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Novel Pentamidine Analogs in the Treatment of Experimental *Pneumocystis carinii* Pneumonia

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We have recently demonstrated that substitution of imidazoline moieties for the amidine groups of pentamidine produces a molecule that is effective against rat *Pneumocystis carinii* pneumonia and that is apparently less toxic than pentamidine. For this reason, 10 novel imidazoline substituted compounds were evaluated for their effect against rat *P. carinii* pneumonia. While several of the new compounds were observed to have advantages over pentamidine in the treatment of disease in the rat model, only one compound stood out as a potential new clinical agent. Treatment for 2 weeks with intravenous (i.v.) doses of 1,3-di(4-imidazolino-2-methoxyphenoxy)propane (DIMP) at 1 mg/kg per day produced an anti-*P. carinii* pneumonia effect equivalent to i.v. doses of pentamidine at 10 mg/kg per day. Although pentamidine and one of the test drugs, 1,3-di(4-imidazolinophenoxy)propane, showed no activity against *P. carinii* pneumonia when administered per os, DIMP exhibited potent anti-*P. carinii* pneumonia activity when given by daily gavage doses of 40 and 25 mg/kg. DIMP retained significant activity when given every other day by a gavage dose of 25 mg/kg. No toxicity was observed with the drug at any of the dose levels or by either of the routes of administration. However, the low solubility of the drug prevented testing at higher i.v. doses. Our conclusion is that DIMP has the potential of providing a safer and more effective alternative to pentamidine for the treatment of *P. carinii* pneumonia.

Since the 1930s the aromatic diamidine compound pentamidine has been known to be an effective agent for the treatment of parasitic infections (6, 8). Although it was first discovered to be efficacious against *Pneumocystis carinii* pneumonia in 1958 (4), numerous side effects limited the use of the drug against *P. carinii* pneumonia prior to the acquired immunodeficiency syndrome (AIDS) epidemic. Because trimethoprim-sulfamethoxazole, the drug of choice for use in patients with *P. carinii* pneumonia not associated with AIDS, was found to cause a high frequency of adverse reactions in the treatment of AIDS-related disease (3, 5), a sharp increase was seen in the reliance on pentamidine for *P. carinii* pneumonia therapy. Recent studies have shown that the toxic side effects can be greatly decreased if the drug is given by aerosol administration (7). Despite these encouraging developments, there is still an urgent need for an effective and nontoxic alternative drug that can be administered per os or by the parental route for the treatment of *P. carinii* pneumonia associated with AIDS. Recent observations by ourselves (8a) and others (10) demonstrated that replacement of the amidine groups of pentamidine analogs with imidazoline moieties results in compounds with increased anti-*P. carinii* pneumonia activity and reduced toxicity. This discovery provided guidance in the search for new anti-*P. carinii* pneumonia agents.

In the work described here, our on-going screening studies for new therapeutic compounds against *P. carinii* pneumonia (8a, 9) have been extended to include a series of 10 novel imidazoline-substituted compounds. These compounds were examined for their ability to reduce the mean histologic lung scores in *P. carinii*-infected rats when the compounds were administered intravenously (i.v.). Two compounds chosen from this study and a promising new derivative of pentamidine (butamidine) taken from a previous screening study (8a) were examined for their effects against *P. carinii* pneumonia in dose-response experiments. In addition, two compounds were tested for their therapeutic effects against *P. carinii* pneumonia when administration of the drug was per os.

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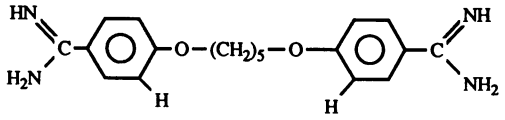
MATERIALS AND METHODS

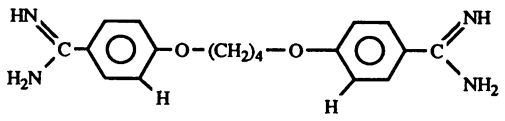
Pentamidine analogs. All of the compounds tested in this study were synthesized as the mono- or dihydrochloride salts in our laboratory by previously described methods (2). The purity of each drug was determined by high-performance liquid chromatography, elemental analysis, and proton magnetic resonance. Structures of the imidazoline derivatives are shown in Fig. 1 and Table 1, and the melting points and elemental analyses for the novel compounds are given in Table 2.

Animal protocol. The induction and treatment of *P. carinii* pneumonia was carried out with only minor alterations to previously published methods (1, 9, 10), in the following manner. Male Sprague-Dawley rats (weight, 150 to 200 g) that were barrier raised and not certified to be virus-free were obtained from Hilltop Laboratories (Scottsdale, Pa.). The individually caged animals were begun on a low-protein (8%) diet (ICN Biomedicals, Cincinnati, Ohio) and drinking water containing tetracycline (0.5 mg/ml) and dexamethasone (1.0 μg/ml) immediately upon arrival in the laboratory. This regimen was continued for the next 8 weeks, with fluid intake monitored daily and animals weighed weekly. At the beginning of week 6, animals were divided into groups of eight or more animals per group and the test compounds were administered by one of the following protocols: (i) i.v. injection at a daily dose of 10 mg/kg or the next highest soluble, nontoxic dose for 14 days, (ii) oral administration by



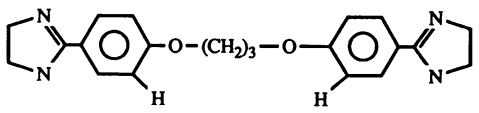
Pentamidine

1,5-Di(4-amidinophenoxy)pentane



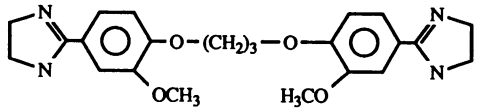
Butamidine

1,4-Di(4-amidinophenoxy)butane



DIPP

1,3-Di(4-imidazolinophenoxy)propane



DIMP

1,3-Di(4-imidazolino-2-methoxyphenoxy)propane

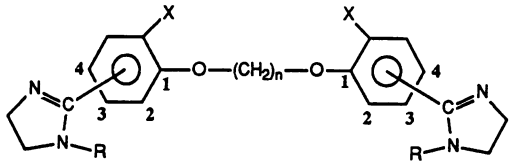
FIG. 1. Structures of pentamidine analogs.

gavage daily for 14 days, or (iii) oral administration by gavage every other day for 14 days. Saline- and pentamidine-treated groups were included as negative and positive controls, respectively.

Assessment of the drugs against *P. carinii* pneumonia. Animals were sacrificed at the end of week 8 by chloroform inhalation. The right lung was inflated in situ with 10% Formalin and fixed for histologic examination. The lung tissue was sectioned longitudinally and stained with Grocott methenamine silver stain. The Grocott methenamine silver stain selectively stains the walls of the *P. carinii* cysts. Stained sections were coded, and each section was scored by two examiners using a blinded protocol. Sections were read and scored by the following system: 0.5, less than 10 cysts counted per two fully examined sections; 1, scattered cysts with less than 10% of 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 very intense focal areas of involvement having greater than 50% of lung tissue involved.

Evaluation of toxicity. Toxicities of the test compounds were evaluated at concentrations of 10 mg/kg or the next highest soluble or nontoxic dose by the following criteria: 0, no local, clinical, or histologic toxicity; +, all animals survived the test dose without observable distress, minimal or no signs of hypotension were observed, some excess weight loss and/or mild signs of local toxicity at the injection site were noted, and no histopathology was noted; ++, all or most animals survived the test dose with marked signs of hypotension, all animals were observed to have other clinical side effects and/or some histopathology, and many animals had severe lesions at the injection site; +++, an acute toxic effect was seen after a single dose with symptoms compatible with severe hypotension and/or a sharp decrease

TABLE 1. Structures of imidazoline derivatives of pentamidine and their activities against *P. carinii* pneumonia



Compound no.	Structure component			Position of imidazoline	No. of animals with the following histologic scores[a]:					Mean histologic score	Toxicity[b]
	n	X	R		0.5	1	2	3	4		
Saline					1	2	14	41	58	3.3	0
Pentamidine					27	36	19	5	0	1.2	++
1	3	−H	−H	4	7	0	0	0	0	0.5	0
2	3	−H	−H	3	1	2	4	3	0	2.0	0
3[c]	3	$-OCH_3$	−H	4	8	5	2	0	0	0.9	0[d]
4[e]	3	$-OCH_3$	−H	3	7	3	0	0	0	0.7	+++
5	4	−H	−H	4	1	2	3	1	0	1.6	+
6[c]	4	−H	−H	3	0	0	1	4	5	3.4	+
7[c]	4	$-OCH_3$	−H	4	0	2	2	1	1	2.2	0[d]
8[c]	4	−H	$-CH_3$	4	0	1	1	3	3	3.0	++++
9	5	−H	−H	4	5	3	0	0	0	0.7	0
10[e]	5	$-OCH_3$	−H	4	1	3	1	2	0	1.6	+++
11[c]	5	−H	$-CH_3$	4	0	0	2	4	2	3.0	++++

[a] Activity was tested at 10 mg/kg unless noted otherwise.
[b] Toxicity was tested at 10 mg/kg or the highest soluble dose. See the text for an explanation of the symbols.
[c] Tested at 2.5 mg/kg.
[d] Toxicity was determined at 2.5 mg/kg.
[e] Tested at 5.0 mg/kg.

TABLE 2. Physical data of novel imidazoline derivatives of pentamidine

Compound no.	Formula	Melting point (°C)[a]	Elemental analysis (calculated/found [%])		
			C	H	N
1	$C_{21}H_{24}N_4O_2 \cdot 2HCl \cdot 2H_2O$	Dec at 106	49.47/49.21	6.13/6.28	10.99/10.80
2	$C_{21}H_{24}N_4O_2 \cdot 2HCl \cdot 1.9H_2O$	119–139	53.48/53.51	6.37/6.34	11.88/11.81
3	$C_{23}H_{28}N_4O_4 \cdot 1.75HCl \cdot 2H_2O$	252	52.69/52.59	6.49/6.38	10.60/10.46
4	$C_{23}H_{28}N_4O_4 \cdot 2HCl \cdot 2.2H_2O$	253–255	51.44/51.49	6.46/6.54	10.43/10.29
5	$C_{22}H_{26}N_4O_2 \cdot 2HCl \cdot 2.2H_2O$	249–250	53.81/53.80	6.65/6.70	11.41/11.36
6	$C_{22}H_{26}N_4O_2 \cdot 2HCl \cdot 2H_2O$	>300	54.21/53.83	6.62/6.65	11.49/11.31
7	$C_{24}H_{30}N_4O_4 \cdot 2HCl \cdot 2H_2O$	Dec at 268	52.65/52.76	6.63/6.42	10.23/10.15
8	$C_{24}H_{30}N_4O_2 \cdot 1HCl \cdot 1.5H_2O$	203–205	61.33/61.17	7.29/7.40	11.92/11.75
10	$C_{25}H_{32}N_4O_4 \cdot 2HCl \cdot 2.5H_2O$	175	52.63/52.47	6.89/6.85	9.82/9.97
11	$C_{25}H_{32}N_4O_2 \cdot 2HCl \cdot 1H_2O$	203–204	58.71/58.73	7.09/7.16	10.95/10.88

[a] Dec, Decomposes.

in the animals' health status after multiple doses, and death occurred in less than 50% of the animals, resulting in a reduced screening dose; and ++++, death occurred in greater than 50% of the animals with a resulting reduction in screening dose.

Statistical studies. Student's *t* test was used to calculate the *P* values of each test group when it was compared with the saline-treated and pentamidine-treated groups. The statistical analysis was carried out by using a software package (StatView 512+; Brainpower, Inc., Calabasas, Calif.) on a personal computer (Macintosh II; Apple Computers).

RESULTS AND DISCUSSION

Structure-activity studies. Compound structures, activities against *P. carinii* pneumonia when administered i.v., and toxicities of the 11 diimidazoline-substituted molecules are given in Table 1. The activities and toxicities of the test compounds were compared with those of the pentamidine- and saline-dosed control groups. In general, variation of the central alkyl bridge of from three through five carbons demonstrated that the compounds with an odd number of carbons in the central link produced lower mean histologic scores in the *P. carinii*-infected rats. The mean histologic scores produced by daily i.v. injections of the three- and five-carbon-chain-unsubstituted compounds (compounds 1 and 9) were 0.5 and 0.7, respectively, while dosing with the corresponding four-carbon compound (compound 5) gave a score of 1.6. Unfortunately, direct comparisons between activity and chain length variation were not possible in the methoxy-substituted compounds (compounds 3, 7, and 10) or derivatives with imidazoline groups in the 3 position (compounds 2 and 6). This circumstance was the result of the necessity of using reduced screening doses of some of the compounds because of either their high toxicities or low solubilities in saline.

The addition of methoxy groups on the aromatic ring in the position *meta* to the imidazoline moiety (compounds 3, 7, and 10) gave compounds that appeared to be more active, less soluble, and more toxic than the corresponding unsubstituted analogs. However, statistical comparisons were not possible because of differences in dosing levels. One compound (compound 3) in this series proved to be especially interesting. Compound 3 gave a mean histologic score of 0.9 at a relatively low daily dose of 2.5 mg/kg. Although no toxicity was observed at this dose, toxicity could not be evaluated at higher i.v. doses because of the low solubility of the compound. The result of moving the imidazoline moiety from the position *para* to the ether bridge to the *meta* position could only be ascertained with the three-carbon-chain analogs (compounds 1 and 2). In this case, the derivative with the imidazoline group in the *para* position (compound 1) proved to be significantly more active ($P = 0.001$) than the corresponding *meta*-substituted compound (compound 2). Substitution of a methoxy group *para* to the imidazoline moiety (compound 4) appeared to increase the

TABLE 3. Dose-response activity of butamidine versus that of pentamidine against *P. carinii* pneumonia by daily i.v. injection

Compound	Dose (mg/kg per day)	No. of animals with the following histologic scores:					Mean histologic score	Toxicity[a]
		0.5	1	2	3	4		
Saline		0	0	2	5	5	3.3	0
Pentamidine	10.0	3	5	2	1	0	1.2	++
	1.0	1	0	3	5	3	2.8	0
	0.1	0	0	0	7	5	3.4	0
Butamidine	10.0	10	2	0	0	0	0.6	0
	1.0	0	1	3	5	3	2.8	0
	0.1	0	0	4	5	2	2.8	0

[a] See the text for an explanation of the symbols.

TABLE 4. Dose-response activity of DIPP versus that of pentamidine against *P. carinii* pneumonia by daily i.v. or oral administration

Compound	Dose (mg/kg per day)	No. of animals with the following histologic scores:					Mean histologic score	Toxicity[a]
		0.5	1	2	3	4		
Saline[b]		0	0	4	14	24	3.5	0
Pentamidine[b]	10.0	6	13	8	1	0	1.3	++
	5.0	1	3	7	1	0	1.7	0
	1.0	1	0	3	5	3	2.8	0
	0.1	0	0	0	7	5	3.4	0
DIPP[b]	10.0	7	0	0	0	0	0.5	0
	5.0	2	5	3	0	0	1.2	0
	1.0	0	0	4	4	2	2.8	0
	0.25	0	1	1	5	3	3.0	0
DIPP[c]	20.0	0	1	1	6	2	2.9	0

[a] See the text for an explanation of the symbols.
[b] Administered i.v.
[c] Administered orally.

TABLE 5. Dose-response activity of DIMP against *P. carinii* pneumonia by daily i.v. administration

Compound	Dose (mg/kg per day)	No. of animals with the following histologic scores:					Mean histologic scores	Toxicity[a]
		0.5	1	2	3	4		
Saline		0	0	1	4	3	3.3	0
DIMP	2.5	6	3	2	0	0	0.9	0
	1.0	3	6	3	0	0	1.1	0
	0.5	1	2	7	2	0	1.9	0
	0.25	0	1	2	4	4	3.0	0

[a] See the text.

activity and toxicity of compound 4 relative to those of the unsubstituted analog (compound 2).

Finally, placement of a methyl group on an imidazoline nitrogen (compounds 8 and 11) produced highly toxic compounds that showed no activity at the highest nontoxic dose.

Dose-response of butamidine. A comparison of the dose-response data of 1,4-di(4-amidinophenoxy)butane (butamidine) and pentamidine is presented in Table 3. At the highest daily dose level evaluated in the dose-response experiment, 10 mg of butamidine per kg was found to be effective ($P < 0.001$) when compared with saline controls and significantly more potent ($P = 0.002$) and less toxic than pentamidine. However, reduction of the daily dose of butamidine to 1 mg/kg resulted in the loss of the drug's anti-*P. carinii* pneumonia properties. A corresponding reduction of the pentamidine dose resulted in a similar loss in activity. Close examination of the data in Table 3 reveals that the dose-response curves for pentamidine and butamidine are very similar.

Dose-response studies of DIPP. Table 4 outlines the dose-response studies with 1,3-di(4-imidazolinophenoxy)propane (DIPP). DIPP was significantly more effective than pentamidine ($P = 0.001$) in reducing the mean histologic score of *P. carinii*-infected rats with a daily i.v. dose of 10 mg/kg. As with pentamidine, the anti-*P. carinii* pneumonia activity of DIPP was greatly reduced and not statistically significantly different from that in saline-treated controls when given at a daily i.v. dose of 1.0 mg/kg. Both DIPP and pentamidine were observed to retain some activity against *P. carinii* pneumonia in rats when the daily i.v. dose was 5 mg/kg. The drug was found to be inactive against *P. carinii* pneumonia when given by gavage at 20 mg/kg per day.

Dose-response studies of DIMP. The data from the dose-response experiments for i.v. administration of 1,3-di(4-imidazolino-2-methoxyphenoxy)propane (DIMP) are given in Table 5. The data for administration of the drug per os are given in Table 6. Because of the low solubility of the drug, the highest dose administered by the i.v. route in the dose-response study was 2.5 mg/kg. The mean histologic score achieved by this dose was 0.9. The dose-response study revealed that DIMP was only slightly less active when administered at 1.0 mg/kg per day and that the anti-*P. carinii* pneumonia effect remained significant ($P < 0.001$) down to a daily dose of 0.5 mg/kg. Activity against *P. carinii* pneumonia was not significant when the daily i.v. dosage was reduced to 0.25 mg/kg. In addition to the potent activity seen with i.v. injection, DIMP was found to be highly active when it was administered by gavage at daily doses of 40 and 25 mg/kg (Table 6). No drug toxicity was observed at the highest gavage dose. Surprisingly, DIMP was observed to be nearly as active when it was administered per os at 25 mg/kg every other day for 2 weeks (Table 6).

TABLE 6. Dose-response activity of DIMP against *P. carinii* pneumonia by oral administration daily or every other day

Dosing time and compound	Dose (mg/kg)	No. of animals with the following histologic scores:					Mean histologic scores	Toxicity[a]
		0.5	1	2	3	4		
Daily								
Saline		1	0	10	9	9	2.9	0
DIMP	40.0	4	6	1	0	0	0.9	0
	25.0	4	8	3	1	0	1.2	0
	10.0	0	6	7	5	1	2.1	0
Every other day								
Saline		0	0	3	4	9	3.4	0
Pentamidine	10.0	0	1	1	4	10	3.4	0
DIMP	25.0	1	7	3	4	1	1.8	0
	10.0	0	3	8	3	2	2.3	0

[a] See the text.

In summary, our initial screening studies indicated that three compounds, the diamidine, butamidine, and the two diimidazolines, DIPP and DIMP, appear to be more effective and less toxic than pentamidine in the rat model of *P. carinii* pneumonia. The dose-response studies revealed, however, that the anti-*P. carinii* pneumonia activity of butamidine and DIPP were greatly reduced when drug dosage was decreased 10-fold. In contrast, DIMP given i.v. daily at 1 mg/kg produced an anti-*P. carinii* pneumonia effect equivalent to that of pentamidine given at 10 mg/kg per day. Significant activity remained when DIMP was given i.v. at 0.5 mg/kg per day. Furthermore, DIMP was highly effective when it was given orally, even when it was administered on an alternate-day dosing schedule. No toxicity was observed at any of the i.v. or oral doses used. Thus, the diimidazoline compound DIMP appears to be safer and more effective than pentamidine in the rat model and warrants further detailed investigations to explore its future as an alternative drug for the treatment of *P. carinii* pneumonia.

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