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Topical Review

Amiloride and Its Analogs as Tools in the Study of Ion Transport

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I. Introduction

The synthesis of amiloride and amiloride analogs was first described by Cragoe et al. in 1967 [21]. Amiloride was initially demonstrated to inhibit the Na^+ channel present in urinary epithelia. Amiloride and its analogs were subsequently shown to inhibit other ion transport processes as well [3]. As more ion transport systems are studied, it becomes increasingly clear that amiloride analogs are "specific" inhibitors of relatively few ion transporters. They inhibit a large number of membrane transport processes and enzymes. They have also been shown to interact with specific drug and hormone receptors and to inhibit cellular metabolism and DNA, RNA, and protein synthesis. This review addresses the structure-activity relationships of amiloride and its analogs on the large number of ion transporters and other cellular processes that are inhibited by amiloride, and examines the use of these drugs as probes for the characterization of transport proteins.

II. Pharmacology of Inhibition of Ion Transporters, Enzymes, and Receptors by Amiloride and Amiloride Analogs

The structure of amiloride is shown in Fig. 1. Amiloride is pyrazinoylguanidine bearing amino groups on the 3- and 5-positions and a chloro group on the 6-position of the pyrazine ring. Approximately one thousand analogs have been synthesized [19], and representative members have been examined for their inhibitory activity on the Na^+ channel, Na^+/H^+ exchanger, and the Na^+/Ca^{2+} exchanger. Amiloride analogs that have been thoroughly studied are listed in Tables 1–7, along with their effects on inhibition of Na^+ transport in these systems. This section reviews the structure-activity relationships for amiloride and its analogs on ion transport systems, enzymes, drug and hormone receptors, DNA, RNA, and protein synthesis, and cellular metabolism.

A. Epithelial Na^+ Channel

High resistance (or "tight") epithelia that transport Na^+ have been found to possess Na^+ channels which are functionally restricted to the apical plasma membrane. Na^+ crosses the apical membrane by passive diffusion through this channel, and is actively extruded from the cell by a Na^+/K^+-ATPase present on the basolateral plasma membrane. The diuretic amiloride, at a concentration less than 10^{-6} M, inhibits Na^+ transport when added to the solution bathing the apical plasma membrane. The inhibition is rapidly reversed by removing amiloride from the apical solution. Addition of a low concentration of amiloride to the solution bathing the basolateral plasma membrane has no effect on Na^+ transport [8, 72]. Subsequent observations of single-channel recording using the patch-clamp technique have shown that amiloride reduces the mean open time of the Na^+ channel, but has no effect on single-channel conductance [36, 65].

A large number of amiloride analogs have been synthesized, and the effect of many of these analogs

Key Words amiloride · amiloride analogs · ion transport · Na channel · Na/H exchanger · Na/Ca exchanger

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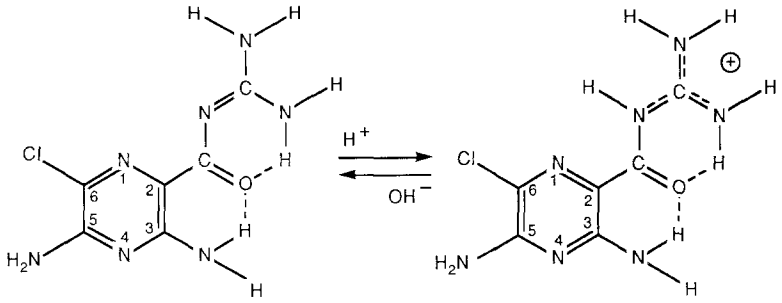
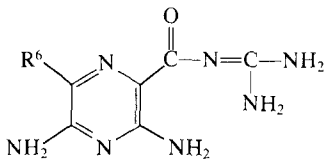


Fig. 1. Ground state structure of amiloride in the unprotonated and protonated form

Table 1.



No.	R^6	IC_{50} (μM) for amiloride and potency relative to amiloride		
		Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
1	Cl— (Amiloride)	(0.34) 1 (0.35)* 1*	(83.8) 1	(1100) 1
2	H—	0.006	0.08	1
3	F—	0.085	0.12	0.1
4	Br—	1	1.9	1
5	I—	0.14	4.6	1

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261**:2839 (1978), or *Verrey et al., *J. Cell Biol.* **104**:1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30**:112 (1986). (c) Kaczorowski et al., *Biochemistry*, **24**:1394 (1985).

on Na^+ transport across tight epithelia has been studied. The most thorough studies have involved use of frog skin or toad urinary bladder as model "tight" epithelia, monitoring transepithelial Na^+ transport electrically as short-circuit current (I_{sc}) with tissue mounted in an Ussing chamber. The concentration of an amiloride analog required to achieve 50% inhibition of I_{sc} (the IC_{50}) was used as a measure of its apparent affinity for the Na^+ channel. This measurement is dependent on a number of variables including:

a. Extracellular Na^+ Concentration. Lowering the Na^+ concentration of the solution bathing the apical membrane of tight epithelia results in a decrease in Na^+ transport. In addition, as the Na^+ concentration is lowered, the IC_{50} for inhibition of Na^+ transport by amiloride decreases [27].

b. Extracellular pH. Amiloride is a weak base (pK_a = 8.7 in 30% ethanol, 8.8 in H_2O) (*see* Table 8) and interacts only in its protonated form with the Na^+ channel to inhibit I_{sc} [7, 23]. As the pH of a solution increases, the fraction of amiloride in the protonated form will decrease.

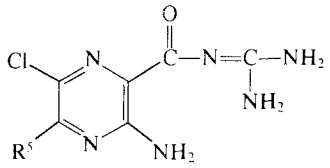
c. Intracellular Accumulation of Amiloride. The unprotonated form of amiloride and many of its analogs are quite lipid soluble. They may bind to and easily cross cell membranes, accumulate within cells, and alter a number of cellular processes (*see below*).

d. Apical Plasma Membrane Potential. The extent of inhibition of the channel by amiloride has been shown to be dependent on the apical plasma membrane potential. It is the charged form of amiloride that interacts with the channel in a manner sensitive to the membrane potential [36, 64].

1. Structure-Activity Relationships for Amiloride and Its Analogs with the Na^+ Channel

The effects of modification of the structure of amiloride on the inhibition of the Na^+ channel are sum-

Table 2.



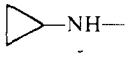
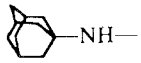
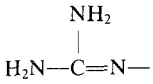
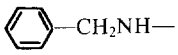
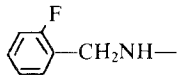
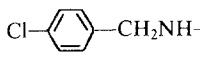
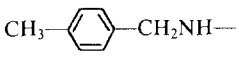
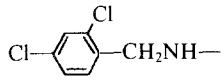
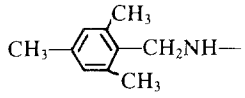
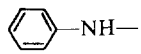
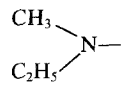
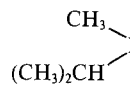
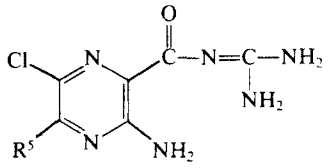
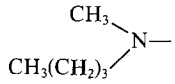
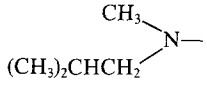
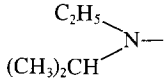
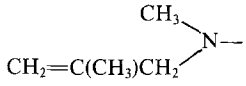
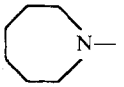
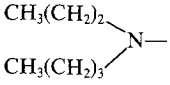
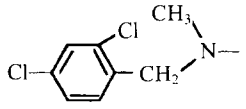
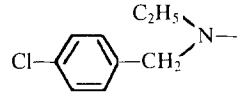
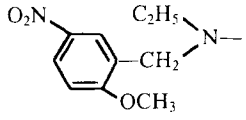
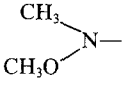
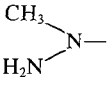
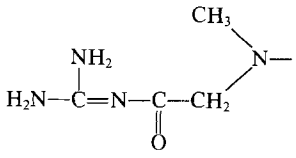
No.	R^6	IC_{50} (μM) for amiloride and potency relative to amiloride					
		Na^+ channel[a]	Na^+/H^+ antiporter				Na^+/Ca^{2+} exchanger[f]
			b	c	d	e	
1	H_2N—(amiloride)	(0.34) 1 (0.35)* 1*	(83.8)	(7) 1	(3) 1	(4) 1	(1100) 1
6	H—	<0.035*	0.5				1.5
7	C_2H_5NH—	0.0003	7.4	1.8		1.5	
8	$CH_3(CH_2)_2NH$—	0.0004		2.3		2	
9		0.002	6.3				
10	$CH_2{=}CHCH_2NH$—	0.0001	7.4				
11	$(CH_3)_3CNH$—	<0.035*	26				11
12	$CH_3(CH_2)_5NH$—		26				
13			5.1				54
14	$HOCH_2(CHOH)_4CH_2NH$—	<0.035*	13	0.7			<0.5
15	$(CH_3)_2N(CH_2)_2NH$—		0.6				
16	$H_2NCH_2C(CH_3)_2CH_2NH$—		4.1				
17			3.6	3.5			
18		<0.035*	6.6	3.5			45
19			9	1.4			50
20			10				55
21		<0.0035*	9.6	0.35	1.6		55
22							115
23			3.3				50
24			22		20	67	
25	$(CH_3)_2N$—	<0.035*	12	23	20	24	2
26		0.0005	133	35			
27		0.0005	349	35	50	13	

Table 2 continued next page

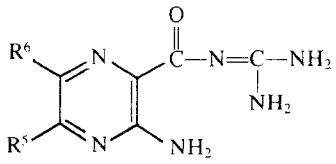
Table 2. (continued)

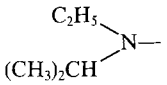


No.	R^6	IC_{50} (μM) for amiloride and potency relative to amiloride					
		Na^+ channel[a]	Na^+/H^+ antiporter				Na^+/Ca^{2+} exchanger[f]
			b	c	d	e	
28			123	7		13	
29			349	14			
30			190				8.5
31		<0.035*	223	140			8.5
32			102				
33		<0.035*	524				11
34			226				55
35			29				75
36			61				75
37		<0.035*	125				60
38			13	0.4			
39			8.7	3.5			
40		<0.035*	62				0.7

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261:**2839 (1978), or *Verrey et al., *J. Cell Biol.* **104:**1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30:**112 (1986). (c) Vigne et al., *Mol. Pharmacol.* **25:**131 (1985). (d) L'Allemain et al., *J. Biol. Chem.* **259:**4313 (1984). (e) Zhuang et al., *Biochemistry* **23:**4481 (1984). (f) Kaczorowski et al., *Biochemistry*, **24:**1394 (1985).

Table 3.



No.	R^6	R^5	IC_{50} (μM) for amiloride and potency relative to amiloride		
			Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
1	Cl—	H_2N—(amiloride)	(0.34) 1 (0.35)* 1*	(83.8)	(1100)
41	H—	H—	<0.035*	0.08	0.08
42		H—		21.2	
43	H—	$(CH_3)_3CNH$—		1.7	
44	Br—	H—	0.4	1	0.15
45	Br—			566	
46	I—	$(CH_3)_2N$—		68	
47	I—			313	1.2

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261**:2839 (1978), or *Verrey et al., *J. Cell Biol.* **104**:1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30**:112 (1986). (c) Kaczorowski et al., *Biochemistry*, **24**:1394 (1985).

marized. These are grouped according to the structural changes at various sites in the molecule (*see* Tables 1–7).

a. 2-Carbonylguanidino Substituents. Substitution of hydrophobic groups on the terminal nitrogen atom of the guanidino moiety has produced a number of analogs with markedly enhanced IC_{50} values [26]. Several of these analogs appear to have the highest affinity and specificity for the Na^+ channel. These include the benzyl (No. 54, benzamil) and substituted benzyl analogs (Nos. 55–66), phenyl (No. 76, phenamil), phenethyl (No. 71) and substituted phenyl (No. 77) and phenethyl (No. 72) analogs, as well as straight and branched chain alkyl groups (Nos. 48 and 49). This increase in the potency has been shown to be due to changes in both the on- and off- rate constants of these amiloride analogs [56].

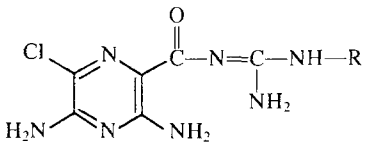
The introduction of two hydrophobic groups on a single terminal guanidino nitrogen atom has not been studied extensively. Two alkyl substituents on a single terminal guanidino nitrogen atom (Nos. 78 and 79) increases activity. However, a methyl and a benzyl group (No. 80) located in this position decreases activity by an order of magnitude.

The carbonylguanidino moiety of amiloride is required for inhibition of the Na^+ channel. Replacement of the imidocarbonyl group of the guanidino moiety with C=S (No. 84) or deletion of the guanidino moiety and replacement with HO— (No. 85) results in a loss of activity. The insertion of a NH group between the carbonyl group and guanidino moiety of amiloride (No. 83) results in a 40% decrease in activity.

b. 5-Position Substituents. An unsubstituted 5-amino group is required for optimal inhibition of the Na^+ channel. Amiloride analogs bearing one or two substituents on the 5-amino nitrogen atom exhibit substantial loss in activity. This is true whether one or both substituents are alkyl (Nos. 7, 8, 11, 25–27, 31), substituted alkyl (Nos. 14, 40), cycloalkyl (Nos. 9, 33), or aralkyl (Nos. 18, 21, 37). Analogs where the amino group has been replaced with H— (No. 6), alkoxy (i.e., CH_3O—, C_2H_5O—), HS—, CH_3S—, HO—, or Cl— have a substantial loss of activity, as measured by saliuretic and antikaliuretic activity in whole animal studies [19, 20].

c. 6-Position Substituents. Optimal activity is observed when the 6-position is occupied by a chloro

Table 4.



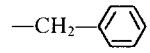
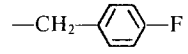
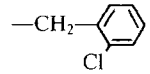
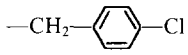
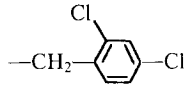
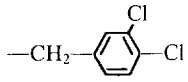
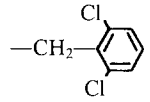
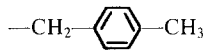
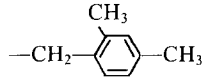
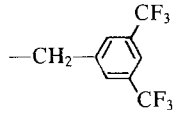
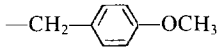
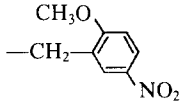
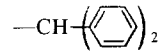
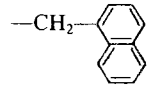
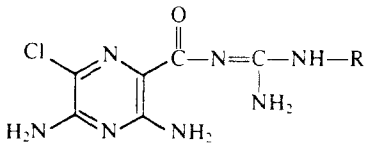
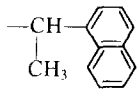
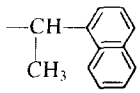
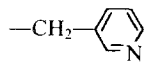
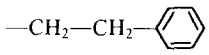
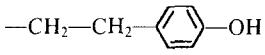
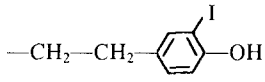
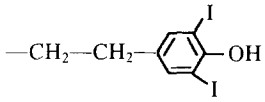
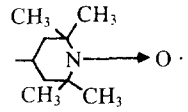
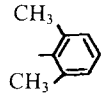
No.	—R	IC_{50} (μM) for amiloride and potency relative to amiloride		
		Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
1	—H (amiloride)	(0.34) 1 (0.35)* 1	(83.8) 1	(1100) 1
48	$-C(CH_3)_2CH_2C(CH_3)_3$	13	<0.08	7.9
49	$-(CH_2)_{11}CH_3$	2*		9
50	$-CH_2CH_2OH$	3		<0.5
51	$-CH_2CF_3$	1		1
52	$-(CH_2)_3COOH$	0.6*		0.5
53	$-(CH_2)_3COOC_2H_5$	2*		<4
54		9	<0.08	11
55		10		11
56		13		55
57		9		15
58		2.5	<1	92
59		4		37
60		47*		75
61		13		16
62		4		110
63				42
64		10		8.5
65		7*		28
66		18*		115
67		4		31

Table 4. (continued)



No.	—R	IC_{50} (μM) for amiloride and potency relative to amiloride		
		Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
68	 R(+)			94
69	 S(−)			94
70		1.2		1
71		13	<0.008	12
72		18*		1
73				16
74				6
75		1.6		
76		17	<0.008	5.5
77		23		7.3

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261**:2839 (1978), or *Verrey et al., *J. Cell Biol.* **104**:1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30**:112 (1986). (c) Kaczorowski et al., *Biochemistry*, **24**:1394 (1985).

(No. 1), amiloride) or bromo (No. 4) group. The 6-iodo (No. 5), 6-fluoro (No. 3), and the 6-H compounds (No. 2) are 7-, 12-, and 170-fold less potent than amiloride, respectively. The decrease in the IC_{50} is due to an increase in the off-rate constant [55]. Analogs with replacement of the halogen by CH_3O—, C_6H_5S—, and CN— have significant loss of activity, as measured in whole animal studies [19, 20].

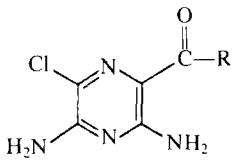
d. Replacement of the Pyrazine Nucleus. Replacement of the pyrazine ring with a hydrophobic moiety results in a loss of activity (Nos. 89 and 91).

e. 3-Position Substituents. The effect of substitutions at this site on inhibition of the Na^+ channel have received limited study. Replacement of the 3-amino group with H— results in substantial loss of activity [19, 20].

2. Specificity of Amiloride and Its Analogs for the Na^+ Channel

The Na^+ channel is the only ion transporter inhibited by amiloride with an IC_{50} of less than 1 μM. In the presence of a physiologic Na^+ concentration, the IC_{50} for inhibition of Na^+ transport is in the

Table 5.



No.	—R	IC_{50} (μM) for amiloride and potency relative to amiloride		
		Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
	—N=C—NH_2 (amiloride) | NH_2	(0.34) 1		
1		(0.35)* 1*	(83.8) 1	(1100) 1
78	—N=C—$N(C_2H_5)_2$ | NH_2	3		<0.5
79	—N=C—$N[(CH_2)_3CH_3]_2$ | NH_2	3		3.3
80	—N=C—N(CH_3)—CH_2—(C$_6$H$_5$) | NH_2	0.1		7.3
81	—N=C—N=C—$N(CH_3)_2$ | | NH_2 $NHCH_3$	0.12		<0.08
82	—N(CH_3)—C—$NHCH_2$—(C$_6$H$_5$) | $NHCH_3$			2.2
83	—NHN=C—NH_2 | NH_2	0.6	<0.08	<0.08
84	—NH—C—NH_2 ‖ S	0.001	<0.08	
85	—OH	<0.035		<0.08

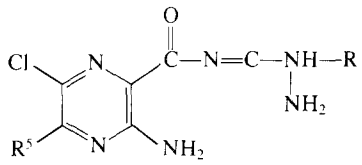
Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261:**2839 (1978), or *Verrey et al., *J. Cell Biol.* **104:**1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30:**112 (1986). (c) Kaczorowski et al., *Biochemistry,* **24:**1394 (1985).

range of 0.1 to 0.5 μM. The IC_{50} obtained from studies of different transport processes are difficult to compare, since the interaction of amiloride with the various Na^+ transporters may be effected by a number of variables including extracellular pH and $[Na^+]$, and the apical transmembrane potential. The IC_{50} for Na^+/H^+ exchange is as low as 3 μM when measured in the presence of a low external $[Na^+]$ [50] but as high as 1 mM in the presence of a high $[Na^+]$. Amiloride is a weak inhibitor of the Na^+/Ca^{2+} exchanger, with an IC_{50} of 1 mM.

The most specific inhibitors of the epithelial Na^+ channel are amiloride analogs bearing hydrophobic substituents on the terminal nitrogen atom of the guanidino moiety. The benzyl and phenyl analogs have been most extensively studied. These analogs inhibit I_{sc} with IC_{50} of approximately 10 nM. This is more than three orders of magnitude lower than that reported for inhibition of Na^+/H^+ and Na^+/Ca^{2+} exchangers, Na^+/K^+-ATPase, and Na^+-glucose and Na^+-alanine cotransporters (*see below*). The effect of these analogs on protein kinases has not been reported.

Several amiloride analogs have proved useful in

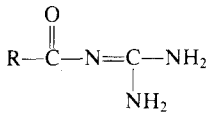
Table 6.



No.	R^5	—R	IC_{50} (μM) for amiloride and potency relative to amiloride		
			Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
1	H_2N—(amiloride)	—H	(0.34) 1 (0.35)* 1*	(83.8) 1	(1100) 1
86	$CH_3(CH_2)_2$ $CH_3(CH_2)_3$ N—	—CH_2—(2-Cl-phenyl)—Cl			300
87	Cl—(phenyl)—CH_2—NH—	—CH_2—(2-CH_3-phenyl)—CH_3	<0.035*		150

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261:**2839 (1978), or *Verrey et al., *J. Cell Biol.* **104:**1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30:**112 (1986). (c) Kaczorowski et al., *Biochemistry,* **24:**1394 (1985).

Table 7.



No.	R—	IC_{50} (μM) for amiloride and potency relative to amiloride		
		Na^+ channel[a]	Na^+/H^+ antiporter[b]	Na^+/Ca^{2+} exchanger[c]
1	(amiloride)	(0.34) 1 (0.35)* 1*	(83.8) 1	(1100) 1
88	N—(pyridin-2-yl)—CH=CH—		1	<0.1
89	(diphenyl)CH—	<0.035*	1.2	11
90	(anthraquinone-2-yl)		11	<0.1
91	Cl—(phenyl)—CH=C(CH$_3$)—	<0.035*	23	11

Evaluated by the method of (a) Cuthbert et al., *Br. J. Pharmacol.* **261:**2839 (1978), or *Verrey et al., *J. Cell Biol.* **104:**1231 (1987). (b) Simchowitz et al., *Mol. Pharmacol.* **30:**112 (1986). (c) Kaczorowski et al., *Biochemistry,* **24:**1394 (1985).

Table 8. pK_a and lipophilicity data

Compound number	pK_a[a]	Lipophilicity[b]	Compound number	pK_a[a]	Lipophilicity[b]
		1			
1	8.7	5[c]	49		99.4
2	9.2	1	50	8.5	2[c]
3	8.9	7	54	8.1	56
4	8.6	8[c]	56	7.4	98
5	8.8	10	59	7.63	>99.8
6	7.0	2[c]	62	7.45	99
10	8.8	4[c]	70	4.4	47
14	8.9	0.4	71	8.2	90
15	7.8 9.2	0.7	75	7.4	99
21	9.0	93	76	7.8	98.7
25	8.8	6	77	7.5	99.5
30	8.1	94	78	7.6	78
33	8.5	93	81	10.5	4
37	3.68 8.30	99.5	83	9.0	1[c]
40	6.4 9.2	6			

[a] Determined as the half neutralization point in water containing 30% ethanol.
[b] Percent distributed in octanol or (c) chloroform when equilibrated between equal volumes of the solvent and pH 7.4 aqueous phosphate buffer (0.1 M).

biochemical characterization of the Na^+ channel. Tritiated benzamil, phenamil, and 6-bromomethylamiloride (where the methyl group is located on the amide nitrogen atom) have been used to study binding of amiloride analogs to channel containing membranes [2, 25, 46, 71]. The binding of radiolabeled amiloride analogs has recently been used to follow the channel during solubilization and purification [2, 5]. Photoactive analogs of benzamil (6-bromobenzamil and the benzamil derivative No. 65) and 6-bromomethylamiloride have been used to identify components of the Na^+ channel [6, 44, 46] (*see* section III.C). A spin-labeled analog (No. 75) has recently been used as a probe for the Na^+ channel [18].

Finally, it has been observed that it is difficult to reverse the inhibition of Na^+ transport due to phenamil by removing phenamil from the solution bathing the apical plasma membrane of frog skin or toad urinary bladder [34]. This may be due to hydrophobic interactions of the drug with the channel. Studies with [^{3}H]phenamil show clearly that the drug is not binding covalently to the channel [2].

B. Na^+/H^+ Antiporter

A plasma membrane transporter that exchanges Na^+ for H^+ in an electroneutral fashion has been described in cells and plasma membranes derived from a variety of species [74]. This exchanger appears to be involved in regulation of intracellular pH and may provide a major pathway for Na^+ entry into specific cells. Amiloride inhibition of the Na^+/H^+ exchanger was first described in 1976 [40], and has been used subsequently as an inhibitor not only of Na^+/H^+ exchange, but of subsequent events that might be altered through regulation of this transporter. The effects of a large number of amiloride analogs on Na^+/H^+ exchange have been studied in a number of different cell types including Chinese hamster lung fibroblasts [50], human fibroblasts [62], the epidermoid cell line A431 [86], chick skeletal muscle cells [82], human neutrophils [75], and the pig kidney epithelial cell line LLC-PK [35]. The inhibition of Na^+/H^+ exchange by amiloride analogs has also been studied in rabbit renal microvillus membrane vesicles [51].

The methods used for measurement of Na^+/H^+ exchange are numerous and are described in detail elsewhere [35, 50, 51, 62, 75, 82, 86]. A number of factors may influence the inhibition of transport by amiloride and its analogs, including:

a. Extracellular pH. Amiloride and its analogs inhibit the Na^+/H^+ exchanger in the protonated form [50]. Therefore, the pH of the extracellular (or extravesicular) solution must be well below the pK_a of the amiloride analog (*see* Table 8).

b. Collapse of pH Gradient. Amiloride and a large number of its analogs (in particular, a number of analogs that have been shown to be potent inhibitors of Na^+/H^+ exchange) bear hydrophobic substituents and are quite lipophilic, especially when the analogs are unprotonated. These amiloride analogs are permeable weak bases, and may accumulate in acidic compartments and collapse a pH gradient across plasma membranes, organelles, or membrane vesicles (*see below*). We[1] and others [31] have observed that amiloride and several of its analogs will collapse a pH gradient across membrane vesicles. This is dependent, at least in part, upon the hydrophobicity of the amiloride analog.

c. Intracellular Accumulation of Amiloride. Amiloride has been shown to accumulate within cells. This may potentially influence inhibition of Na^+/H^+ exchange by a number of mechanisms, such as inhibition of intracellular proteins (such as protein kinases) which may be involved in the regulation of Na^+/H^+ exchange [61, 70], or through increasing intracellular pH which may inactivate the Na^+/H^+ exchanger [1]. The Na^+/H^+ exchanger is regulated by intracellular pH. Lowering intracellular (or intravesicular) pH activates the exchanger by increasing its affinity for internal H^+ without changing the K_m for external Na^+ or K_i for amiloride [66, 67]. Among several cell lines that have been studied, either an increase in V_{max} [12, 83] or no change in V_{max} has been observed [66, 67] following a decrease in intracellular pH.

d. Extracellular Na^+ Concentration. The extracellular (or extravesicular) Na^+ concentration has been shown to effect the interaction of amiloride analogs with the Na^+/H^+ exchanger. Increasing the extracellular (or extravesicular) Na^+ concentration decreases the apparent affinity of amiloride analogs for the Na^+/H^+ exchanger. In addition, amiloride increases the K_m for Na^+ without changing the V_{max}. Amiloride analogs and Na^+ share a common binding site on the Na^+/H^+ exchanger [43]. In addition, amiloride has been shown to inhibit the Na^+/H^+ exchanger by binding to a second site which is independent of the Na^+ binding site [39].

e. Extracellular anions. Studies with brush border membrane vesicles derived from rabbit kidney cortex have demonstrated that the IC_{50} of amiloride for the Na^+/H^+ exchanger is reduced in the presence of a gluconate containing buffer when compared with a chloride containing buffer [84].

1. Structure-Activity Relationships for Amiloride Analogs with the Na^+/H^+ Exchanger

A large number of amiloride analogs have been used to inhibit Na^+/H^+ exchange in different cell types and in membrane vesicles by a variety of methods. A consensus on the structural modifications that enhance the affinity of amiloride analogs for the Na^+/H^+ exchanger is apparent from these studies, although the absolute K_i varies, in part dependent upon the method used to study Na^+/H^+ exchange. It is clear that the most potent and specific inhibitors of the Na^+/H^+ exchanger are amiloride analogs with hydrophobic groups on the 5-amino nitrogen atom. A representative list of amiloride analogs that have been studied appears in Tables 1–7. General observations on the effect of these amiloride analogs on Na^+/H^+ exchange are discussed in relation to changes at various sites on the pyrazine ring utilizing data obtained in studies of the human neutrophil Na^+/H^+ exchanger [75]. The IC_{50} for inhibition of the exchanger by amiloride is 84 μM in human neutrophils, and as low as 3 to 7 μM in other tissues. This variation is likely due, in part, to differences in the concentration of Na^+ used in the assay. The IC_{50} for inhibition of Na^+/H^+ exchange, relative to the IC_{50} for inhibition by amiloride are, in general, similar among the various cells studied.

a. 5-Position Substituents. The most potent inhibitors of Na^+/H^+ exchange are amiloride analogs in which the 5-amino nitrogen atom bears one or two substituents. Two substituents are generally superior to one and the substituents are generally hydrophobic in nature. However, some hydrophilic groups (e.g. polyhydroxyalkyl) and basic moieties capable of protonation at a second site in the molecule (e.g. aminoalkyl (No. 14), guanidino (No. 17) and guanidinoacylalkyl (No. 40)) confer considerable activity to the molecule. In several cell lines, the most potent compounds in this class exhibit IC_{50} values for inhibition of Na^+/H^+ exchange below 100 nM.

[1] Kaczorowski, G., Garcia, M., Kleyman, T., and Cragoe, E.J., Jr. (*unpublished observations*).

The generalities summarized above are illustrated in Table 2. Among the more potent analogs which have received a considerable amount of study in a variety of tissues are the 5-(N-methyl-N-isopropyl) (No. 27), the 5-(N-methyl-N-isobutyl) (No. 30), the 5-(N-ethyl-N-isopropyl) (No. 31), the 5-(N,N-hexamethylene) (No. 33) and the 5-(N,N-dimethyl) (No. 25) analogs of amiloride which exhibit 10- to 500-fold greater potency than amiloride.

The 5-H analog of amiloride (No. 6) is only half as potent as amiloride itself, indicating that an unsubstituted 5-amino group does not confer much of an enhancing effect on the molecule.

b. 6-Position Substituents. The 6-Br (No. 4) and 6-I (No. 5) are, respectively, two- and fivefold more potent than amiloride, whereas the 6-H (No. 2) and 6-F (No. 3) analogs of amiloride are, respectively, 13- and eightfold less potent.

c. 2-Carbonylguanidino Substituents. The carbonylguanidino moiety is required for activity. When this group is replaced by the carbonylaminoguanidino (No. 83) or the carbonylthioureido (No. 84) group, activity is lost. Substitution on the terminal guanidino nitrogen atom with hydrophobic groups such as alkyl (No. 48), benzyl (No. 54), substituted benzyl (No. 58), phenethyl (No. 71) or phenyl (No. 76) decreases activity.

d. 5- and 6-Position Substituents. The 5-H, 6-H compound (No. 41) is a poor inhibitor of Na^+/H^+ exchange, as is the 5-NH_2, 6-H compound (No. 2). The 5-H, 6-*p*-chlorophenyl analog (No. 42) is 21-fold more potent than amiloride. The 5-(N,N-dimethyl)-6-I (No. 46) is approximately fivefold more potent than its 6-Cl counterpart (No. 25), just as 6-I amiloride (No. 5) is fivefold more potent than amiloride (No. 1).

e. Replacement of the Pyrazine Ring. Compounds have been synthesized with cyclic or heterocyclic groups in place of the substituted pyrazine ring. Several of these compounds are more potent than amiloride in inhibiting the Na^+/H^+ exchanger. Replacement of the 3,5-diamino-6-chloropyrazinyl moiety by 2-anthraquinolyl (No. 90) or 1-(4-chlorophenyl)2-propenyl (No. 91) produced compounds 11- and 23-fold more active, respectively, than amiloride. Replacement by 2-(4-pyridyl) ethenyl (No. 88) or diphenylmethyl (No. 89) provided compounds as active as amiloride.

f. 3-Position Substituents. The effect on the Na^+/H^+ exchanger of amiloride analogs with substitutions at this site has not been studied.

2. Specificity of Amiloride and Its Analogs for Inhibition of the Na^+/H^+ Exchanger

Amiloride analogs bearing hydrophobic substituents on the 5-amino group of the pyrazine ring have both the highest activity and specificity for the Na^+/H^+ exchanger. When studied in the presence of a low [Na^+], $IC_{50} < 100$ nM have been observed for a number of amiloride analogs. This is 100-fold less than the IC_{50} observed with these analogs for inhibition of the epithelial Na^+ channel [26], Na^+/Ca^{2+} exchanger [41], Na^+/K^+-ATPase [86], and Na^+-D-glucose cotransporter [16].

The effect of amiloride analogs on protein kinases has not been extensively studied. Amiloride and 5-(N,N-dimethyl)amiloride are 9 and 100 times more potent, respectively, in inhibiting Na^+/H^+ exchange than in inhibiting protein kinase C [9]. Amiloride has also been shown to inhibit a number of other kinases with IC_{50} similar to that reported for protein kinase C [29]. Amiloride inhibits adenylate cyclase with an IC_{50} similar to the IC_{50} for inhibition of Na^+/H^+ exchange [58]. The effect of 5-amino substituted amiloride analogs on adenylate cyclase has not been examined.

C. Na^+/Ca^{2+} Exchanger

Changes in the intracellular [Ca^{2+}] have been shown to mediate a variety of cellular processes. The intracellular [Ca^{2+}] is tightly regulated in eukaryotic cells by a variety of mechanisms, including transport of Ca^{2+} across plasma membranes, transport across membranes surrounding intracellular organelles, and binding to negatively charged groups. The Na^+/Ca^{2+} exchanger is a major mechanism of transport of Ca^{2+} across plasma membranes [52] and has been described in a variety of cell types, including electrically excitable cells such as giant squid axon, cardiac muscle, skeletal muscle, and pituitary cells. The exchanger has also been found in both leaky and tight epithelia, and in erythrocytes.

The Na^+/Ca^{2+} exchanger is a reversible electrogenic transporter that exchanges 3 Na^+ ions for each Ca^{2+} ion. Amiloride is a poor inhibitor of the Na^+/Ca^{2+} exchanger, with an IC_{50} of approximately 1 mM [41]. It is a reversible inhibitor of the exchanger and the inhibition is competitive with respect to Na^+. Amiloride interacts with more than one Na^+ binding site on the transporter [41]. Amiloride analogs have been shown to block Na^+/Ca^+ exchange in intact cardiac myocytes [11, 42]. The pharmacology of amiloride inhibition of the Na^+/Ca^{2+} exchanger has been studied by measuring the inhibition of Na^+ or Ca^{2+} fluxes across membrane vesicles. As with other amiloride-sensitive trans-

porters, the IC_{50} is dependent on the extracellular (or extravesicular) Na^+ concentration, and it is the protonated form of the drug which interacts with the exchanger [41, 73].

1. Structure-Function Relationships for Amiloride and Its Analogs with the Na^+/Ca^{2+} Exchanger

a. 2-Carbonylguanidino moiety. The introduction of hydrophobic groups on the terminal guanidino nitrogen atom (Nos. 48 and 49) increases the activity over that of amiloride. A benzyl substituent (No. 54) increases activity by an order of magnitude. Appropriate substitution of the benzyl group further enhances activity. The 2′-Cl (No. 56), 2′,4′-Cl_2 (No. 58), 3′,4′-Cl_2 (No. 59), 2′,6′-Cl_2 (No. 60), and the 2′,4′-$(CH_3)_2$ (No. 62) analogs are between 40- to 110-fold more active than amiloride. Addition of phenyl to the methylene portion of the benzyl group (No. 66) enhances the activity of benzamil (No. 54) by another order of magnitude. The 2-phenethyl compound (No. 71) is as potent as benzamil (No. 54). The phenyl (No. 76, phenamil) and 2,6-dimethylphenyl (No. 77) substituents are, respectively, 5- to 7- fold more active than amiloride. The introduction of two small alkyl groups (No. 78) on a terminal guanidino nitrogen atom decreases activity; however, two large alkyl groups (No. 79) slightly enhances activity.

The guanidino moiety is required for inhibition of the exchanger. Analogs with substitution of the guanidino moiety with an aminoguanidino group (No. 83) or deletion of the guanidino moiety and replacement with HO— (No. 85) are inactive.

b. 5-Amino Substituents. Amiloride analogs with hydrophobic substituents on the 5-amino moiety have enhanced activity against the Na^+/Ca^{2+} exchanger. The introduction of adamantyl (No. 13), benzyl (No. 18), substituted benzyl (Nos. 19–23), dialkyl (No. 34), or an alkyl and a substituted benzyl (No. 35–37) are between 50- to 115- fold more potent than amiloride.

c. 6-Position Substituents. Replacement of the 6-Cl group of amiloride by H (No. 2), Br (No. 4) or I (No. 5) does not alter activity. However, the 6-F (No. 3) compound is an order of magnitude less active.

d. 5-Position and Terminal Guanidino Substituents. Introduction of a substituted benzyl group on the terminal guanidino nitrogen atom and a substituted benzyl group (No. 87) or two alkyl groups (No. 86) on the 5-amino nitrogen atom produced compounds 150- to 300-fold more potent than amiloride. This structural maneuver enhances activity against the Na^+/Ca^{2+} exchanger, but markedly diminishes activity against the Na^+ channel.

e. Replacement of the Pyrazine Ring. As seen with the Na^+/H^+ exchanger, the replacement of the 3,5-diamino-6-chloropyrazinyl moiety by certain other groups can be accomplished and still achieve enhancement of Na^+/Ca^{2+} exchange inhibitory activity over that observed with amiloride. The diphenylmethyl and 2-(4-chlorophenyl)propenyl substituted analogs (Nos. 89 and 91) are 11-fold more active than amiloride. The 2-(4-pyridyl)ethenyl (No. 88) and the 2-anthraquinolyl (No. 90) analogs are inactive against the Na^+/Ca^{2+} exchanger, but maintain or enhance activity against the Na^+/H^+ exchanger.

f. 5- and 6-Position Substituents. Replacement of the 5- and 6-substituents of amiloride by H— (No. 41) results in a profound decrease in activity. Replacing the 6-Cl group of 5-(N-ethyl-N-isopropyl) amiloride (No. 31) by 6-I (No. 47) decreases activity.

g. 3-Position substituents. The effect of amiloride analogs with substitutions at this site on the Na^+/Ca^{2+} exchanger has not been studied.

2. Specificity of Amiloride Analogs for Inhibition of the Na^+/Ca^{2+} Exchanger

Relatively high concentrations of amiloride are required to inhibit the Na^+/Ca^{2+} exchanger. Although proper substituents on either the 5-amino or the terminal guanidino nitrogen atoms increase the activity of these analogs by up to 100-fold, the IC_{50} is only in the range of 10 μM. This is a considerably greater concentration than is required for inhibition of a number of other transporters, such as the epithelial Na^+ channel and the Na^+/H^+ exchanger. It is important to note that of the amiloride analogs examined, those active against the Na^+/Ca^{2+} exchanger are equal or more potent inhibitors of the Ca^{2+} channel (Nos. 1, 25, 30, 31, 54, 59, 76)[2] [11].

Amiloride analogs bearing substituents on both the 5-amino and terminal guanidino nitrogen atoms have increased activity for the exchanger, with IC_{50} of 3.5 to 7 μM. These analogs do not inhibit the Na^+ channel at this concentration and therefore do show some specificity for the Na^+/Ca^{2+} exchanger. However, the effects of these analogs on other transport

[2] Kaczorowski, G., and Garcia, M. (*unpublished observations*).

systems and on kinases has not been examined. Also these compounds are quite hydrophobic and are difficult to use at concentrations above 10 μM.

D. Na^+/K^+-ATPase

The Na^+/K^+-ATPase is present in many eukaryotic cells, transporting Na^+ out of and K^+ into cells creating transcellular chemical gradients for both Na^+ and K^+. The Na^+/K^+-ATPase is inhibited by cardiac glycosides and is also inhibited by amiloride. The inhibition of the Na^+/K^+-ATPase by amiloride has been studied by a number of investigators using intact cells, membrane vesicles, and partially purified pump [37, 69, 78, 86]. It is unclear if amiloride interacts with the pump by binding to a Na^+ binding site, an ATP binding site, or by an alternative mechanism. It is also unclear if amiloride binds to an extracellular or intracellular site on the protein.

The pharmacology of amiloride inhibition of the pump has been characterized most extensively using partially purified pump from dog kidney. The IC_{50} for inhibition of ATPase activity by amiloride is greater than 3 mM. Appropriate substitution of the 5-amino moiety of amiloride decreases the IC_{50} to as low as 200 μM [86]. 5-(N,N-Dimethyl)amiloride inhibits the pump with an IC_{50} of 3 mM, which is four orders of magnitude greater than the IC_{50} for inhibition of Na^+/H^+ exchanger [86]. The effect of introduction of substituents on the terminal nitrogen atom of the guanidino moiety has not been studied except for benzamil, which has an IC_{50} less than 1 mM when studied in membrane vesicles from rabbit kidney [37].

E. Na^+-Coupled Solute Transport

The transport of a number of solutes across the apical plasma membrane of epithelia has been shown to be tightly coupled to Na^+. The Na^+-D-glucose, Na^+-L-alanine, and Na^+-PO_4^{3-} transporters are inhibited by amiloride at concentrations greater than 1 mM [16, 37]. The interaction of amiloride with the Na^+-D-glucose transporter has been studied in brush border membrane vesicles from rabbit kidney cortex and in LLC-PK1/CL4 cells, a cell line with transport characteristics resembling renal proximal tubular cells. The IC_{50} of amiloride is 2 mM [37]. The inhibition is binding to a Na^+ binding site on the transporter [16, 37]. Amiloride is also a competitive inhibitor of Na^+-dependent [^{3}H]phlorizin binding [37]. This transporter is inhibited by amiloride analogs bearing hydrophobic substituents on the 5-amino moiety (Nos. 30, 31) or on terminal nitrogen of the guanidino moiety (Nos. 59, 76) with IC_{50} between 0.1 and 0.3 mM [16], when assayed in the presence of a low concentration of Na^+.

Na^+-dependent L-alanine and PO_4^{3-} transporters are inhibited by amiloride at concentrations greater than 1 mM. Introduction of hydrophobic groups on the 5-amino moiety or the terminal nitrogen atom of the guanidino moiety slightly decreases the IC_{50} [37].

F. Other Ion Transporters

a. Voltage-Gated Na^+ Channel. A voltage-gated Na^+-selective ion channel is present in electrically excitable cells and is inhibited specifically by the toxins tetrodotoxin and saxitoxin in the nanomolar range. It has recently been shown that amiloride inhibits this channel in both synaptosomes and heart membrane vesicles, as measured by inhibition of veratridine-activated $^{22}Na^+$ flux across membrane vesicles [80]. The IC_{50} for inhibition of the $^{22}Na^+$ flux by amiloride is 600 μM. Amiloride analogs bearing hydrophobic substituents on the 5-amino moiety or the terminal nitrogen of the guanidino moiety have lower IC_{50} (6 μM for 5-(N-ethyl-N-isopropyl)amiloride (No. 31) and 37 μM for benzamil (No. 54)). It is unknown if varying the Na^+ concentration used in the assay affects the IC_{50}. Amiloride and analogs with hydrophobic substituents on the 5-amino moiety or on the terminal nitrogen atom of the guanidino moiety inhibit the binding of [^{3}H]batrachotoxin-A 20-alpha-benzoate and [^{3}H]tetracaine to the channel [80].

b. Voltage-Gated Ca^{2+} Channel. The voltage-gated Ca^{2+} channel in sarcolemma vesicles from pig heart is inhibited by amiloride with an IC_{50} of 90 μM. Amiloride analogs bearing hydrophobic substituents on the 5-amino moiety or the terminal nitrogen of the guanidino moiety (Nos. 25, 30, 31, 54, 59, 76) are between five- to 75-fold more active than amiloride.[3] Amiloride analogs bind to a cation binding site in the pore of the Ca^{2+} channel and allosterically alter binding of dihydropyridines, aralkylamines and benzothiazepines [33]. Electrophysiologic studies with intact frog atrial myocytes have shown that the amiloride 3',4'-dichlorobenzamil (No. 59) inhibits the voltage-gated Ca^{2+} channel with an IC_{50} of 0.8 μM and is a more potent inhibitor of the Ca^{2+} channel than of the Na^+/Ca^{2+} exchanger [11].

c. K^+ channel. Electrophysiologic studies with intact frog atria myocytes suggest that 3',4'-dichloro-

[3] Kaczorowski, G., and Garcia, M. (*unpublished observations*).

benzamil (No. 59) inhibits the delayed rectifier K^+ channel with 30 to 40% inhibition of the K^+ current at a concentration of 5 μM [11].

d. Nicotinic Acetylcholine Receptor. Amiloride has been observed to inhibit the nicotinic acetylcholine receptor isolated from *Torpedo*[4]. The IC_{50} is approximately 100 μM.

G. Enzymes and Receptors

a. Protein Kinases. Phosphorylation of cellular proteins by protein kinases has been shown to be involved in the regulation of a variety of cellular processes including metabolism, transport, growth and differentiation. Amiloride has been shown to inhibit a number of protein kinases, including types I and II cAMP-dependent protein kinase [68], protein kinase C [9], and protein kinase activity associated with the insulin receptor, epidermal growth factor (EGF) receptor, and the platelet-derived growth factor receptor [29]. The IC_{50} for inhibition of EGF receptor protein kinase is 350 μM. The inhibition is competitive with ATP, suggesting that amiloride binds to an ATP binding site on the enzyme. Amiloride is a noncompetitive inhibitor of substrate (histone) phosphorylation [29]. Both amiloride and 5-(N,N-dimethyl)amiloride inhibit purified protein kinase C with IC_{50} of approximately 1 mM [9]. Amiloride inhibits types I and II cAMP-dependent protein kinases with an IC_{50} of approximately 1 mM [68]. The interaction of other amiloride analogs with protein kinases has not been studied.

b. Adenylate Cylcase. Amiloride inhibits protein kinases through competition with ATP for an ATP binding site, and therefore might inhibit other ATP-dependent processes. Adenylate cyclase catalyzes the conversion of ATP to cAMP. The generation of cAMP in fish erythrocytes is inhibited by amiloride in a dose-dependent manner, with an IC_{50} of 6 μM [58]. The effect of amiloride analogs in this system have not been examined.

c. Monoamine Oxidase. Amiloride is a comparative inhibitor of monoamine oxidase activity measured in rat brain homogenate [63].

d. Acetylcholinesterase. Amiloride is a noncompetitive inhibitor of acetylcholinesterase, with an IC_{50} in the range of 20 to 60 μM [28].

e. Urokinase-Type Plasminogen Activator. Amiloride inhibits urokinase-type plasminogen activator with an IC_{50} of 7 μM [79]. Amiloride does not inhibit tissue-type plasminogen activator, plasma kallikrein, or thrombin.

f. Renal Kinins. Amiloride is a noncompetitive inhibitor of rat and human renal kallekrein, with an IC_{50} in the 85 to 230 μM range [59].

g. Muscarinic Acetylcholine Receptor. Amiloride has been shown to inhibit the muscarinic acetylcholine receptor. Studies with rabbit pancreatic acini have shown that amiloride inhibits amylase secretion induced by carbachol with an IC_{50} of 40 μM, $^{45}Ca^{2+}$ efflux induced by carbachol with an IC_{50} of 80 μM, and is a competitive inhibitor of [^{3}H]quinuclidinyl benzylate binding to the muscarinic acetylcholine receptor [49].

h. Alpha and Beta Adrenergic Receptors. Amiloride in a competitive inhibitor of [^{3}H]prazocin binding to $alpha_1$ receptors in membrane vesicles from rat renal cortex or bovine carotid artery with an IC_{50} of 24 to 33 μM [10, 38]. Amiloride is also a competitive inhibitor of [^{3}H]rauwolscine binding to $alpha_2$ receptors and [^{125}I]iodocyanopindolol binding to beta adrenergic receptors in rat renal cortical membranes with IC_{50} of 14 and 84 μM, respectively [38]. Both benzamil and 5-(N-ethyl-N-isopropyl)-amiloride are between two- to 25-fold more potent than amiloride in inhibiting specific ligand binding to $alpha_1$, $alpha_2$, and beta adrenergic receptors. Amiloride blocks both veratridine and norepinephrine-stimulated smooth muscle contraction. The inhibition of smooth muscle contraction by amiloride may be due to inhibition of the $alpha_1$ adrenergic receptor or due to inhibition of a number of other cellular processes [10].

i. Atrial Natriuretic Factor (ANF) Receptor. ANF is a peptide hormone secreted by atrial myocytes and binds to specific cell surface receptors. Recent studies have shown that ANF binds to both low and high affinity receptors, and that amiloride (100 μM) increases the number of high affinity sites in membranes obtained from adrenal zona glomerulosa [60]. This change in the number of binding sites is associated with a conformational change in the 15,000-Da receptor, as shown by a change in the elution profile on steric exclusion chromatography [60].

H. Other Cellular Effects

a. DNA and RNA Synthesis. Cells grown in the absence of growth factors may arrest in G0. Addition of serum or defined growth factors stimulates

[4] Karlin, A. (*personal communication*).

DNA, RNA, and protein synthesis. In intact cells, addition of amiloride inhibits the growth factor-induced DNA, RNA, and protein synthesis [48, 50]. The effect of amiloride on protein synthesis is a direct effect (*see below*). The effect of amiloride on DNA and RNA synthesis may be indirect. Lowering the extracellular Na^+ concentration inhibits DNA and RNA synthesis, suggesting that entry of Na^+ into cells may be a requirement for these events. Amiloride analogs inhibit DNA replication with IC_{50} similar to that observed for inhibition of Na^+/H^+ exchanger [50], suggesting that amiloride inhibition of DNA synthesis is indirect, possibly through inhibition of Na^+/H^+ exchange.

Amiloride also blocks RNA synthesis, as measured by incorporation of [^{3}H]uridine into RNA. Following stimulation of cells with growth factors, two peaks of incorporation of [^{3}H]uridine into RNA are observed. Amiloride abolishes the late peak of [^{3}H]uridine incorporation, while only slightly decreasing the initial peak [48]. It is unknown if this is direct or an indirect effect of amiloride. The effect of amiloride analogs on RNA synthesis has not been examined.

b. Protein Synthesis. In both intact cells and cell free retriculocyte lysate, amiloride inhibits protein synthesis as measured by incorporation of radiolabeled amino acids into proteins [48, 54, 57, 86]. The IC_{50} for inhibition of protein synthesis in reticulocyte lysate is approximately 300 to 400 μM [54, 57, 86]. Amiloride inhibits protein synthesis in intact cells with an IC_{50} between 100 and 400 μM, and varies with the cell type studied. Fibroblasts (Swiss 3T3 cells (IC_{50} = 100 μM) [57] are more sensitive than hepatocytes and A431 cells (IC_{50} = 400 μM) [54, 57, 86]. Amiloride both lowers the initial rate of incorporation of [^{35}S]methionine into proteins and the plateau levels of [^{35}S]methionine labeled proteins [54]. The IC_{50} for inhibition of synthesis of albumin in hepatocytes is 30 μM, and is 14-fold more sensitive to amiloride inhibition than is the rate of overall protein synthesis, suggesting that the IC_{50} for inhibition of synthesis of individual proteins may vary [54]. The effect of amiloride analogs on the inhibition of protein synthesis has been studied. In cell free systems, amiloride analogs bearing hydrophobic substituents on the 5-amino moiety showed little variation in their IC_{50} for inhibition of protein synthesis. However, when protein synthesis was studied in intact cells, the IC_{50} of these analogs varies over 25-fold. The rank order of inhibition of protein synthesis by these amiloride analogs correlated with the rank order of decreasing Rb content of cells, although the absolute IC_{50} was 5–7 times higher for inhibition of protein synthesis. These data suggest that amiloride analogs may inhibit protein synthesis in intact cells indirectly through inhibition of ion transport or metabolism [86]. The mechanism by which amiloride directly inhibits protein syntheses in cell free systems in unclear. As protein synthesis requires ATP, amiloride inhibition may be occurring through competition with ATP and inhibition of ATP-dependent enzymes.

c. Metabolism. It has been recently demonstrated that amiloride analogs deplete intracellular levels of ATP [77, 86]. Addition of analogs with substituents on the 5-amino moiety (at concentrations >30 μM) to A431 cells or to proximal tubular cells in suspension results in a fall in the intracellular levels of ATP, whereas addition of either amiloride or 5-(N,N-dimethyl)amiloride (A431 cells) at concentrations of 2 mM had no effect [86].

The depletion of intracellular ATP levels may be due in part to inhibition of oxidative phosphorylation. The consumption of O_2 in renal proximal tubular cells is tightly coupled to the rate of ion transport. Addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) uncouples O_2 consumption from ion transport. Amiloride analogs bearing hydrophobic substituents on the 5-amino moiety inhibit O_2 consumption in FCCP-treated renal proximal tubular cells in suspension with IC_{50} of 250 to 500 μM. The IC_{50} of amiloride is greater than 1 mM. These data suggest that amiloride analogs have a direct effect on oxidative phosphorylation, independent of their effects on ion transport [77].

III. Use of Amiloride Analogs of Probes for Characterizing Transport Proteins

A. Radiolabeled Amiloride Analogs

a. Amiloride. [^{14}C]Amiloride has been synthesized with a specific activity of 54 mCi/mmol by the reaction of [^{14}C]guanidine with methyl 3,5-diamino-6-chloropyrazinecarboxylate [21]. [^{3}H]Amiloride has not been synthesized, as all of amiloride's protons are freely exchangeable.

b. Amiloride Analogs Bearing Substituents on the 2-Carbonylguanidino Moiety. A number of tritium-labeled amiloride analogs have been synthesized, including [^{3}H-phenyl]phenamil, [^{3}H-benzyl]benzamil [24], and [^{3}H-benzyl]6-bromobenzamil, with specific activities between 2 and 21 Ci/mmol. These analogs have been synthesized by the reaction of synthon I (*see* Fig. 2) 1-methyl-2-(3,5-diamino-6-

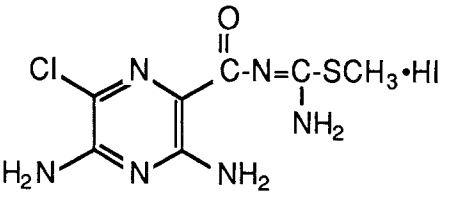


Fig. 2. Synthon for the synthesis of amiloride analogs bearing substituents on the terminal guanidino nitrogen atom

chloropyrazinoyl)pseudothiouronium iodide or the 6-Br synthon I with [^{3}H]aniline or [^{3}H]benzylamine [22]. [^{3}H-Methyl]-6-bromomethylamiloride has been synthesized by the reaction of bromoamiloride with [^{3}H] CH_3I and has a specific activity of 1 Ci/mmol [53]. The methyl group is attached to the guanidino nitrogen atom adjacent to the carbonyl moiety.

Amiloride has been coupled to albumin via the N-hydroxysuccinimide ester of the amiloride analog bearing a 5-carboxypentyl group on the terminal guanidino nitrogen [45]. This was then radioiodinated by conventional techniques placing the ^{125}I on albumin. A new amiloride analog bearing a 4′-hydroxyphenethyl moiety (No. 72) should prove useful in synthesis of radioiodinated amiloride analogs (No. 73 and 74).

c. Amiloride Analogs Bearing Substituents on the 5-Amino Group. Both 5-([N-ethyl-N-[^{3}H]propyl)amiloride [81] and 5-([N-methyl-N-[^{3}H]isobutyl)amiloride[5] have been synthesized with specific activities of 2 and 28 Ci/mmol, respectively.

d. Amiloride Analogs Bearing Substituents on the 6-Position of the Pyrazine Ring. A new method has recently been described for replacing an H- in the 6-position with ^{125}I [15]. 6-Iodoamiloride and a number of 6-iodoamiloride analogs have been synthesized with this procedure.

B. Binding Studies Using Radiolabeled Amiloride Analogs

A number of studies have been published recently using radiolabeled amiloride analogs to identify and characterize binding sites of amiloride analogs, with plasma membranes or microsomal membranes derived from cells known to have amiloride-sensitive transporters. Analogs bearing substituents on the 5-amino group, including 5-(N-methyl-N-[^{3}H]isobutyl)amiloride and 5-(N-ethyl-N-[^{3}H]isopropyl)amiloride have been used to characterize binding to the putative Na^+/H^+ exchanger [30, 81]. [^{3}H]Benzamil, [^{3}H]phenamil, and [^{3}H]6-bromomethylamiloride have been used to characterize binding to epithelial Na^+ channel [2, 5, 25, 46, 71].

C. Photoaffinity Labels

Three major photoactive groups have been used in the development of photoreactive amiloride analogs. These include arylhalides, arylazides, and aromatic ethers.

a. Aryl Halides. Photolysis of 6-bromo, 6-iodo, or 6-chloro analogs of amiloride can lead to the formation of a free radical, and subsequent covalent incorporation into adjacent proteins. This approach has been used to identify putative subunits of the epithelial Na^+ channel, using [^{3}H]6-bromobenzamil and [^{3}H]6-bromomethylamiloride as photoactive amiloride analogs that bind to the Na^+ channel with both high affinity and specificity [6, 46]. Amiloride and 6-bromobenzamil have a major absorption peak at 360 nm, and we have used this wavelength light to photoactive 6-bromobenzamil [46]. [^{14}C]5-(N-ethyl-N-isopropyl)-6-bromoamiloride has been used as a photoaffinity label for the Na^+/H^+ exchanger [32].

b. Arylazides. 4-Azidophenamil has been synthesized; however, conditions to demonstrate incorporation of this amiloride analog into the Na^+ channel have not been defined.

c. Aromatic Ethers. Aromatic ethers have been shown to undergo photoactivation and photoincorporation into proteins by the mechanism of aromatic nucleophilic photosubstitution [17]. Photoactivation leads to formation of a reactive intermediate with a half life of less than one msec, at which time nucleophiles can bind covalently to the amiloride analog. If no incorporation occurs, the drug returns to the ground state.

Photoreactive amiloride analogs have been synthesized with a 2′-methoxy-5′-nitrobenzyl moiety located either on the terminal nitrogen atom of the guanidino moiety or on the 5-amino moiety (which also bears either a hydrogen or an ethyl group). These drugs undergo photoactivation with 313 nm wavelength light. The 2′-methoxy-5′-nitrobenzamil has been used to photolabel and identify putative components of the Na^+ channel [44]. Anti-amiloride antibodies were used to detect the photolabel after

[5] 5-(N-methyl-N-^{3}H-isobutyl)amiloride was prepared by New England Nuclear by the catalytic tritiation of 5-[N-methyl-N-(2-methylallyl)]amiloride.

photoincorporation into the channel. An amiloride analog with the photoactive group on the 5-amino moiety has been used to label the Na^+/H^+ exchanger [85].

D. Affinity Matrices

Amiloride has been coupled to support matrices through either the terminal nitrogen atom of the guanidino moiety or through the 5-amino group of the pyrzine ring. Three separate methods have been used to couple amiloride to a matrix through the guanidino moiety. One method has been used to couple through the 5-amino moiety.

The first method involves a reactive amiloride synthon, 1-methyl-2-(3,5-diamino-6-chloropyrazinoyl)pseudothiouronium iodide (*see* Fig. 2). The synthon reacts with primary or secondary amines to form the corresponding pyrazinoylguanidine. The synthon was allowed to react with aminohexylsepharose in the presence of a hindered base (triethylamine). The product was amiloride coupled to sepharose through a six-carbon spacer arm [47].

The second method involves the use of an amiloride analog bearing a 5'-carboxypentyl group on the terminal guanidino nitrogen atom. A mixed anhydride was synthesized using isobutyl chloroformate, which reacts with primary amines to form the corresponding amide. Amiloride was coupled to albumin using this procedure, and coupling to aminohexylsepharose by this method should be straightforward. The amiloride-albumin complex was subsequently coupled to sepharose and has been used to affinity purify anti-amiloride antibodies [45].

A third method involves the reaction of amiloride with cyanogen bromide-activated sepharose. The site on the amiloride which binds to the resin is unclear. Coupling may occur on the terminal nitrogen atom of the guanidino moiety. The amiloride sepharose resin was subsequently used as an affinity resin to purify the epithelial Na^+ channel [5].

A method for coupling amiloride through the 5-amino group utilized an amiloride analog bearing an isothiocyanato group. 5-[N-(3-aminophenyl)] amiloride was converted to its corresponding isothiocyanate and then coupled to dextran [14].

E. Anti-Amiloride Antibodies

An amiloride analog bearing a 5-carboxypentyl group on the terminal nitrogen of the acylguanidino moiety of amiloride was coupled to albumin by generation of a mixed anhydride. Approximately 10 moles of amiloride were bound per mole of albumin. The amiloride-bovine serum albumin was used to raise anti-amiloride antibodies in rabbits, which were subsequently affinity purified with an amiloride-rabbit serum albumin affinity column [45].

F. Methodologic Problems

a. Solubility Characteristics. Amiloride and many of its analogs are soluble in aqueous solutions at concentrations less than 1 to 10 mM. The solubility of the analogs usually decreases with addition of hydrophobic groups. Stock solutions of amiloride and many of the amiloride analogs may be made in DMSO at concentrations of 1 M. We generally make stock solutions of amiloride analogs at a concentration of 10 mM in DMSO and store the solutions at −20°C protected from light.

b. pK_a. The guanidino moiety of amiloride is protonated at physiologic pH, and it is this positively charged species that appears to be required for inhibition of ion transport. The pK_a of amiloride is 8.7 in 30% ethanol of 8.8 in water. The pK_a tends to decrease with substitution of electron-withdrawing groups on the terminal nitrogen of the guanidino moiety. Table 8 lists of pK_a's of amiloride and several amiloride analogs.

c. Lipid Solubility. Both amiloride and amiloride analogs bearing hydrophobic substituents on the 5-amino and acylguanidino moieties of amiloride are hydrophobic when uncharged, and dissolve easily in organic solvents, such as octanol. The protonated (i.e., charged) species are more soluble in aqueous solutions, and have varying solubility in octanol. The lipophilicity of amiloride and its analogs has been determined by measuring the distribution between a buffered aqueous phase and octanol or chloroform. The results for several amiloride analogs are listed in Table 8.

d. Intracellular Accumulation. Several studies have shown that amiloride accumulates within cells. Amiloride and its analogs may diffuse across membranes in the unprotonated or protonated form, or may be transported across. Amiloride uptake into hepatocytes was shown to be dependent on the extracellular Na^+ concentration and on temperature [54]. The $t_{1/2}$ for cellular uptake was 5 to 10 min. These data suggest that amiloride uptake in hepatocytes was due, in part, to transport across the plasma membrane. The intracellular concentration of amiloride was 10-fold greater than the extracellular concentration, assuming that cellular amiloride was free in solution and not bound to lipid or compartmentalized. Amiloride was also shown to accumulate within frog skin epithelial cells [13] and

within A431 cells [29] at intracellular concentrations greater than the extracellular concentration. Amiloride has been shown to diffuse across red blood cell and neutrophil plasma membranes with a permeability coefficient of approximately 10^{-7} cm · sec^{-1} [4, 76]. The uptake of amiloride was shown to be temperature dependent and independent of the extracellular [Na^+]. In neutrophils 75% of the intracellular amiloride was considered to be in the lysozomal compartment [76]. In other cell types, the extent to which intracellular amiloride localized to intracellular compartments or is bound to membranes is uncertain.

e. Absorption Characteristics. The absorption characteristics of amiloride and a number of its analogs have been studied. In general, three major absorption peaks have been observed at approximately 360 to 370 nm, 265 to 290 nm, and 215 to 235 nm. The absorption peaks are broad and appear to vary slightly among the different analogs and with the solvent system used. The extinction coefficients at these wavelengths are in the range of 10,000 M^{-1} cm^{-1} to 25,000 M^{-1} cm^{-1}.

f. Fluorescence Characteristics. Amiloride and its analogs are aromatic compounds and are highly fluorescent. Amiloride has excitation maxima at 286 and 360 nm, and an emission maximum at 410 to 414 nm. Both the fluorescence and absorption properties of the analogs may interfere with techniques that utilize fluorescent probes to measure intracellular pH and intracellular Ca^{2+}. Amiloride may quench the fluorescence of both acridine orange and carboxyfluorescein, probes that have been used to measure intravesicular and intracellular pH. The absorption peak of amiloride at 360 nm may interfere with the absorption of quin 2 (excitation at 342 nm) and fura 2 (excitation at 340 and 380 nm), probes that have been used to measure intracellular calcium ions.

IV. Summary

Amiloride inhibits most plasma membrane Na^+ transport systems. We have reviewed the pharmacology of inhibition of these transporters by amiloride and its analogs. Thorough studies of the Na^+ channel, the Na^+/H^+ exchanger, and the Na^+/Ca^{2+} exchanger, clearly show that appropriate modification of the structure of amiloride will generate analogs with increased affinity and specificity for a particular transport system. Introduction of hydrophobic substituents on the terminal nitrogen of the guanidino moiety enhances activity against the Na^+ channel; whereas addition of hydrophobic (or hydrophilic) groups on the 5-amino moiety enhances activity against the Na^+/H^+ exchanger. Activity against the Na^+/Ca^{2+} exchanger and Ca^{2+} channel is increased with hydrophobic substituents at either of these sites. Appropriate modification of amiloride has produced analogs that are several hundred-fold more active than amiloride against specific transporters. The availability of radioactive and photoactive amiloride analogs, anti-amiloride antibodies, and analogs coupled to support matrices should prove useful in future studies of amiloride-sensitive transport systems.

The use of amiloride and its analogs in the study of ion transport requires a knowledge of the pharmacology of inhibition of transport proteins, as well as effects on enzymes, receptors, and other cellular processes, such as DNA, RNA, and protein synthesis, and cellular metabolism. One must consider whether the effects seen on various cellular processes are direct or due to a cascade of events triggered by an effect on an ion transport system.

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