl sulfonyl fluoride 1:10 v/v) for 1 min using a Biospec homogenizer (Bartlesville, OK, USA). The homogenate was centrifuged at $600 \times g$ for 10 min at 4°C. The resulting pellet was washed with solution A, discarded and the supernatant fractions were combined and centrifuged at $12\,000 \times g$ for 30 min. This pellet was washed then resuspended in cold buffer B (50-mM Tris/HCl, pH 7.4). Using a Bio-Rad (Hercules, CA, USA) assay kit, the protein concentration was determined using bovine serum albumin as the standard according to the assay of Bradford (1976). The preparation was stored at −80°C at a concentration of 20-mg protein/ml.

2.2. Saturation binding studies

Binding studies were performed in triplicate in 1-ml buffer B containing [^{3}H]idazoxan (0.1–50 nM). The addition of rat liver membranes at a final concentration of 500-μg protein/ml initiated binding, and the samples were incubated to equilibrium at 22°C for 45 min. Nonspecific binding was defined as the amount of [^{3}H]idazoxan bound in the presence of 10-μM cirazoline.

2.3. Competition binding studies

Competition experiments were performed in 1-ml buffer B containing 5-nM [^{3}H]idazoxan. Increasing concentrations of compound were added (1 nM–10 mM) and binding was initiated by the addition of rat liver membranes at a final concentration of 500-μg protein/ml. In all experiments, bound and free radioligand were separated by vacuum filtration through Whatman GF/C filters and washed with 2×10-ml cold buffer B. Radioactivity on filters was determined by liquid scintillation spectroscopy using a Packard Tri-Carb 2100TR. Samples were run in triplicate and the results were derived from at least three experiments with the exception of Compounds 4 and 8, which were run twice due to limited quantities.

2.4. Anti-Pneumocystis carinii activity

Induction, treatment and evaluation of *P. carinii* pneumonia in the rat was carried out according to methods described previously (Jones et al., 1990). To summarize the scoring procedure, the mean histologic scores were determined by two examiners using a blinded protocol. *P. carinii* cysts were counted in the stained lung sections and assigned the following values: 0.5, less than 10 cysts counted per two fully examined sections; 1, scattered cysts with less than 10% of the lung tissue involved; 2, scattered cysts with limited intense focal involvement and 10 to 25% of lung tissue involved; 3, scattered cysts with numerous intense areas of focal involvement and 26 to 50% of lung tissue involved; 4, cysts found throughout the tissue with numerous intense focal areas of involvement having greater than 50% of lung tissue involved.

2.5. Chemical synthesis

The procedure for the synthesis of 4-methoxybenzamidine was adapted from Tidwell et al. (1990). The procedure was altered in the following way: a solution of 1,4-dioxane was cooled to −10°C and presaturated with hydrogen chloride (HCl) gas. 4-Methoxybenzonitrile and anhydrous ethanol were added and the reaction mixture was saturated with HCl gas. The reaction flask was sealed and stirred at ambient temperature for twelve days. The imidate was collected and dried under high vacuum for 20 min and then dissolved in ethanolic ammonia at −10°C. The reaction mixture was stirred at room temperature overnight. The purification of 4-methoxybenzamidine was followed according to the published procedure. All other compounds were synthesized as previously described (Berger et al., 1991; Tidwell et al., 1990). Final compounds were analyzed using high-performance liquid chromatography, proton nuclear magnetic resonance and elemental analysis.

2.6. Drugs

[^{3}H]Idazoxan (specific activity 45 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA), cirazoline from Research Biochemicals International (Natick, MA, USA), phenylmethyl sulfonyl fluoride from Boehringer-Mannheim (Indianapolis, IN, USA), sucrose from Fisher (Fair Lawn, NJ, USA) and Tris/HCl from Schwartz/Mann Biotech (Cleveland, OH, USA). Benzamidine, yohimbine, phentolamine, epinephrine and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). 1,4-Dioxane and 4-methoxybenzonitrile were purchased from Aldrich (Milwaukee, WI, USA), and HCl gas and ammonia from Matheson (Secaucus, NJ, USA). All other compounds were synthesized in our laboratory. Buffers were prepared using deionized water from Dracor Water Systems (Kensington, MD, USA).

2.7. Data analysis

Binding analyses, statistics and graphics were performed using Prism 2.01 (Graphpad Software, San Diego, CA, USA). Binding parameters for inhibitors are given as mean ± S.D. except Compounds 4 and 8, which are expressed as mean ± 95% confidence intervals due to limited supply of compound. The calculated log of the octanol:water partition coefficient (ClogP) was calculated using MedChem Software 3.5 (Daylight Chemical Information Systems, New Orleans, LA, USA).

3. Results

3.1. [^{3}H]Idazoxan saturation binding in rat liver membranes

Rat liver membranes, prepared according to the method of Zonnenchein et al. (1990) were reported to be rich in imidazoline I_2 sites while excluding α_2-adrenoceptors. Saturation binding analyses reported a B_{max} of 438 ± 30 fmol/mg protein with non-specific binding representing 10–15% of total binding, which conforms with the results reported by Zonnenchein et al. (1990). [^{3}H]Idazoxan bound

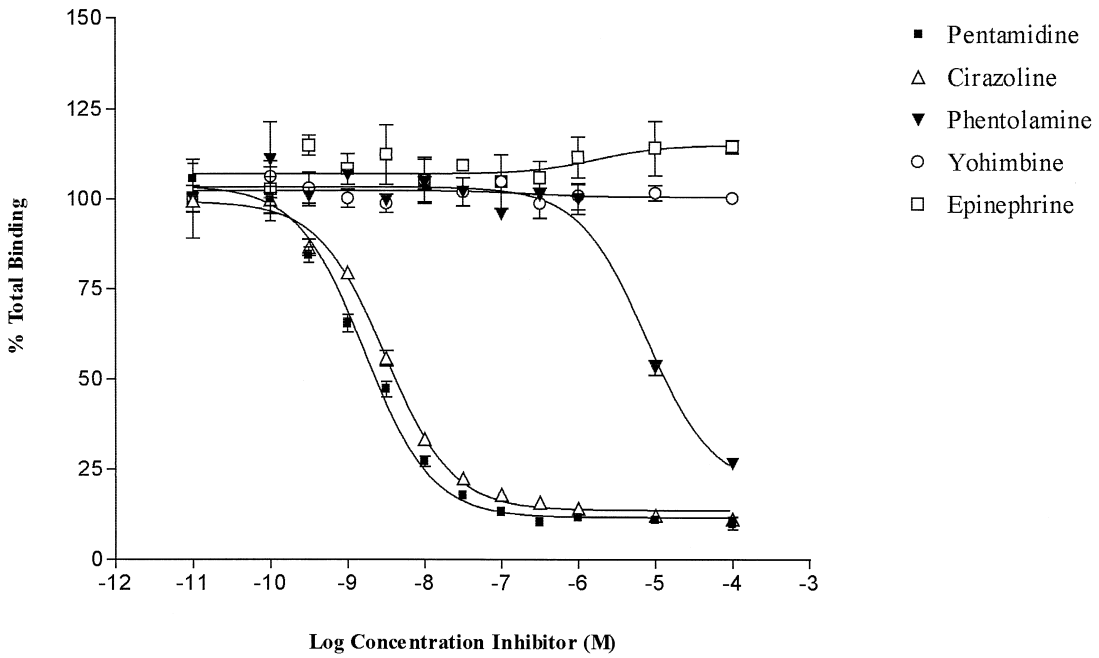
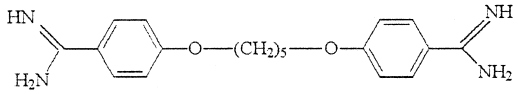
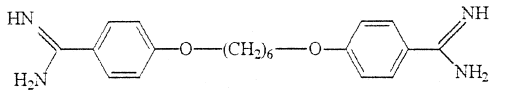
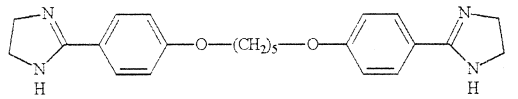
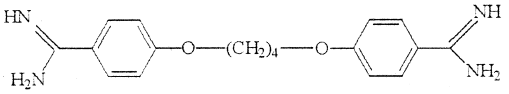
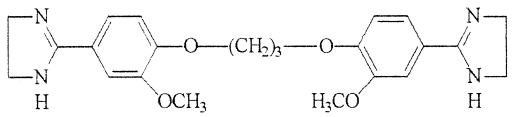
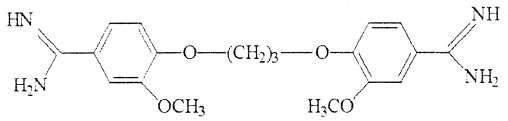
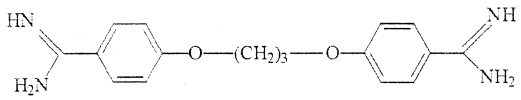
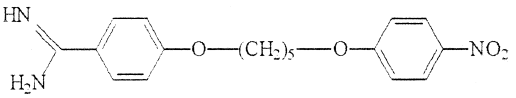
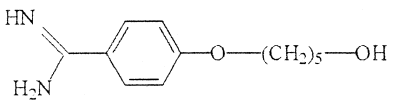
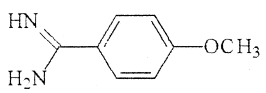
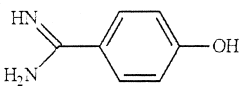
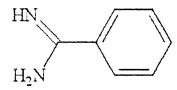


Fig. 1. Inhibition of 5-nM [^{3}H]idazoxan (K_d = 14 ± 2 nM) binding to rat liver membranes by α_2-adrenoceptor ligands and pentamidine. Cirazoline, K_i = 3.0 ± 0.4 nM; Phentolamine, K_i > 5000 nM; Epinephrine, K_i > 25 000 nM; Yohimbine, K_i > 25 000 nM; Pentamidine, K_i = 1.4 ± 0.22 nM.

the rat liver membranes with a $K_d = 14 \pm 2$ nM for saturable binding. To confirm that α_2-adrenoceptors were absent from the preparation, displacement experiments were performed using yohimbine, a selective α_2-adrenoceptor antagonist; epinephrine, a selective α_2-adrenoceptor agonist; phentolamine, an α_2-adrenoceptor antagonist; and cirazoline, an α_2-adrenoceptor antagonist as competitive inhibitors of [^{3}H]idazoxan binding. Fig. 1 shows that selec-

Table 1
Affinity for I_2 binding sites, anti-*P. carinii* activity and ClogP values for pentamidine (Compound 1) and various novel analogs

Compound	Structure	K_i (nM)	Hill Slope	Anti-PCP activity[a]	ClogP
1.		1.4 ± 0.22	-0.8	1.3	2.3
2.		3.2 ± 0.36	-1.2	0.9	2.8
3.		4.3 ± 1.09	-0.8	0.7	6.0
4.		23.4 ± 6.8	-0.8	0.6	1.8
5.		50.1 ± 1.06	-0.9	0.6	4.0
6.		~3500	-0.9	0.6	0.09
7.		27.2 ± 7.63	-0.8	1.4	1.3
8.		62.0 ± 25	-0.8	ND	3.7
9.		319 ± 39.5	-0.8	3.9	0.8
10.		228 ± 36.5	-0.8	ND	0.7
11.		~30,000	-0.3	3.7	0.08
12.		580 ± 61.3	-0.8	ND	0.6

[a] Mean Histologic Score (compared to saline control; mean histologic score saline = 3.8 out of a possible 4.0). ND = not done.
Binding in the presence of 5 nM [^{3}H]idazoxan ($K_d = 14 \pm 2$ nM). $B_{max} = 438 \pm 30$ fmol/mg protein.
K_i values are the result of at least three experiments expressed as mean $K_i \pm$ SD except Compounds 4 and 8, which were derived from two experiments due to limited quantities of compound. These results are stated as mean $K_i \pm$ 95% confidence intervals.

tive α_2-adrenoceptor ligands did not compete for radioligand binding, whereas phentolamine displaced [^{3}H]idazoxan binding with low affinity ($K_i > 5000$), and cirazoline with high affinity ($K_i = 3.0 \pm 0.4$ nM).

3.2. Competition binding studies

Fig. 1 shows a representative competitive inhibition curve using pentamidine as the inhibitor of [^{3}H]idazoxan binding. The K_i of 1.4 ± 0.22 nM demonstrates that pentamidine binds to imidazoline I_2 sites with very high affinity, comparable to that of the most potent ligands. Several interesting structure/activity relationships were observed from the data in Table 1. Taking pentamidine (Compound 1) as the lead compound, no change in affinity was observed when the central alkyl link was increased to six carbons (Compound 2) or the amidine groups replaced with imidazoline moieties (Compound 3). Moderate reductions in affinity (10–100 nM) were observed when the alkyl chain was shortened further (Compounds 4 and 7) and a similar reduction was noted when one of the cations was replaced by a nitro group (Compound 8). Loss of one benzamidine moiety (Compound 9) caused a further reduction in affinity; however, when both the amidine phenoxy moiety and four of the carbons from the alkyl chain were removed from pentamidine (Compound 10), there was no further reduction in affinity when compared to Compound 9.

Interestingly, methoxy substitution meta to the cation (Compounds 5 and 6) produced varied results depending on the nature of the cationic moiety. When compared to the unsubstituted analog (Compound 7), methoxy substitution (Compound 6) resulted in over a two log decrease in imidazoline receptor binding. However, methoxy substitution of the imidazoline analog (Compound 5) resulted in a compound with similar affinity to Compound 7. The low affinity of Compound 6 may be a result of its very low ClogP. Likewise, a comparison of Compounds 10–12 shows that Compound 11 with the lowest ClogP of all the compounds evaluated also had the lowest affinity. The low affinity of Compound 11 may be due to the existence of contributing resonance structures to form a zwitterion. The basicity and therefore the binding property of the amidines are greatly influenced by the presence of different substituents on the benzamidine ring. Both benzamidine (Compound 12) and 4-methoxybenzamidine (Compound 10) display resonance stabilization of the positive charge throughout the molecule; however, the phenoxide ion of 4-hydroxybenzamidine neutralizes the positive charge, therefore making it unavailable for receptor binding. This molecule lends support to the theory that a strong cation is an important factor in receptor binding.

3.3. ClogP

ClogP increases as compound lipophilicity increases. Table 1 shows that Compound 3 is the most lipophilic (ClogP = 6.0) and Compound 11 is the least lipophilic (ClogP = 0.08) of the compounds tested. There is no significant correlation for the group as a whole between K_i and ClogP; Pearson correlation coefficient $r = -0.38$, $P = 0.22$. However, it is interesting to note that the ClogP and K_i values for Compounds 5 and 6 differ considerably. The low affinity of Compound 6 ($K_i \cong 3500$ nM) may be attributed to its hydrophilic nature, denoted by the very low ClogP (0.09). Similarly, when Compounds 10–12 are compared, the derivative with the lowest ClogP (Compound 11) shows weakest affinity for the imidazoline I_2 site ($K_i \cong 30{,}000$ nM, ClogP = 0.08).

3.4. Anti-Pneumocystis carinii activity

Table 1 shows that pentamidine exhibits anti-*P. carinii* activity with a mean histologic score of 1.3. This is a moderately effective antimicrobial agent, having activity against a variety of organisms (Blagburn et al., 1991; Bell et al., 1991; Lindsay et al., 1991). Five of the compounds have anti-*P. carinii* activity greater than pentamidine (Compounds 2, 3, 4, 5, 6), while two of the compounds have no activity at all (Compounds 9, 11). Compounds 5 and 6 are equally effective anti-*P. carinii* agents, yet their respective affinities for the imidazoline I_2 site differ 70-fold. These compounds provide an example that affinity for the imidazoline I_2 site does not correlate to anti-*P. carinii* activity; Pearson correlation coefficient $r = 0.60$ $P = 0.09$.

4. Discussion

The series of compounds tested display a wide range of affinities for the imidazoline I_2 binding site, the affinity varying according to their structure. Compounds 1 and 3, and 5 and 6 differ only by the presence of an imidazoline ring vs. an amidine group on the aromatic ring. Since the binding sites are so named because of their affinity for ligands having imidazoline moieties, one would expect that compounds having an imidazoline ring would have a somewhat higher affinity for the binding site. It is surprising, however, that this should result in a difference in K_i of such magnitude for compounds 5 and 6, since other diamidines demonstrate high affinity for the site, and Compounds 1 and 3 appear unaffected by the change in cation groups. A comparison of the ClogP for these two compounds offers a possible explanation for the difference in binding affinities. Although there is no significant correlation between K_i and ClogP for the group of compounds as a whole ($P = 0.22$), those compounds which are hydrophilic (ClogP < 1), appear to bind the receptor to a much lesser degree. Since the binding sites are located in the lipophilic medium of the plasma and mitochondrial membranes, extreme hydrophilicity may prevent com-

pound binding to the site, suggesting a contributing factor to receptor/ligand binding characteristics.

We have determined that pentamidine has a high affinity for the imidazoline I_2 site; however, this does not address whether the compound is a selective ligand. We are currently investigating the effects of pentamidine on imidazoline I_1 sites and α_2-adrenoceptors. It is important to determine the effects of pentamidine on these receptors if the potential mechanisms of toxicity are to be elucidated. Clinical toxicity in patients receiving pentamidine therapy for *P. carinii* pneumonia includes hypoglycemia and hypotension (O'Brien et al., 1997), both of which may be attributed to binding to either imidazoline or adrenoceptors.

There is considerable evidence to support the theory that certain compounds with an imidazoline moiety can stimulate insulin secretion from pancreatic islets, which results via blockade of a K^+_{ATP} mediated by an imidazoline binding site, which is atypical from both I_1 and I_2 sites (Brown et al., 1993; Chan et al., 1993). Since pentamidine has such a high affinity for the imidazoline I_2 site, and we know that one of the more debilitating side effects of pentamidine therapy is hypoglycemia, it is reasonable to hypothesize that this toxicity is due to an interaction between pentamidine and a pancreatic imidazoline site, which regulates the K^+_{ATP} channel resulting in insulin release. It is important to study how the K^+_{ATP} channel in the pancreas is affected by pentamidine and potential antimicrobial agents in an effort to determine whether this is the mechanism which leads to hypoglycemia in the patient.

Drugs such as moxonidine and rilmenidine that are selective for the imidazoline I_1 binding site in the rostral ventrolateral medulla are promising hypotensives since they decrease arterial blood pressure, yet minimize the side effects typically observed with α_2-adrenoceptor antagonists, including sedation and dry mouth (Dubar and Pillion, 1995; Schafer et al., 1995). Pentamidine causes acute hypotension in patients receiving pentamidine therapy for *P. carinii* pneumonia, an effect which may be mediated by either the imidazoline I_1 site or adrenoceptors. By determining the effect of pentamidine on these receptors, an important step may be made in elucidating mechanisms of toxicity.

In an effort to find alternatives to pentamidine against *P. carinii* and other organisms, we are committed to synthesizing compounds which have improved antimicrobial activity, while minimizing toxic side effects. An insight into the binding characteristics and mechanisms of toxicity will enable us to design compounds which contain the appropriate moieties to attack the organism while avoiding those which interact with the host cell machinery thereby reducing the risk of toxicity. The importance of the current work is that it demonstrates that pentamidine and related compounds interact with high affinity to imidazoline binding sites in a range that would be likely to have a physiological effect and may be related to pentamidine toxicity. The levels of pentamidine administered for treatment and prophylaxis of *P. carinii* pneumonia are more than sufficient to promote binding to imidazoline sites (Anonymous, 1997). It is highly encouraging to discover that binding is not related solely to the cationic nature of pentamidine which is necessary for DNA binding, but rather it is influenced by the structure of the cation. This finding could become a valuable tool in the design of future pentamidine-related compounds as useful new antimicrobial agents.

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