Small Atrial Natriuretic Peptide Analogues: Design, Synthesis, and Structural Requirements for Guanylate Cyclase Activation†

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Structure/activity studies on atrial natriuretic peptide ANP(1-28) have highlighted three portions of the native molecule as necessary for its biological responses. We have linked these three regions and excised the remaining segments to produce a family of small analogues (less than half the size of the parent) which demonstrate the full range of ANP's actions. Importantly, these compounds act at both major types of ANP receptor. Two critical modifications lead to more potent analogues; both involve expanding the cyclic portion of the molecule. Further optimization of one of these modified structures leads to A68828, a full ANP agonist which shows promise as a preventative agent against acute renal failure.

The atrial natriuretic peptides (ANPs) are a family of peptide hormones released from atrial cardiocytes in response to increases in central venous pressure.[1-3] They are intimately involved in the regulation of blood pressure and fluid volume status and, for this reason, have attracted recent attention as possible therapeutic agents.[4,5] ANPs (Figure 1) are known to have a variety of microscopic and macroscopic actions; they stimulate sodium and water excretion by the kidneys, cause a shift of fluid from the circulation into extravascular space, enhance the production of guanosine cyclic monophosphate (cGMP) by the membrane-bound form of guanylate cyclase while lowering intracellular adenosine cyclic monophosphate (cAMP) levels, inhibit aldosterone biosynthesis, and oppose the actions of the renin–angiotensin system.

This complex set of responses is mediated through a number of different receptors.[6-11] In particular three major classes of peripheral ANP receptors have been closely studied. ANP-R1, the high molecular weight or type A receptor, has been shown to be functionally coupled to the production of cGMP. There is strong physical evidence for this linkage; the receptor has been sequenced and found to consist of an extracellular binding domain and an intracellular domain which bears close homology to soluble guanylate cyclase.[12] ANP-R2, the C-receptor, is also membrane-bound but contains only a vestigial intracellular domain. This receptor, which predominates in most cell lines, has been suggested by Lewicki et al. to serve a clearance function;[13] it has been tentatively linked to both phosphoinositide (PI) turnover[14] and the adenylate cyclase/cAMP system[15] via guanine nucleotide binding proteins. More recently McEnroe et al.[16] have reported the identification of a second guanylate cyclase-coupled receptor, localized in smooth muscle cells. This B-receptor is selectively activated by members of the C-type natriuretic peptide family,[17] which are homologous to but not identical with ANPs.

The initial reports of ANP's actions prompted a number of groups to initiate synthetic studies aimed at reducing the size of the native peptide. In general these efforts have met with limited success. While some truncation of the N- and C-termimi is permitted, and can even lead to superactive analogues,[46] further deletions result in substantial decreases in biological activity[18]. In particular the disruption of[47] or removal of residues from within[19,20] the disulfide-enclosed ring has a profound impact on the

† Abbreviations for unnatural amino acids: Amcca, *trans*-4-aminocyclohexanecarboxylic acid; Aoa, 8-aminooctanoic acid; apa, 5-aminopentanoic acid; β-Ala, 3-aminopropionic acid; Cha, cyclohexylalanine; Mapaa, *m*-aminophenylacetic acid; Mpa, 3-mercaptopropionic acid; Naa, 2-naphthylacetic acid; Papaa, *p*-aminophenylacetic acid; Sar, sarcosine = *N*-methylglycine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

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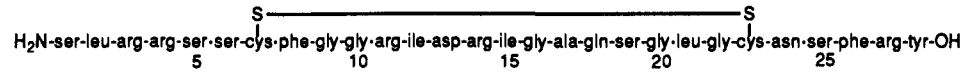

Figure 1. Atrial natriuretic peptide (rANP[1-28]).

binding characteristics and vasorelaxant properties of the analogues. Notable exceptions to these general rules are found in the work of Scarborough and co-workers at California Biotechnology,[21,22] and also of Bovy et al. at Searle.[23] These groups have reported that a class of small peptides containing residues 8–15 of ANP bind potently and selectively to the C-type receptor. These "C-peptides" are reported to exhibit some ANP-like activity in vivo, presumably because of their ability to elevate circulating ANP levels by displacing the native peptide from this "clearance receptor". However, they do not act directly at the critical cGMP-coupled receptors and so cannot function as full ANP agonists.

In this report, we describe the results of our own studies directed toward the development of small atrial peptide analogues. We have uncovered several families of compounds which, although less than half the size of ANP[1–28], have affinity for both cyclase-coupled and "clearance" ANP receptor subtypes. As expected, these compounds are efficacious in a variety of assays measuring ANP-like activity; in particular they are able to induce dramatic increases in cGMP levels in vitro and in vivo. We have performed structure–activity studies that have enhanced our understanding of ANP's mode of action and also have aided in the design of more potent analogues. One such analogue, A68828, has been evaluated as an ANP agonist in vivo in the dog.

Chemistry

The peptides described in this study were prepared by solid-phase peptide synthesis methodology using Boc Chemistry and a standard side chain protecting-group strategy. All protecting groups were cleaved simultaneously and the peptide was removed from the resin support by treatment with anhydrous hydrogen fluoride containing 10% anisole as a cation scavenger. The crude linear peptides were cyclized by iodine oxidation at high dilution at neutral pH. After desalting by passage through an XAD-16 adsorption column, the cyclized products were purified by reversed-phase HPLC.

The homogeneity of the peptide products was established by analytical HPLC; their identity was confirmed through a combination of NMR, FAB-MS, and amino acid composition and/or sequence analysis.

Table I. Vasorelaxant Effect of Small Linear ANP Fragments[a]

fragment	intrinsic vasorelaxation, 10^{-4} M
rANP[1-28]	1.00 ($EC_{50} = 4 \times 10^{-10}$ M)
[5-9][b]	0.03
[5-11][c]	0.00
[8-15]	0.57
[11-16]	0.70
[17-27][c]	0.00
[24-27]	0.16

[a] Peptides are tested in a standard tissue bath prep using rabbit aortic rings preconstricted with histamine. [b] Cysteine blocked by carboxymethyl (Cm) group. [c] Cysteine blocked by acetamidomethyl (Acm) group.

Table II. Vasorelaxant Effect of Small Cyclic ANP Analogues[a]

	analogue	vasorelaxant potency: EC_{50}, nM
A61772	ANP[7–15]Cys	3800
1	ANP[5–15]Cys	5200
2	ANP[7–15]Cys-Phe-Arg	4900
A62555	ANP[5–15]Cys-Phe-Arg	3000
3	ANP[5–15]Cys-Asn-Ser	>10000

[a] Dose-response curves are recorded over a peptide dosing range from 10^{-8} to 10^{-4} M using rabbit aortic rings preconstricted with histamine.

Biology

Because of the complexity of the in vitro and in vivo effects of the atrial peptides, a number of different biological assay systems were employed to establish and confirm the ANP-like activity of our analogues. Our primary screening assay measured the peptides' ability to displace radiolabeled ANP from rabbit adrenal plasma membranes, which contain ANP(A) and ANP(C) receptors. We have also evaluated our compounds for the ability to stimulate cGMP formation, a critical marker of ANP action,[24,25] in bovine aortic endothelial cells (BTAEC). The stimulation of guanylate cyclase in these cells is taken as an indication of A-receptor activation.[16] Vasorelaxant potency was evaluated in a standard tissue bath prep using rabbit aortic rings. We have studied the hypotensive and natriuretic/diuretic effects of a selection of our peptides in the rat, using either bolus injections or the stepped-dose

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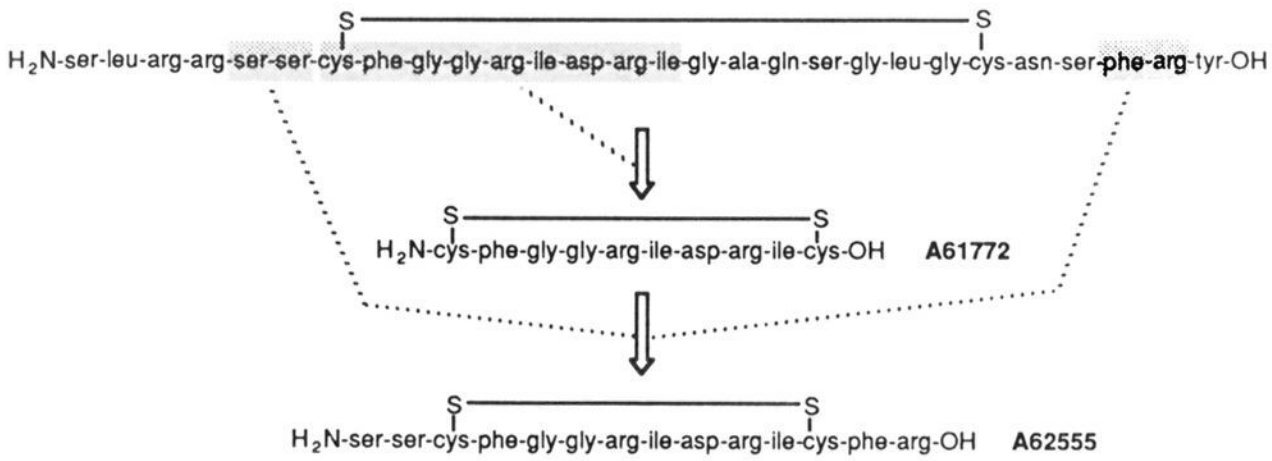

Figure 2. Three critical regions of ANP[1–28] are joined together to give the small ANP analogue A62555 = ANP[5–15]Cys-Phe-Arg.

infusion protocol that we have previously described.[26] A more extensive study of the actions of A68828 was performed in the dog.

Results and Discussion

Development of a Small Peptide Lead. Our earliest efforts were directed at identifying a small linear fragment of ANP capable of mimicking the biological activities of the parent peptide. Such localization of function has been observed in a number of other peptide systems. To this end we prepared a series of linear fragments of varying lengths spanning the ANP sequence. These linear peptides (Table I) were evaluated for their ability to relax preconstricted aortic smooth muscle. All of the compounds were much less active than ANP itself in this assay. Several fragments, however, show some modest intrinsic vasorelaxant potential. The two most "active" of these fragments lie within the Phe8–Gly16 region of the native molecule.

While the above results argued against a convenient localization of ANP-like function, they did highlight residues 8–16 as a region of potential interest. Like the CalBio and Searle groups, we focused on this [8–16] region as the critical portion of the ANP ring. We speculated that these residues might represent one of several loops formed by the cyclic portion of the molecule upon interaction with its receptor. In order to mimic the spatial orientations within such a loop, ANP[8–15] was cyclized by including cysteine-7 from the native sequence and appending a second cysteine at the end of the chain. The resulting analogue, A61772 = ANP[7–15]Cys, is 30× more potent as an in vitro vasorelaxant agent than ANP[8–15] (Table II). A61772 does not function as an ANP agonist in vivo, however. In fact, a 300 μg/kg bolus of the peptide raises blood pressure 30% in the rat (Figure 3). The origin of this hypertensive effect is obscure.

In order to provide our peptides with the full range of ANP-like activities, we found it necessary to attach fragments from the N- and C-termini of the native hormone onto A61772 (Figure 2). A seryl–seryl dipeptide fragment was first appended to the N-terminus; this fragment was chosen by analogy with APIII = ANP[5–28], a naturally occurring active ANP analogue.[27] The C-terminus was also extended by the addition of a dipeptide, in this case a Phe–Arg derived from the carboxy terminus of ANP. The resulting analogue, A62555 = ANP[5–15]Cys-Phe-Arg, is similar in potency to A61772 in the in vitro vasorelaxant

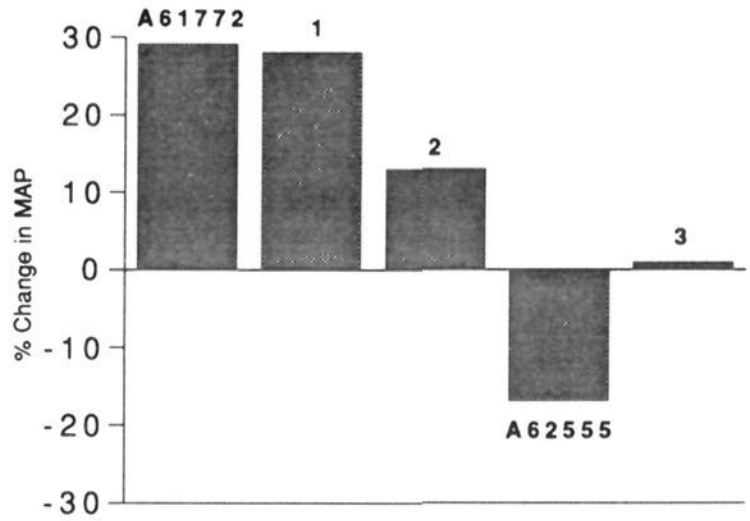

Figure 3. Blood pressure effects of small ANP analogues. Male Sprague-Dawley rats received 300 μg/kg bolus injections of each test peptide related to A61772. Only A62555, containing both N- and C-terminal tails, gave the desired hypotensive effect.

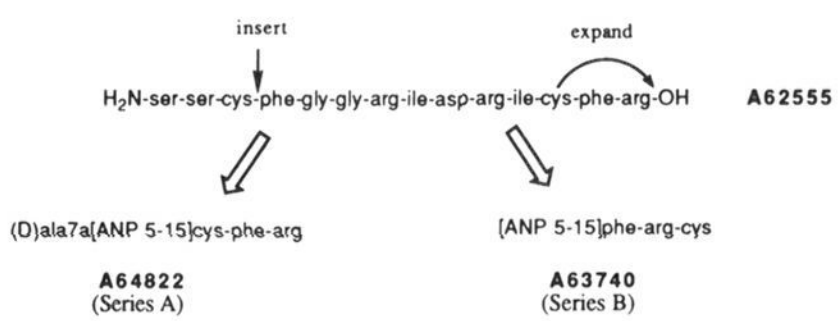

Figure 4. Modifications which improve activity of small ANP analogues.

assay (Table II); it now produces the expected hypotensive effect in vivo (Figure 3). It appears that both of the tail fragments are required to produce the desired in vivo response; **1** and **2**, which contain only the N- and C-terminal fragments, respectively, both give pressor responses similar to A61772 upon bolus injection. Our results are consistent with the suggestion of Murad et al.[28] that the Phe–Arg fragment seems to be the critical portion of the ANP carboxy-terminal tail; compound **3**, in which the Asn–Ser dipeptide replaces Phe–Arg, has no effect on blood pressure.

Modifications That Improve Binding and in Vivo Potency. With A62555 taken as an important lead, our initial efforts centered around improving the potency and protease stability of this new class of ANP analogue. We have described the results of our preliminary studies of the structure/activity relationships within this class of molecules.[29,30] Ironically, several unsuccessful attempts at

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Table III. The Effect of "Stabilizing" Substitution on Small ANP Analogues[a]

	compound	EC_{50}, nM (rabbit aorta)	K_L, nM (rabbit adrenal)	stability: $t_{1/2}$,[b] min
A62555	[ANP5–15]Cys-Phe-Arg	3000	2000	4.7
4	D-Phe8[ANP 5–15]Cys-Phe-Arg	>10000	4400	5.8
A64822	D-Ala7a[ANP 5–15]Cys-Phe-Arg	360	710	5.3
5	Gly7a[ANP 5–15]Cys-Phe-Arg	120	410	3.2
6	D-Val7a[ANP 5–15]Cys-Phe-Arg	24	130	
7	βAla7a[ANP 5–15]Cys-Phe-Arg	1800	6500	
8	D-Cha7a[ANP 5–15]Cys-Phe-Arg	430	180	
9	Sar7a[ANP 5–15]Cys-Phe-Arg	>1000	410	
A63740	[ANP 5–15]Phe-Arg-Cys	2000	790	<2

[a] While "7a" substitutions (A64822 and peptides 5–9) and C-terminal internalization (A63740) have relatively little effect on the compounds' stability in a kidney homogenate, they do improve potency in the binding and vasorelaxant assays. [b] Reference 31.

stabilization[31] led to the discovery of two new, more active series of compounds (Figure 4).

Our early studies of ANP metabolism suggested that the enzyme primarily responsible for degrading the native peptide is neutral metalloendopeptidase EC 3.4.24.11, or "enkephalinase". This result has subsequently been confirmed by other workers,[32-34] and inhibitors of this enzyme are presently being studied as ANP potentiators.[35-37] The peptidase cleaves preferentially on the amino-terminal side of hydrophobic residues; thus, one major site of degradation is the Cys-7–Phe-8 bond. Substitution of a D amino acid at this junction might be expected to stabilize A62555 against similar proteolysis. However, residue 8 has been demonstrated[38] to be critical for agonist activity; accordingly, peptide 4, with a D-Phe8 substitution, is significantly less active than A62555. Insertion of a D amino acid *between* residues 7 and 8, on the other hand, provides an analogue (A64822) that is 1 order of magnitude more potent as a vasorelaxant than the parent. This fortuitous result is apparently *not* due to an increase in stability, however. The in vitro half-life of A64822 in a crude homogenate of rabbit renal cortex, a rich source of the neutral endopeptidase ($t_{1/2}$ = 5.3 min, Table III) is not appreciably different from that of A62555 ($t_{1/2}$ = 4.7 min). Other D amino acid substitutions at position "7a" yield potent analogues (compounds 5–9, Table III). Insertion of glycine provides a similar result, suggesting that the added residue may be allowing flexibility in the ring as well as introducing a favorable conformational bias. Consistent with this suggestion, replacement of the 7a substituent by either sarcosine (*N*-methylglycine) or β-alanine (3-aminopropionic acid) increases potency, but to a lesser degree.

The C-terminus of A62555 contains several possible protease sites as well. In one attempt at stabilizing this

Table IV. Biological Activities of Series A ("7a Substituted") Small ANP Analogues in a Binding Assay (Rabbit Adrenal Membranes)[a]

	compound	K_L, nM
	ANP5–28	2.2
A64822	D-Ala7a[ANP 5–15]Cys-Phe-Arg	710
10	D-Ala7a[ANP 7–15]Cys-Phe-Arg	2700
11	Lys6,D-Ala7a[ANP 6–15]Cys-Phe-Arg	390
12	Arg6,D-Ala7a[ANP 6–15]Cys-Phe-Arg	240
13	Apa6,D-Ala7a[ANP 6–15]Cys-Phe-Arg	280
14	Aoa6,D-Ala7a[ANP 6–15]Cys-Phe-Arg	790
15	Gly7a[ANP 5–15]Cys-Pro-Arg	440
16	D-Ala7a[ANP 5–15]Cys-L-Tic-Arg	540
17	D-Ala7a[ANP 5–15]Cys-D-Tic-Arg	960
18	Gly7a[ANP 5–15]Cys-Phe-Gly	3100
19	Gly7a[ANP 5–15]Cys-Phe-Lys	5900
20	Gly7a[ANP 5–15]Cys-Phe-Harg	320
21	Gly7a[ANP 5–15]Cys-D-Phe-Arg	780
22	Gly7a[ANP 5–15]Cys-Phe-D-Arg	620
23	D-Ala7a,Trp8[ANP 5–15]Cys-Phe-Arg	720
24	D-Ala7a,(NMe)Phe8[ANP 5–15]Cys-Phe-Arg	290
25	D-Ala7a,$(MeO)_2$Phe8[ANP 5–15]Cys-Phe-Arg	13000
26	D-Ala7a,Tyr8[ANP 5–15]Cys-Phe-Arg	10000
27	D-Ala7a,Papaa9,10[ANP 5–15]Cys-Phe-Arg	1400
28	D-Ala7a,Mapaa9,10[ANP 5–15]Cys-Phe-Arg	810
29	D-Ala7a,Amcca9,10[ANP 5–15]Cys-Phe-Arg	1000
30	D-Ala7a,Ser13[ANP 5–15]Cys-Phe-Arg	510
31	D-Ala7a,Gly13[ANP 5–15]Cys-Phe-Arg	740
32	D-Ala7a,Ala13[ANP 5–15]Cys-Phe-Arg	1000
33	Gly7a,Asp(OMe)13[ANP 5–15]Cys-Phe-Arg	1400
34	D-Ala7a,Asn13[ANP 5–15]Cys-Phe-Arg	540
35	D-Ala7a,Arg13,Asp14[ANP 5–15]Cys-Phe-Arg	7800
36	D-Ala7a,Cit11[ANP 5–15]Cys-Phe-Arg	6900
37	D-Ala7a,Ala14[ANP 5–15]Cys-Phe-Arg	7100
38	D-Ala7a,Leu12,15[ANP 5–15]Cys-Phe-Arg	680

[a] K_L measures binding at the low-affinity site, presumably ANP-R1.

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Table V. Biological Activities of Series B ("Phe-Arg-Cys") Small ANP Analogues in a Binding Assay (Rabbit Adrenal Membranes[a])

	compound	K_L, nM
A63740	[ANP 5–15]Phe-Arg-Cys	790
39	Arg6[ANP 6–15]Phe-Arg-Cys	660
A68828	Arg6,Cha8[ANP 6–15]Phe-Arg-Cys-NH_2	140
40	Ac-Arg6,Cha8[ANP 6–15]Phe-Arg-Cys-NH_2	370
41	His6,Cha8[ANP 6–15]Phe-Arg-Cys-NH_2	190
42	Mpa7,Cha8[ANP 7–15]Phe-Arg-Cys-NH_2	300
43	Arg6,Cha8[ANP 6–15]Ala-Arg-Cys	280
44	Arg6,Cha8[ANP 6–15]L-Tic-Arg-Cys-NH_2	270
45	Arg6,Cha8[ANP 6–15]D-Tic-Arg-Cys-NH_2	96
46	Arg6,Cha8[ANP 6–15]Gly-Ala-Cys-NH_2	1200
47	Mpa7,Cha8[ANP 7–15]D-Phe-Arg-Cys-NH_2	6500
48	Mpa7,Cha8[ANP 7–15]Phe-D-Arg-Cys-NH_2	380
49	Mpa7,Cha8[ANP 7–15]Phe-Arg-D-Cys-NH_2	1900
50	Cha8[ANP 5–15]Phe-Arg-Cys	160
51	Arg6,Cha8[ANP 6–15]Phe-Arg-Cys	460
52	Arg6,(I_2)Tyr8[ANP 6–15]Phe-Arg-Cys-NH_2	1700
53	Arg6,Cha8,D-Ala9,Ala10[ANP 6–15]Phe-Arg-Cys-NH_2	200
54	Arg6,Leu13[ANP 6–15]Phe-Arg-Cys	680
55	Arg6,Cha8,Gly13[ANP 6–15]Phe-Arg-Cys-NH_2	260
56	Mpa7,Cha8,Glu13[ANP 7–15]Phe-Arg-Cys-NH_2	430
57	Arg6,Cha8,Cyclo(Lys11–Asp13)[ANP 6–15]Phe-Arg-Cys-NH_2	58
58	Mpa7,Cha8,Lys11[ANP 7–15]Phe-Arg-Cys-NH_2	150
59	Cha8,Ala14[ANP 7–15]Phe-Arg-Cys-NH_2	8900
60	Arg6,Cha8,Phe12[ANP 6–15]Phe-Arg-Cys-NH_2	680
61	Arg6,Cha8,Ala15[ANP 6–15]Phe-Arg-Cys-NH_2	230
62	Cha8,Ala15[ANP 7–15]Phe-Arg-Cys-NH_2	800

[a] K_L measures binding at the low-affinity site, presumably ANP-R1.

region of the molecule, the C-terminal cysteine was moved to expand the ring and enclose the Phe-Arg dipeptide (Figure 4). While the resultant analogue, A63740 = [ANP 5–15]Phe-Arg-Cys, is even less stable to proteases than the parent ($t_{1/2}$ < 2 min), it binds better and is a more potent vasorelaxant agent (Table III). It also proves (vide infra) to be a useful scaffold on which to build further improvements. This increase in potency may again be due to an increase in conformational flexibility resulting from the ring expansion. The conformational freedom in the ring has been significantly restricted by the removal of seven residues from the native sequence; a slight "loosening" of this ring appears to be beneficial. A63740 demonstrates that ring expansion may be accomplished without increasing the overall size of the peptide.

Structure/Activity Studies. Amino acid replacement studies involving both of the lead series were undertaken concurrently. In general these studies paralleled each other; however, some structure/function correlations were unique to each series.

Amino Terminus. Our initial results with A62555 and peptide **2** suggested that the N-terminal region plays an important role in determining the binding activity of the small peptide analogues. An extensive study of this region, using the "D-Ala" (series A) and "FRC" (series B) peptides as starting templates, suggests that this is not necessarily the case (Tables IV and V). A variety of substitutions in this region led to analogues with similar binding affinities. In fact, in series B the N-terminal region may be removed entirely (e.g. compound **42**) with relatively little deficit. These observations are consistent with the results of earlier studies on the full-sized peptide.[18,19] There is some tendency for charged amino acids (e.g. Arg, His) to provide a significant boost in binding (peptides **12** and **41**).

The N-terminal region has more dramatic effects on the in vivo actions of the peptides. In particular, the compounds in Tables IV and V vary significantly in the number of cationic residues they contain (i.e. three positive charges on peptide **42** at pH 7, four on A63740, and five for **41**). It is known that highly positively charged compounds can trigger histamine release from mast cells; our group has reported[39] that atrial peptide analogues with greater numbers of basic residues (e.g. ANP[1–28], six positive charges at pH 7) similarly act as histamine secretagogues. In vivo we find that these highly charged analogues tend to produce sudden and dramatic decreases in blood pressure when infused at higher concentrations in rats. This sudden hypotension, which is reminiscent of side effects reported during clinical trials of ANP[1–28], may be due to histamine release. Replacement of the charged residues in the N-terminal region may offer some control over these parameters.

Ring Residues. Early SAR studies on large ANP derivatives[38,40] suggested the importance of Phe-8, and led us to study a number of substitutions at this position. While tryptophan (peptide **23**) is an acceptable replacement, oxygenated aromatics (**25** and **26**) consistently decrease potency in the binding assay. N-Methylation (**24**), which stabilizes the Cys–Phe bond against protease action, improves potency slightly. It appears that the Phe pocket contains hydrophobic, as opposed to aromatic, recognition elements; replacement with cyclohexylalanine also increases binding affinity.

Attempts to rigidify Gly-9,10, presumably a spacer region, met with limited success. A variety of linear and slightly bent spacers (**27–29**) are tolerated; none provides a noticeable advantage. The D-Ala-9, Ala-10 combination previously reported by Williams et al.[41] is also acceptable (compound **53**) but offers no advantage over the native sequence.

Systematic replacement studies on ANP[5–28][38] have suggested that the basic arginine residues in the ring are relatively inviolate, though an early study by Nutt and coworkers[40] demonstrated that Arg-11 may be substituted by alanine in ANP[3–28]. We find that replacement of Arg-11 by citrulline (**36**) or of Arg-14 by alanine (**37** and **59**) reduces activity dramatically in our smaller series. Lysine is tolerated at position 11 (**58**), suggesting that a charge may be the key feature of this residue.

For Asp-13 the results are less straightforward. Replacement of the carboxylate moiety (**33** and **34**) or removal of the entire sidechain (**31**, **32**, and **55**) has relatively little effect on the potency of an analogue in the binding assay. Despite this apparent insensitivity to substitution at this position, Asp-13 may be involved in a conformation-restricting hydrogen bond with Arg-11. We find that replacement of residues 11–13 with the H-bond mimic cyclo-[Lys-Ile-Asp] provides a substantial boost in binding potency (compound **57**). Our results provide the first example of a transannular cyclization which improves the activity of an ANP analogue. On the other hand, switching residues 13 and 14 to test for a 13–14 salt bridge gives the very weak **35**.

None of the peptides substituted at position 13, however, shows a significant level of in vivo activity. A similar loss of functional activity was first noted in a larger series by Balasubramanian et al.,[45] although other groups have re-

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ported conflicting results.[27,38] We have determined that Asp-13 is critically involved in the signal transduction process for the guanylate cyclase-coupled receptor; in fact, compounds perturbed at this amino acid act as ANP antagonists. We have examined this effect in some detail, and have reported the results of these studies elsewhere.[26]

Carboxy Terminus. The essential difference between series A and series B analogues, as described above, centers on the positioning of the critical carboxy-terminal dipeptide. This difference results in some divergence in activity upon C-terminal substitution. Series A, with the C-terminus placed outside of the ring, is relatively inert to D amino acid replacement in this region (**21** and **22**), suggesting that the backbone of the tail is free to accommodate the conformational changes enforced on the molecule upon receptor binding. In series B, with this tail internalized, such conformational changes necessarily have an effect on the rest of the ring. Stereochemical inversion in the C-terminal region has much more dramatic effects in this series (**47–49**). In particular the accommodation of the D-phenylalanine side chain seems difficult. On the other hand, replacement of arginine, with its flexible tether linking the guanidine moiety to the backbone, by D-Arg is accomplished without a loss in binding potency.

Because of the importance of Phe-8 as a binding residue, we anticipated that replacement of the C-terminal Phe would also have a significant effect on binding. Surprisingly these effects are quite minimal; even complete removal of the aromatic group (**15** and **43**) has relatively little impact. As in the case of Asp-13, these Phe-substituted analogues show reduced activity in vitro, in the cGMP assay, and also as in vivo agonists; in fact, they act as potent antagonists of ANP-induced cGMP stimulation, and also of ANP-induced natriuresis. In-depth studies have shown that these two residues are jointly involved in the ANP/cGMP signal transduction process.[26]

The terminal arginine, on the other hand, behaves in a straightforward fashion as a binding element. Removal of the side chain (**18**) or replacement with lysine (**19**) decreases activity 7- and 14-fold, respectively, suggesting not only that the residue is required, but that the guanidine moiety is critical. The latter result is *not* simply an effect of chain length; when the lysine ϵ-amine is converted to guanidine (**20**) the activity returns. Other workers have noted the importance of this terminal arginine residue; the Merck group has suggested that a reverse turn in the C-terminal domain may position the guanidine moiety near the hydrophobic residues of the central core in the native peptide.[49]

Effects on Cyclic GMP. In an attempt to understand the origin of its unusual in vivo profile, A61772 was evaluated in a variety of functional assays of ANP action. Significantly, it was observed that the peptide has no effect on cGMP levels in a cultured endothelial cell line (Figure 5). In contrast, ANP[1–28] increases cGMP levels >100-fold at low doses in this system. This result is not surprising in light of the work of the groups at CalBio[21] and Searle[23] on C-receptor selective analogues. Containing only residues 8–15 of ANP, A61772 has a resemblance to compounds that are reported to be selective for this noncyclase-coupled receptor. Compounds **63** and **64**, linear peptides described in the CalBio work as typical C-selective agonists, give a similar response, as does **1**, an analogue of A61772 that includes only the N-terminal dipeptide fragment.

Analogues containing the carboxy-terminal fragment, on the other hand, produce dose-dependent increases in cGMP levels in BTAEC, indicating that they bind to type

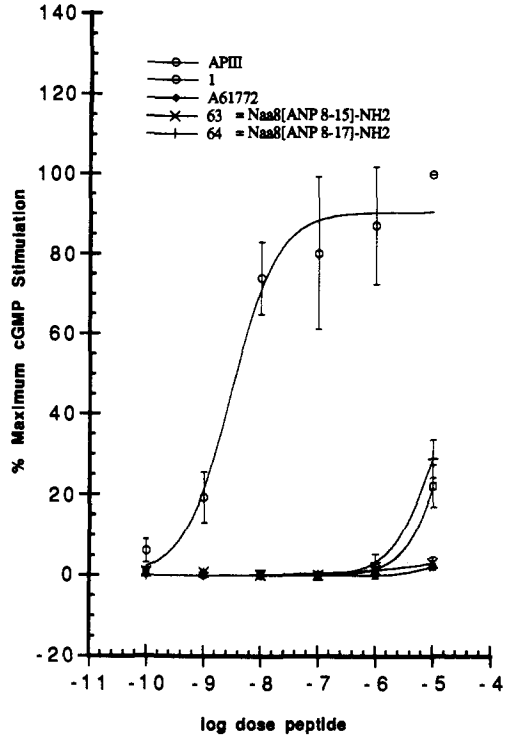

Figure 5. Small ANP analogues without N- and C-terminal fragments do not stimulate synthesis of cGMP in bovine aortic endothelial cells (BTAEC).

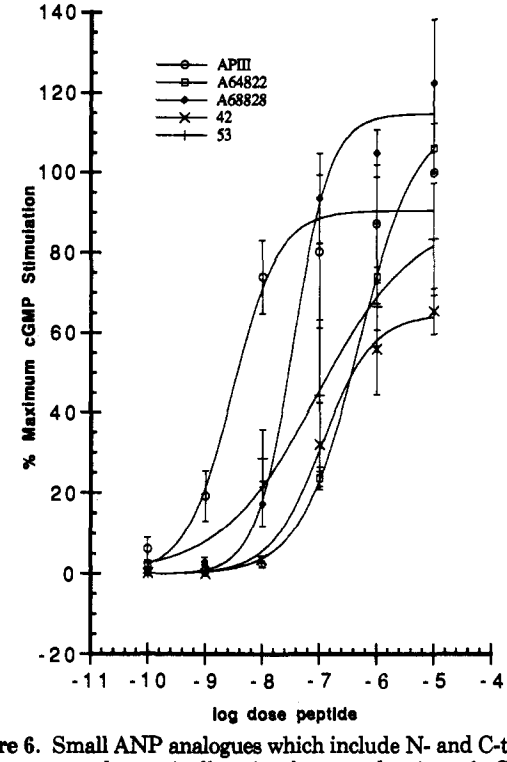

Figure 6. Small ANP analogues which include N- and C-terminal tail fragments dramatically stimulate production of cGMP by BTAEC.

A receptors. This result is demonstrated in Figure 6 for a number of series A and series B analogues. The compounds function as partial to full agonists in this assay, albeit with less potency than ANP[1–28]. In fact, A68828 in particular proves more efficacious than the native peptide at stimulating this second messenger system. While this hyperstimulation does not achieve statistical significance in the experiment reported here, we have

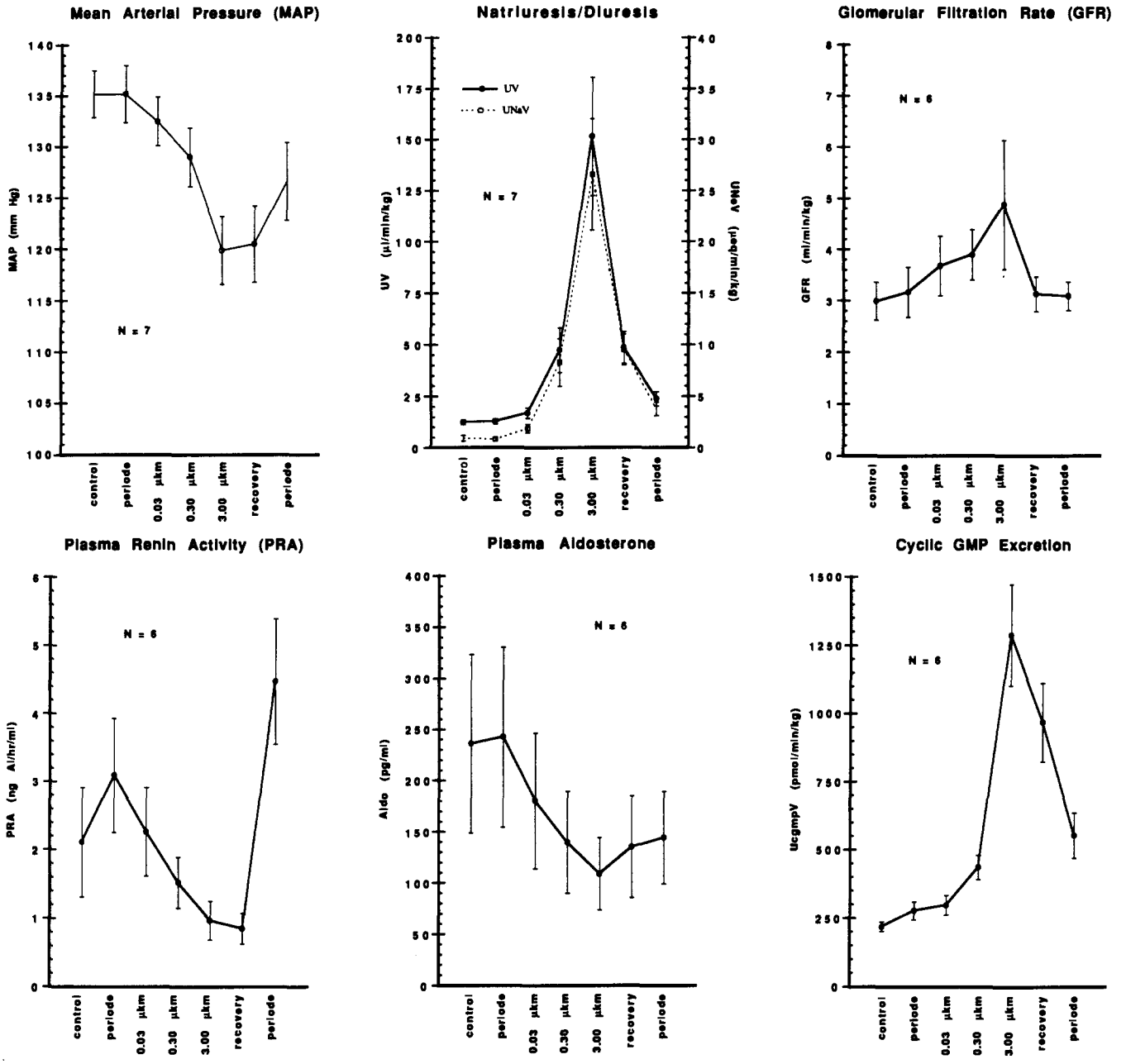

Figure 7. In vivo effects of small ANP analogue A68828 in the normotensive dog. A68828 causes a dose-dependent decrease in blood pressure and a large increase in sodium and water excretion, when infused at doses of 0.03, 0.3 and 3.0 $\mu g\ kg^{-1}\ min^{-1}$ (μkm) in the dog. Glomerular filtration rate increases, while plasma renin activity (PRA) and plasma aldosterone decrease. (Note the rebound in PRA after peptide infusion ceases.) Urinary cGMP increases dramatically. All of these effects are consistent with an ANP agonist.

consistently observed the same phenomenon in other, related studies. The correlation of the cyclic GMP response and in vivo effects with the presence of the terminal tail fragment is noteworthy. The compounds we describe here are unique in being the first class of small ANP analogues (50) capable of binding to guanylate cyclase-coupled receptors and of duplicating the full range of ANP's actions. It appears that the presence of the C-terminal dipeptide Phe–Arg is critical in obtaining this full range of responses.

Evaluation of a Small ANP Agonist. A68828, a series B 13-mer incorporating a number of the advantageous replacements uncovered during our structure–activity studies, was selected for more extensive evaluation as an ANP agonist. In our mixed-receptor assay A68828, with a K_L of 140 nM, is 65-fold less potent than ANP[5–28]. As described above, it is a full agonist in stimulating cGMP production in BTAEC, roughly 10-fold less potent than ANP[5–28] and 20% more efficacious.

The in vivo profile of A68828 (Figure 7) is strongly indicative of an ANP agonist. When infused intravenously in the anesthetized dog A68828 has an effect on mean arterial pressure (MAP) at a dose of 0.03 $\mu g\ kg^{-1}\ min^{-1}$, and decreases MAP by 15% at 3 $\mu g\ kg^{-1}\ min^{-1}$. At the same time A68828 causes a marked increase in urine flow rate, from 12.8 to 157 $\mu L\ min^{-1}\ kg^{-1}$ at the higher dose. This combination of decreased blood pressure and increased renal function is unusual, but is typical of the atrial peptides. The quality of urine is relatively unchanged; thus, diuresis results in a corresponding increase in sodium excretion. The natriuretic/diuretic effect we observe is triggered, at least in part, by an increase in glomerular filtration rate (GFR) from 3.1 to 4.9 $mL\ min^{-1}\ kg^{-1}$. In addition A68828 has the anticipated effects on a number of ANP-related functional markers. Plasma renin activity decreases by about 60% in dose-dependent fashion, and plasma aldosterone decreases by about 50%. Most im-

portantly, A68828 stimulates the production of cGMP in vivo as well as in vitro.

The above results are generally descriptive of a compound with about $^1/_{10}$ of the potency and all of the efficacy of ANP[5–28]. It thus appears that A68828 is more potent in vivo than would be predicted based on the results of the in vitro assays. The one significant difference in in vivo profiles may suggest an advantage for A68828 over other atrial peptides as a therapeutic agent. The 60% increase in GFR produced by the analogue at the 3.0 $\mu g\ kg^{-1}\ min^{-1}$ dose is greater than that usually achieved with pharmacological doses of ANP. This ability to stimulate hyperfiltration may make the compound uniquely suitable for the treatment of disorders such as acute renal failure (ARF). It is tempting to speculate that this effect relates to the hyperstimulation of guanylate cyclase which we observed with A68828.

To further study the possibilities for a small ANP agonist as a therapeutic agent, we have examined A68828 in several models of ARF. In experiments in which ARF is induced in rats by clamping the renal arteries for 30 min, or by injection of the nephrotoxic agent cisplatin, intravenous infusions of A68828 lead to significant improvements in kidney function (refs 42 and 43; data not shown). In fact, in the former model a 1-h infusion of A68828 improves both renal morphology and function up to 4 days later.[44] These experiments, which we have previously reported the details of, support the idea that our small analogues may serve as the pharmacological equivalents of ANP in a variety of disease states.

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Summary

It appears that the atrial natriuretic peptide sequence includes several critical fragments that are jointly responsible for its biological actions. By combining these critical fragments, and eliminating other segments, we have been able for the first time to produce small ANP analogues that have *all* of the native peptide's characteristics. Structure/activity studies have highlighted a number of modifications that lead to increases in potency. A68828, a 13-mer which incorporates several such modifications, has been demonstrated to behave like ANP in several in vitro and in vivo models. A68828 and its congeners contain fewer cationic residues than ANP[1–28], and thus may be less likely to produce the hypotensive crises which have plagued trials of the native peptide. In addition, the hyperstimulation of GFR produced by A68828 should enhance its utility as a therapeutic agent for the treatment of acute renal failure.

Experimental Section

Peptide Synthesis, Purification, and Characterization. All cyclic peptides are prepared using standard solid-phase techniques on a Biosearch model 9500 automated synthesizer, employing Boc chemistry. Peptide acids are assembled on Merrifield resin preloaded with the first residue; amides are assembled on 4-methylbenzhydrylamine resin. Peptides are removed from the resin and deprotected with anhydrous HF (~20 mL/mmol peptide) containing anisole (10% v/v) for 60 min at 0 °C. After removal of HF in vacuo, the residue is washed with 50 mL of ether, and the crude peptide is extracted with 200 mL of deoxygenated 20% aqueous acetic acid. The extracts are diluted to a concentration of ~50 mg/L and neutralized to pH 7.2 with concentrated ammonia. Iodine (0.01 N in ethanol; ~2 equiv) is added, and disulfide bond formation is allowed to occur for 60 min. Any excess iodine is removed by the addition of aqueous sodium thiosulfate to a colorless endpoint. The solution is poured onto a 500-g column of Amberlyst XAD-16 nonionic adsorbent; after washing with 3 L of distilled water, the crude peptide is eluted with 2 L of 1:1 ethanol/water. The solvents are removed to provide a crude product, which is purified by reversed-phase HPLC using a Dynamax 60-A C18 column and eluting with gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid.

All cyclic peptides are characterized and evaluated for purity using analytical HPLC (Vydac Protein and Peptide C18 column), 300- and 500-MHz ^1H NMR, fast atom bombardment (FAB-MS) or plasma desorption (PD-MS) mass spectrometry, and amino acid composition and/or sequence analysis. Unless otherwise specified, peptides are >95% pure by analytical HPLC analysis.

Linear fragments were prepared and deprotected using standard solution-phase or solid-phase techniques and were purified by passage through a column of Amberlite XAD-16 adsorbent. These peptides were characterized by 300-MHz ^1H NMR and direct chemical ionization (DCI) or FAB-MS.

Vasorelaxant Assays. Rabbit aortic rings (4–5 mm) are suspended in 2-mL jacketed tissue baths containing a Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM D-glucose). The baths are gassed with 95:5 O_2/CO_2, and pH is adjusted and maintained at 7.4. The rings are connected to an isometric force transducer for monitoring tension changes. Baseline tension is adjusted to 2 g and maintained over a 2-h period. To study a test compound as an ANP agonist, the rings are contracted with 3 μM histamine or with 3 μM methoxamine, and then cumulative additions of six half-log concentration increments of peptide to each bath are completed over a period of 90 min. All data points represent triplicate determinations.

Receptor Binding Assays. Rabbit adrenal plasma membranes are incubated with (^{125}I)Tyr28ANP[1–28] (IANP) and various concentrations of competing analogue for 90 min at 4 °C in a total volume of 0.2 mL of assay buffer (50 mM HEPES, 100 mM NaCl, 5 mM EGTA, 1 mM PMSF, 0.01% Brij-35 detergent, 0.01% BSA, 0.1% bacitracin, 5 mM $MnCl_2$, 1 μM leupeptin, 1 μM phosphoramidon, pH 7.4). Bound IANP is separated from free ligand by rapid filtration through 0.1% polyethylenimine-

treated glass fiber filter strips, followed by rapid washing with ice cold saline. The filters are counted for radioactivity. All data points represent duplicate determinations. Binding curves are fit to the data points using the SCAFIT routine and a two-site model. Reported binding constants are for the lower affinity site and are generally presumed to represent A-receptor binding.

Cyclic GMP Assays. Confluent monolayers of BTAEC or rVSMC are incubated in assay medium with a test compound at 37 °C for 2 h. After termination of the incubation by acidification with $HClO_4$ to 1.6%, the supernatant is collected by centrifugation. Cyclic GMP was determined after acetylation by radioimmunoassay. Data are expressed relative to the maximum response (=100%) observed for ANP[5-28]. All data points represent duplicate determinations.

In Vivo Studies. All rat experiments are performed using male Sprague-Dawley rats. The animals are anesthetized with Inactin (100 mg/kg, ip) and catheters placed in the femoral artery and vein for measurement of arterial blood pressure and infusion of drugs, respectively. A bladder catheter is inserted for timed collections of urine. After completion of surgical procedures the animals are allowed to equilibrate for 90 min. Test agonists are studied via a stepped-dose protocol, in which eight half-log incremental doses of peptide are infused sequentially for 15-min intervals. Mean arterial pressure, urine volume, and urinary sodium and cGMP are measured over each infusion period. Alternatively, renal and hemodynamic parameters are recorded following bolus injection of a single dose of peptide.

Male purpose-bred beagle dogs are anesthetized with pentobarbital sodium (30 mg kg^{-1} min^{-1}, iv, followed by 5 mg kg^{-1} h^{-1}), thermostatically regulated, and surgically prepared with femoral artery and vein catheters for blood pressure measurement and drug infusion respectively, ureteral cannulae for bilateral urine collection, and electromagnetic blood flow probes on both renal arteries. Inulin was continuously infused for measurement of glomerular filtration rate. Following equilibration for 1 h, two control clearance periods (30 min each) are followed by three drug infusion periods (0.03, 0.3, and 3.0 μg kg^{-1} min^{-1}, iv) and two recovery periods, in sequence. An arterial blood sample is taken at the midpoint of each clearance period for determination of plasma inulin, renin activity, and aldosterone levels. Urine samples are collected over each 30-min clearance period and are assayed for inulin, sodium, potassium, and cGMP.

Use of animals was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was approved by Abbott's Institutional Animal Care and Use Committee.

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Supplementary Material Available: Chemistry experimentals and analytical data for the compounds investigated in this article (13 pages). Ordering information is given on any current masthead page.

Synthesis and Biological Action of the Aminotetrahydroisoquinocarbazoles and Related Compounds: A New Class of Compounds with Antiarrhythmic Activity

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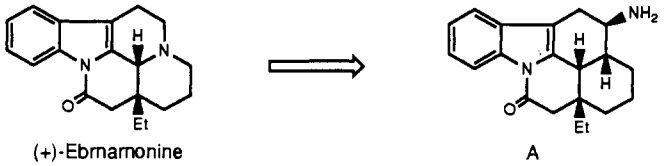
A series of 12-aminotetrahydroisoquinocarbazoles and related compounds were synthesized using an intramolecular Diels–Alder reaction and screened for antiarrhythmic activity in chloroform-induced ventricular arrhythmias in mice. Several compounds showed more potent activity than disopyramide. There was some correlation between substituents on aromatic ring and angular position, and antiarrhythmic activity. An amino group or some functional groups containing an amino group on C-12 seemed to be essential to exhibit the activity. Ring size also influenced the activity. The compound (+)-10 (RS-2135) had the most favorable combination of antiarrhythmic activity and toxicity and was selected for further evaluation.

The problem of the risk of sudden death following myocardial infarction has not been resolved though many antiarrhythmic agents have been developed for the treatment of arrhythmia. Current antiarrhythmic therapy is far from satisfactory, because it is often ineffective and accompanied by serious adverse effects. The development of new antiarrhythmic agents that are effective and safe in the treatment of ventricular arrhythmia has been a major goal of cardiovascular research and development of pharmaceutical firms.[1] In the course of synthetic studies on carbazole derivatives, we found that some pentacyclic fused carbazole derivatives (for example A) which were prepared[2] as eburnamonine analogues[3,4] by an intramolecular Diels–Alder reaction[5] had potent antiarrhythmic activity. Afterward we synthesized a series of related fused carbazole derivatives and tested their antiarrhythmic activities to clarify the structure–activity relationships. In this paper, we wish to report our results on the chemistry and pharmacology of a new class of antiarrhythmic agents, aminotetrahydrocarbazoles, and related compounds.



(+)-Ebrnamonine A

Chemistry

A series of aminoisoquinocarbazole compounds was

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