Effects of [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]alkanoic Acids on Chloride Transport in Primary Astroglial Cultures

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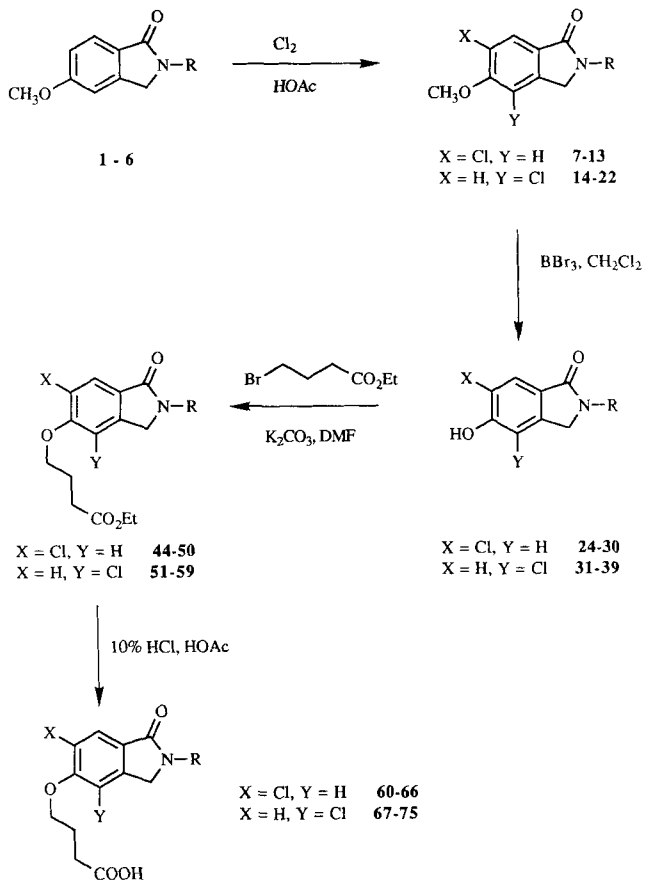
Received April 12, 1993, from the *Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360, and *Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn, AL 36849.* Accepted for publication September 9, 1993®.

Abstract □ It has been demonstrated that agents which inhibit chloride influx and, therefore, lower intracellular chloride levels in the astrocyte, a major cell type in the cerebral gray matter, inhibit astrocytic swelling *in vitro* and *in vivo*. Herein, we report additional examples of a series of [(*N*-alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]alkanoic acids and their effects upon ion transport in primary rat astrocyte cultures. The 4-chloro-substituted 1-oxoisoindolines demonstrated superior astrocytic chloride influx inhibitory activity as compared to the 6-chloro and non-chlorinated analogs. The four-carbon acid side chain derivatives were more active than the three- and two-carbon analogs. The pharmacological profile of these compounds was examined with respect to inhibition of the Cl^-–Cl^-/Cl^-–HCO_3^- anion exchanger and Na^+–K^+–$2Cl^-$ cotransport mechanisms in glia, and the compounds were found to exhibit a similar profile to that of furosemide by inhibiting both transporters.

The National Research Council[1] has emphasized the need for escalating the research effort in the area of injury prevention and treatment. In 1985, the head injury mortality rate was estimated at 25 per 100 000 population and an overall estimate of incidence in the United States is in the range of 200 per 100 000 per year.[2] With mortality rates for patients suffering from severe closed head injury in the vicinity of 40–50%, Becker[3] and Marshall[4] found that the single most frequent cause of death was uncontrolable increase in intracranial pressure due to edema formation. Stroke, the third leading cause of death in the United States, is also accompanied by a significant cytotoxic (intracellular) cerebral edema component.[5]

Edema within the cerebral white matter is primarily of a vasogenic nature (extracellular) while that associated with the cerebral cortex is primarily cytotoxic and occurs mainly as a manifestation of swelling within a major cell type, the astrocyte. Astrocytes are nonsynaptic glial cells of ectodermal origin and, along with oligodendrocytes, compose the macroglia. In addition to various other functions in the central nervous system (CNS), astrocytes serve in a liaison capacity to exchange gases and nutrients between neurons and neurocapillaries as a structural component of the blood–brain barrier.[6] Swelling of astrocytes may lead to detrimental effects upon this function or even lysis resulting in hypoxic/ischemic injury and necrosis.[7] It has been suggested that astrocytic swelling in pathological conditions associated with stroke or closed head injury can result from enhanced activation of the Cl^-–Cl^-/Cl^-–HCO_3^- and Na^+/H^+ ion exchange systems.[8] The ability of certain agents to prevent astrocytic swelling after closed head injury and to inhibit the swelling-induced release of excitotoxic amino acids such as L-glutamate has been found to closely correlate with their ability to predominantly inhibit anion exchange transport mechanisms in astrocytes.[9-11]

We previously reported the activity of a series of [(*N*-alkyl-1,3-dihydro-1,3-dioxo- and 1-oxoisoindolin-5-yl)oxy]alkanoic acids with regard to chloride influx inhibition in primary rat astrocyte cultures.[12] Here, we report the activity of additional



Scheme 1

examples within the 1-oxoisoindoline series with regard to their effect upon chloride influx in cultured rat astrocytes. The derivatives reported herein extend the original series of 1-oxoindolines in that chloro-substituted analogs were prepared which are similar to the halogen substitution present on ethacrynic acid and a series of 1-indanone and fluorene antiswelling agents[9,13] to which these compounds are structurally related. In addition, the substituents on the ring nitrogen have been varied to a greater extent to include homologous ring substituents with increasing lipophilic character without greatly increasing steric bulk in that position. Variation of the acid side chain and its effect upon activity is also reported. Also, effects on specific ion transporters in astrocytes, namely those discussed above, have been examined. Presence of the chlorine at a specific position of the aromatic ring in conjunction with a nitrogen substituent of suitable lipophilicity and steric size produced analogs of significantly greater potency compared to the original series.[12]

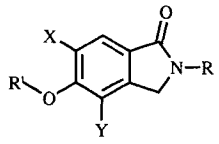
Experimental Section

All chemicals were used as received from the manufacturers. Thin-layer chromatography was performed using silica gel 60 coated plates, and column chromatography was performed using silica gel 60 (70–230

* Abstract published in *Advance ACS Abstracts*, January 15, 1994.

0022-3549/94/1200-571$04.50/0

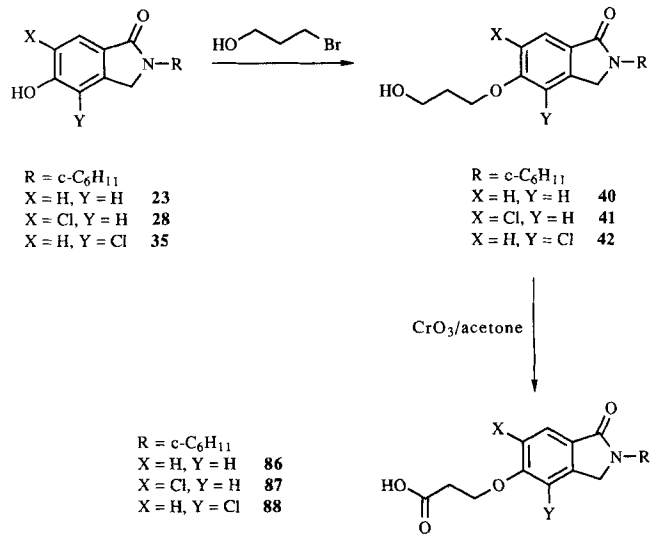
Table 1—Physical Constants for *N*-Alkyl-1,3-dihydro-1-oxoisoindolines



No.	R	R′	X(6)	Y(4)	Mp, °C	Yield, %	Formula
1	c-C_5H_9	CH_3	H	H	93–96	52	$C_{14}H_{17}NO_2$
2	c-C_6H_{11}	CH_3	H	H	113–116	49	$C_{15}H_{19}NO_2$
3	c-C_7H_{13}	CH_3	H	H	Wax	23	$C_{16}H_{21}NO_2$
4	c-C_8H_{15}	CH_3	H	H	Wax	24	$C_{17}H_{23}NO_2$
5	2-CH_3-c-C_6H_{10}	CH_3	H	H	109–111	36	$C_{16}H_{21}NO_2$
6	Adamantyl	CH_3	H	H	181–183	17	$C_{19}H_{23}NO_2$
7	CH_3	CH_3	Cl	H	150–154	47	$C_{10}H_{10}ClNO_2$
8	C_2H_5	CH_3	Cl	H	148–151	28	$C_{11}H_{12}ClNO_2$
9	C_3H_7	CH_3	Cl	H	114–116	33	$C_{12}H_{14}ClNO_2$
10	C_4H_9	CH_3	Cl	H	87–90	41	$C_{13}H_{16}ClNO_2$
11	c-C_6H_{11}	CH_3	Cl	H	169–172	20	$C_{15}H_{18}ClNO_2$
12	c-C_8H_{15}	CH_3	Cl	H	114–117	26	$C_{17}H_{22}ClNO_2$
13	2-CH_3-c-C_6H_{10}	CH_3	Cl	H	130–132	10	$C_{16}H_{20}ClNO_2$
14	C_2H_5	CH_3	H	Cl	85–88	64	$C_{11}H_{12}ClNO_2$
15	C_3H_7	CH_3	H	Cl	Gum	56	$C_{12}H_{14}ClNO_2$
16	C_4H_9	CH_3	H	Cl	73–76	47	$C_{13}H_{16}ClNO_2$
17	c-C_5H_9	CH_3	H	Cl	90–92	45	$C_{14}H_{16}ClNO_2$
18	c-C_6H_{11}	CH_3	H	Cl	104–108	20	$C_{15}H_{18}ClNO_2$
19	c-C_7H_{13}	CH_3	H	Cl	125–128	38	$C_{16}H_{20}ClNO_2$
20	c-C_8H_{15}	CH_3	H	Cl	92–95	52	$C_{17}H_{22}ClNO_2$
21	2-CH_3-c-C_6H_{10}	CH_3	H	Cl	133–135	29	$C_{16}H_{20}ClNO_2$
22	Adamantyl	CH_3	H	Cl	70–75	67	$C_{19}H_{22}ClNO_2$
23	c-C_6H_{11}	H	H	H	228–230	81	$C_{14}H_{17}NO_2$
24	CH_3	H	Cl	H	245–250	66	$C_9H_8ClNO_2$
25	C_2H_5	H	Cl	H	225–231	71	$C_{10}H_{10}ClNO_2$
26	C_3H_7	H	Cl	H	263–265	89	$C_{11}H_{12}ClNO_2$
27	C_4H_9	H	Cl	H	230–234	60	$C_{12}H_{14}ClNO_2$
28	c-C_6H_{11}	H	Cl	H	310 dec	79	$C_{14}H_{16}ClNO_2$
29	c-C_8H_{15}	H	Cl	H	>255	100	$C_{16}H_{20}ClNO_2$
30	2-CH_3-c-C_6H_{10}	H	Cl	H	>290	100	$C_{15}H_{18}ClNO_2$
31	C_2H_5	H	H	Cl	185–188	100	$C_{10}H_{10}ClNO_2$
32	C_3H_7	H	H	Cl	210–212	60	$C_{11}H_{12}ClNO_2$
33	C_4H_9	H	H	Cl	188–190	26	$C_{12}H_{14}ClNO_2$
34	c-C_5H_9	H	H	Cl	223–226	82	$C_{13}H_{14}ClNO_2$
35	c-C_6H_{11}	H	H	Cl	230–232	100	$C_{14}H_{16}ClNO_2$
36	c-C_7H_{13}	H	H	Cl	212–215	73	$C_{15}H_{18}ClNO_2$
37	c-C_8H_{15}	H	H	Cl	205–208	65	$C_{16}H_{20}ClNO_2$
38	2-CH_3-c-C_6H_{10}	H	H	Cl	210–212	70	$C_{15}H_{18}ClNO_2$
39	Adamantyl	H	H	Cl	>280	42	$C_{18}H_{20}ClNO_2$

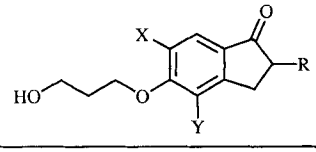
mesh). All melting points were obtained on a Mel-Temp apparatus and are uncorrected. ^1H NMR spectra were obtained on either a Varian FT80A 80-MHz spectrometer or a Bruker AC300 300-MHz spectrometer using tetramethylsilane as an internal standard and were consistent with assigned structures. Intermediates **1–59** were purified by column chromatography or recrystallization and carried on to the next synthetic step without elemental analysis. Elemental analyses were obtained from M-H-W Laboratories, Phoenix, AZ, and are correct within ±0.4% of theoretical values. Protein analyses were performed using a Bausch and Lomb 601 UV-vis spectrometer. Radioisotopes were quantitated using a Packard Tricarb 4000 liquid scintillation spectrometer. Pregnant Sprague-Dawley rats were purchased from Charles River Co., Wilmington, MA.

General Procedure for Preparation of Chlorinated [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]butanoic Acids (60–75)—As shown in Scheme 1, a solution of chlorine (0.01 mol) in 20 mL of glacial acetic acid was added dropwise over 2 min to a cooled solution (15–18 °C) of 0.0083 mol of the appropriate *N*-alkyl-1,3-dihydro-5-methoxy-1-oxoisoindoline[12] in acetic acid with stirring. The reaction was allowed to come to room temperature while stirring was continued for an additional 1.5–2 h. The volatiles were removed *in vacuo* to afford a mixture of the 6-chloro **7–13**, and the 4-chloro isomers **14–22** (Table 1),



Scheme 2

Table 2—Physical Constants for [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]propanols



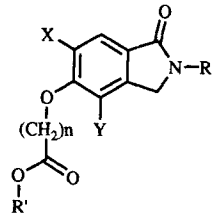
No.	R	X(6)	Y(4)	Starting Material	Mp, °C	Yield, %	Formula
40	c-C_6H_{11}	H	H	23	105–107	59	$C_{17}H_{23}NO_3$
41	c-C_6H_{11}	Cl	H	28	139–141	35	$C_{17}H_{22}ClNO_3$
42	c-C_6H_{11}	H	Cl	35	116–118	45	$C_{17}H_{22}ClNO_3$

which were separated by column chromatography on silica gel (CH_2Cl_2, CH_2Cl_2–Et_2O 9:1). Structural analysis indicated that the 6-chloro isomer had the higher R_f value by thin-layer chromatography. Representative ^1H NMR spectrum for the 6-chloro isomers: ($CDCl_3$, TMS) δ 7.80 (s, 1H, Ar*H*7), 6.95 (s, 1H, Ar*H*4), 4.68 (m, 1H, *N*-c-pentyl, J = 5.5 Hz), 4.30 (s, 2H, CH_2), 3.90 (s, 3H, CH_3), and 1.70 (m, 8H, c-pentyl, J = 3.4 Hz). Representative ^1H NMR spectrum for the 4-chloro isomer: ($CDCl_3$, TMS) δ 7.60 (d, 1H, Ar*H*7, J = 9.1 Hz)), 6.95 (d, 1H, Ar*H*6, J = 9.1 Hz), 4.65 (m, 1H, *N*-c-pentyl, J = 5.5 Hz), 4.30 (s, 2H, CH_2), 3.90 (s, 3H, CH_3), and 1.70 (m, 8H, c-pentyl, J = 3.4 Hz). These intermediates were then converted to the butanoic acids as previously described.[12]

General Procedure for Preparation of [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]propanols (40–42)—As indicated in Scheme 2, the appropriate phenolic intermediate (3.2 mmol) **23**, **28**, or **35** was dissolved in 30.0 mL of dry DMF, and 3.7 mmol of K_2CO_3 was added. The resulting suspension was stirred for 15 min. 3-Bromopropanol (12.9 mmol) was added, and the reaction mixture was then stirred at 50 °C overnight. The DMF was removed *in vacuo*, and the residue was partitioned between CH_2Cl_2 and water. The organic layer was dried with Na_2SO_4 and evaporated *in vacuo* to afford the title compounds usually in >90% purity. When necessary, the product was purified by column chromatography (CH_2Cl_2–ether, 7:3).

General Procedure for Preparation of [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]propanoic Acids (86–88)—As indicated in Scheme 2 the title compounds (Table 3) were prepared by oxidation of the corresponding alcohols **40–42** (Table 2). The alcohol (1.4 mmol) was dissolved in 12.0 mL of acetone. Chromic acid (Jones reagent, 5.0 mL, 1.27 M) was added to the solution, and the reaction mixture was stirred overnight at room temperature. Aqueous methanol (20.0 mL) was added to the solution, and the mixture was extracted with ether. The ether layer was washed with saturated $NaHCO_3$ solution (30.0 mL × 5), and the combined aqueous extracts were acidified with 1.0 N HCl and extracted with ether. Evaporation of the organic layer afforded the crude product which was recrystallized from methanol–toluene to yield the propionic acids.

Table 3—Physical Constants for [(*N*-Alkyl-1,3-dihydro-1-oxoisoindolin-5-yl)oxy]alkanoates



No.	R	R′	X(6)	Y(4)	*n*	Starting Material	Mp, °C	Yield, %	Formula	Analysis
43	c-C_6H_{11}	C_2H_5	H	H	3	**23**	70–72	67	$C_{20}H_{27}NO_4$	ND
44	CH_3	C_2H_5	Cl	H	3	**24**	105–108	98	$C_{15}H_{18}ClNO_4$	ND
45	C_2H_5	C_2H_5	Cl	H	3	**25**	gum	92	$C_{16}H_{20}ClNO_4$	ND
46	C_3H_7	C_2H_5	Cl	H	3	**26**	110–112	80	$C_{17}H_{22}ClNO_4$	ND
47	C_4H_9	C_2H_5	Cl	H	3	**27**	91–93	66	$C_{18}H_{24}ClNO_4$	ND
48	c-C_6H_{11}	C_2H_5	Cl	H	3	**28**	96–97	47	$C_{20}H_{26}ClNO_4$	ND
49	c-C_8H_{15}	C_2H_5	Cl	H	3	**29**	87–89	29	$C_{22}H_{30}ClNO_4$	ND
50	2-CH_3-c-C_6H_{10}	C_2H_5	Cl	H	3	**30**	Oil	70	$C_{21}H_{28}ClNO_4$	ND
51	C_2H_5	C_2H_5	H	Cl	3	**31**	Gum[a]	91	$C_{16}H_{20}ClNO_4$	ND
52	C_3H_7	C_2H_5	H	Cl	3	**32**	56–58	77	$C_{17}H_{22}ClNO_4$	ND
53	C_4H_9	C_2H_5	H	Cl	3	**33**	Gum[a]	100	$C_{18}H_{24}ClNO_4$	ND
54	c-C_5H_9	C_2H_5	H	Cl	3	**34**	Gum[a]	100	$C_{19}H_{24}ClNO_4$	ND
55	c-C_6H_{11}	C_2H_5	H	Cl	3	**35**	82–86	56	$C_{20}H_{26}ClNO_4$	ND
56	c-C_7H_{13}	C_2H_5	H	Cl	3	**36**	Oil	71	$C_{21}H_{28}ClNO_4$	ND
57	c-C_8H_{15}	C_2H_5	H	Cl	3	**37**	42–44	96	$C_{22}H_{30}ClNO_4$	ND
58	2CH_3-c-C_6H_{10}	C_2H_5	H	Cl	3	**38**	71–73	70	$C_{21}H_{28}ClNO_4$	ND
59	Adamantyl	C_2H_5	H	Cl	3	**39**	Oil	100	$C_{24}H_{30}ClNO_4$	ND
60	CH_3	H	Cl	H	3	**44**	170–173	51	$C_{13}H_{14}ClNO_4$	C,H,N
61	C_2H_5	H	Cl	H	3	**45**	180–182	47	$C_{14}H_{16}ClNO_4$	C,H,N
62	C_3H_7	H	Cl	H	3	**46**	130–132	59	$C_{15}H_{18}ClNO_4$	C,H,N
63	C_4H_9	H	Cl	H	3	**47**	141–143	42	$C_{16}H_{20}ClNO_4$	C,H,N
64	c-C_6H_{11}	H	Cl	H	3	**48**	173–175	57	$C_{18}H_{22}ClNO_4$	C,H,N
65	c-C_8H_{15}	H	Cl	H	3	**49**	168–170	100	$C_{20}H_{26}ClNO_4$	C,H
66	2-CH_3-c-C_6H_{10}	H	Cl	H	3	**50**	164–166	100	$C_{19}H_{24}ClNO_4$	C,H
67	C_2H_5	H	H	Cl	3	**51**	130–133	46	$C_{14}H_{16}ClNO_4$	C,H,N
68	C_3H_7	H	H	Cl	3	**52**	150–152	75	$C_{15}H_{18}ClNO_4$	C,H,N
69	C_4H_9	H	H	Cl	3	**53**	140–142	55	$C_{16}H_{20}ClNO_4$	C,H,N
70	c-C_5H_9	H	H	Cl	3	**54**	196–198	49	$C_{17}H_{20}ClNO_4$	C,H,N
71	c-C_6H_{11}	H	H	Cl	3	**55**	169–171	87	$C_{18}H_{22}ClNO_4$	C,H,N
72	c-C_7H_{13}	H	H	Cl	3	**56**	157–159	100	$C_{19}H_{24}ClNO_4$	C,H,N
73	c-C_8H_{15}	H	H	Cl	3	**57**	122–125	100	$C_{20}H_{26}ClNO_4$	C,H
74	2CH_3-c-C_6H_{10}	H	H	Cl	3	**58**	152–154	64[c]	$C_{19}H_{24}ClNO_4$	C,H
75	Adamantyl	H	H	Cl	3	**59**	210–213	100	$C_{22}H_{26}ClNO_4$	C,H
76	C_2H_5	C_2H_5	H	H	1	ref 12[b]	95–96	51	$C_{14}H_{17}NO_4$	C,H,N
77	c-C_6H_{11}	C_2H_5	H	Cl	1	**35**	Gum	91	$C_{18}H_{22}ClNO_4$	
78	CH_3	H	H	H	3	ref 12				
79	C_2H_5	H	H	H	3	ref 12				
80	C_2H_5	H	H	H	1	**76**	209–211	53	$C_{12}H_{13}NO_4$	C,H,N
81	c-C_6H_{11}	H	H	Cl	1	**77**	217–219	100	$C_{16}H_{18}ClNO_4$	C,H,N
82	C_3H_7	H	H	H	3	ref 12				
83	C_4H_9	H	H	H	3	ref 12				
84	c-C_6H_{11}	H	H	H	3	**43**	128–130	100	$C_{18}H_{23}NO_4$	C,H
85	c-C_5H_9	H	Cl	H	3	ref 12				
86	c-C_6H_{11}	H	H	H	2	**40**	168–171	67	$C_{17}H_{21}NO_4$	C,H,N
87	c-C_6H_{11}	H	Cl	H	2	**41**	234–236	78	$C_{17}H_{20}ClNO_4$	C,H,N
88	c-C_6H_{11}	H	H	Cl	2	**42**	189–191	64	$C_{17}H_{20}ClNO_4$	C,H,N

[a] Used without further purification. [b] Starting material was **31**, Table 2, ref 12. [c] >90% *e,e* (trans) isomer.

Preparation of Primary Rat Astrocyte Cultures—Primary rat astrocyte cultures were prepared from neonatal Sprague-Dawley rats.[14] Briefly, neonatal rats were decapitated with surgical scissors, and the brain was removed. A small cortical tissue sample was taken from each cerebral hemisphere, the meninges was removed and the samples were collectively trypsinized (0.1%) for 30 min at 37 °C followed by dispersion into single cells by pipeting. The suspension in Eagle's basic media (BME) was centrifuged at 700 rpm for 10 min to obtain the glial pellet, which was resuspended in fresh BME supplemented with 10% fetal calf serum, glucose, glutamine, and antibiotics and filtered through a 35-μm screen. The filtrate was added in 3.0-mL portions to 60-mm sterile culture dishes, and cultures were grown in an atmosphere of 95% air–5% CO_2 at 37 °C until a confluent monolayer of astrocytes was obtained. The medium was changed every 3 days until the cells were utilized (3–5 weeks). Immunocytochemical staining of such cultures against glial fibrillary acidic protein (GFAP) for astrocytes, fibronectin for fibroblasts, and neuron-specific enolase for neurons using FITC-conjugated antibodies indicates >90% astrocytes, <10% fibroblasts, and no neurons.

Table 4—Effects of Test Compounds on Chloride Transport in Primary Rat Astrocytes

No.	% of Control Astrocytic Intracellular Chloride Concentration ± SD (n = 3 or 4)	
	0.1 mM	1.0 mM
Control	100 ± 5	
Furosemide	79 ± 6[a]	52 ± 5[c]
Ethacrynic acid	78 ± 2[a]	ND
60	93 ± 3	ND
61	87 ± 1	ND
62	78 ± 12	81 ± 2[b]
63	68 ± 14[b]	ND
64	82 ± 10	ND
65	65 ± 10[b]	28 ± 2[c]
66	85 ± 0.1[b]	30 ± 4[c]
67	80 ± 13[a]	85 ± 1[b]
68	72 ± 6[b]	72 ± 6[b]
69	71 ± 7[b]	52 ± 15[b]
70	75 ± 9[a]	57 ± 7[c]
71	47 ± 8[c]	29 ± 3[c]
72	36 ± 1[c]	21 ± 1[c]
73	47 ± 7[b]	37 ± 1[c]
74	50 ± 6[c]	40 ± 2[c]
75	63 ± 6[b]	ND
78	84 ± 4	ND
79	86 ± 10	57 ± 5[b]
80	115 ± 11	ND
81	126 ± 23	ND
82	98 ± 11	ND
83	97 ± 11	ND
84	ND	27 ± 2[c]
85	85 ± 5	ND
86	113 ± 11	79 ± 7
87	111 ± 17	47 ± 5[b]
88	96 ± 12	50 ± 9[b]

[a] $p < 0.01$. [b] $p < 0.005$. [c] $p < 0.001$.

***In Vitro* Astrocyte Chloride Flux Assay**—For assay of test compounds using the above cultures,[12] the BME was replaced by a Cl^-–HCO_3^--containing Hepes-buffered medium and the cells were equilibrated for 60 min at 37 °C in a 95% air-5% CO_2 atmosphere. Test compounds were then added as their sodium salts in distilled water to each dish (n = 3 or 4), distilled water was added to the control group, and the cells were incubated at 37 °C for 15 min. Furosemide at 1.0 mM was included in every assay as a positive control, and the value shown in Table 4 is representative of the inhibition produced by furosemide at this concentration. Following the second incubation, 1.5 μCi of $^{36}Cl^-$ as HCl (New England Nuclear) was added to each dish followed by incubation at 37 °C for 20 min in order to reach steady-state concentration.[9] A time zero control group was included in which the medium was poured off immediately after isotope addition. Isotope quantitated in this group represented noninternalized radioactivity or that present in adherent medium and was substracted from all other groups. All dishes were then washed (6 × 3.0 mL) within 20 s with ice-cold 0.32 M sucrose solution, and the cells from each dish were dissolved in 2.8 mL of 0.5 M NaOH and added to test tubes. A 2.0-mL aliquot from each tube was taken for liquid scintillation counting after quenching, and 0.2-mL aliquots were taken in duplicate for Lowry[15] protein determinations. Intracellular chloride concentrations were determined as millimoles of Cl^- per milligram of cellular protein and expressed as percent of control values ± standard deviation. The p values were determined by the Student's t test.

Results and Discussion

It has been demonstrated that Cl^- may be transported into the astrocyte by either a Na^+–K^+–$2Cl^-$ cotransporter or Cl^-–Cl^-/Cl^-–HCO_3^- anion exchange system.[8-11] Loop diuretics such as furosemide and ethacrynic acid inhibit both systems and therefore swelling *in vitro* and *in vivo*. A series of indanone[9] and fluorene[13] carboxylic acids have also been shown to inhibit chloride influx in primary rat astrocyte cultures as well as in an *in vitro* cerebrocortical tissue slice swelling assay. There is evidence that compounds which inhibit the anion exchanger in erythrocytes are potent inhibitors of cortical swelling *in vitro*.[16] Although rat astrocyte cultures are convenient to prepare, these cells require significantly greater concentrations of transport inhibitors to affect chloride influx and swelling in the *in vitro* assays than do those of the cat or guinea pig which respond in a relatively similar manner.[13]

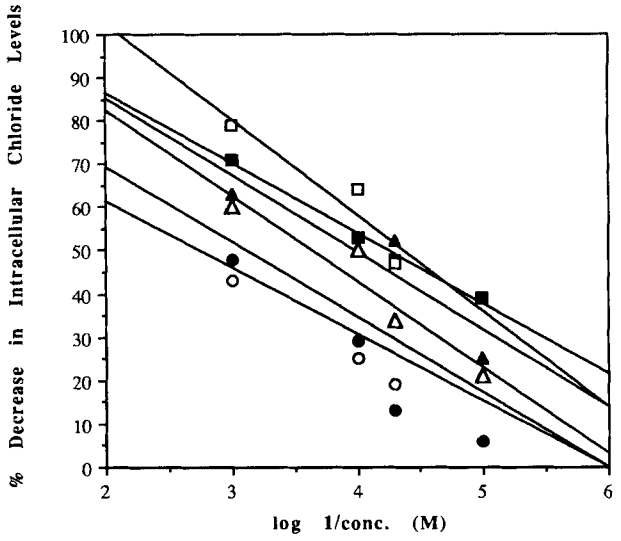


Figure 1—Concentration–response studies for compounds **69** (●), **70** (○), **71** (■), **72** (□), **73** (▲), and **74** (△). Primary rat astrocyte cultures were used. Drugs were administered as their sodium salts at concentrations ranging from 0.01 to 1.0 mM.

Rat Astrocytes—We previously reported the inhibitory effect of **78**, **79**, **82**, **83**, and **85** (Table 3) on intracellular astrocyte chloride levels in primary rat cultures.[12] The activities of the additional analogs **60–75**, **80**, **81**, **84**, and **86–88** are shown in Table 4. Of the 6-chloro analogs (**60–66**, **85**, and **87**, Table 3), only the *N*-*n*-butyl analog **63** (68% of control, $p < 0.005$) and the *N*-cyclooctyl analog **65** (65% of control, $p < 0.005$) both at 0.1 mM demonstrated significant lowering of astrocytic chloride concentrations. The 4-chloro analogs **67–75** demonstrated consistently greater effects than the 6-chloro analogs especially comparing the *N*-cyclohexyl analog **71** (47% of control, $p < 0.001$) at 0.1 mM to the corresponding 6-chloro derivative **64** (82% of control, $p < 0.1$). The 4-chloro analogs substituted with cycloheptyl, cyclooctyl, or 2-methylcyclohexyl substitutions on the ring nitrogen **72–74** demonstrated significant activity at doses of 0.05 (data not shown), 0.1, and 1.0 mM. Within the propanoate series **86–88**, only the 6- and 4-chlorinated analogs displayed significant activity (47% of control, $p < 0.005$ and 50% of control, $p < 0.005$, respectively) at 1.0 mM. The inhibitory activities were, in all cases, considerably less than those of the comparably *N*-alkyl substituted butanoate analogs **64**, **71**, and **84**. Concentration–response studies for several of the most active analogs **69–74** are shown in Figure 1. All six compounds were significantly more inhibitory than was furosemide at all dose levels employed. In general, within the oxoisoindoline series, the 4-chloro analogs are considerably more active than the corresponding 6-chloro analogs which, in turn, are slightly more active than the nonchlorinated analogs **78–80** and **82–84**.

Cl^-–Cl^-/Cl^-–HCO_3^- Anion Exchanger—To ascertain the effect of the oxoisoindolines upon the Cl^-–Cl^-/Cl^-–HCO_3^- anion exchanger, a rat astrocyte chloride influx inhibition assay

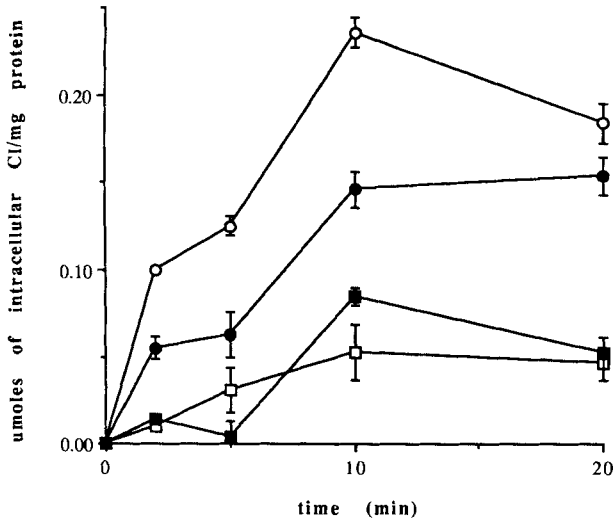


Figure 2—Time course of the inhibition of the anion exchanger mechanism in cultured astrocytes. Astrocytes were incubated with distilled water (○), 1.0 mM SITS (●), 1.0 mM **74** (□), or both SITS and **74** (■). At the designated times, cells were rinsed with 0.32 M sucrose and lysed with 0.5 M NaOH. Values are means ± SD using three culture dishes (triplicate) per time interval in replicate experiments.

involving the use of the selective anion exchanger inhibitor, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), was employed. As shown in Figure 2, the most prominent inhibitory effects of SITS occur prior to steady-state equilibrium and at 1.0 mM SITS reportedly produces complete inhibition of the anion exchanger.[10] One of the two most active compounds in our series, **74**, produced a significantly greater decrease in the amount of pre-steady-state and steady-state intracellular chloride as compared to control levels. Concomitant administration of both SITS and **74** did not produce any enhancement of pre-steady-state chloride influx inhibition compared to **74** alone. These results suggest that compounds of the oxoisoindoline series produce their effects via inhibition of some process other than, or in addition to, the anion exchanger mechanism.

Na^+–K^+–2Cl^- Cotransporter—Bumetanide, a selective inhibitor of the Na^+–K^+–2Cl^- cotransporter, was used to further examine the mechanism of inhibition by the oxoisoindoline compounds. At 0.01 mM, bumetanide produces maximal inhibition of the cotransporter at steady state.[10] At pre-steady state, **74** produced a more pronounced inhibitory effect than bumetanide, but at steady state the difference was statistically insignificant as shown in Figure 3. The results of the effect of **74** upon chloride influx in the presence of SITS and bumetanide suggest that, at steady state, the cotransporter is inhibited as indicated by significantly decreased intracellular chloride levels. At pre-steady state, **74** may inhibit a transport mechanism in addition to the cotransporter–the anion exchanger. Since **74** and SITS in combination produce no greater inhibitory effect than **74** alone at steady state, the anion exchanger must already be inhibited by **74** alone. These results suggest that **74** expresses a similar pharmacological profile to that of furosemide.[10]

Conclusions

The results obtained in tissue culture indicate a sensitivity to the inhibitory effects of the above series of 1-oxoisoindolines in rat astrocytes with regard to inhibition of chloride influx. In general, the 4-chlorooxoisoindolines demonstrated superior astrocytic chloride influx inhibition compared to the 6-chloro analogs in all assays. Preliminary computer-assisted molecular modeling of the compounds[17] indicates that, in the minimum energy conformation for each, the position of the chlorine atom

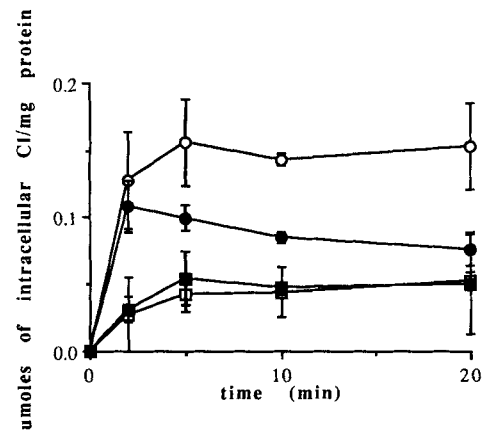


Figure 3—Time course of the inhibition of the Na^+–K^+–2Cl^- cotransport mechanism in cultured astrocytes. Astrocytes were incubated with distilled water (○), 0.01 mM bumetanide (●), 1.0 mM **74** (□), or both bumetanide and **74** (■). At the designated times, cells were rinsed with 0.32 M sucrose and lysed with 0.5 M NaOH. Values are means ± SD using three culture dishes (triplicate) per time interval in replicate experiments.

produces a profound steric orienting effect on the adjacent acid side chain greatly affecting the relative three-dimensional pharmacophoric arrangement of pertinent functional groups. The 4-chloro analogs possess inhibitory activity greater than or equal to that of furosemide. Furthermore, the mechanism of inhibition appears to be similar to that of furosemide with chloride influx inhibitory activity resulting from inhibition of both the Na^+–K^+–2Cl^- cotransporter and the Cl^-–Cl^-/Cl^-–HCO_3^- anion exchanger.[10]

Others have reported the presence of depolarizable chloride channels which open in response to voltage changes and excitatory amino acids such as GABA.[18–21] The effects of these compounds on such channels has not yet been addressed. Also, the chloride transport mechanisms affected by the 1-oxoisoindolines are not specifically limited to astrocytes, and recently the biological activity of a series of structurally similar (aryloxy)-acetic acids was reported with respect to *in vivo* induction of uricosuric diuresis.[22] It is possible that the 1-oxoisoindolines also possess this type of activity.

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Acknowledgments

The authors thank Dr. Kenneth D. McCarthy for assistance with the astrocyte tissue culture work. This work was supported by NIH Biomedical Research Support Grant no. 1-507RR059-01.