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Structure–activity relationship of furosemide-derived compounds as antagonists of cerebellum-specific $GABA_A$ receptors

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Abstract

The Na^+–K^+–$2Cl^-$ cotransporter blocker furosemide inhibits γ-aminobutyric acid (GABA)-gated chloride currents and reverses GABA-mediated inhibition of [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding of the cerebellar $\alpha 6$ subunit-containing $GABA_A$ receptors much more potently than the cerebrocortical non-$\alpha 6$ subunit-containing receptors. Of the 44 compounds studied, all precursors or derivatives of diuretics, one compound [hydrazinosulfonyl-furosemide (PF 1885)] reversed 5-μM GABA-induced inhibition of [^{35}S]TBPS binding to cerebellar and cerebrocortical receptors. Three other compounds, all of which are structurally closely related to furosemide, were selective antagonists for the cerebellar receptors comparable to the lead compound. Still, the diuretic and GABAergic structure–activity relationships differ, since we found potent diuretic structures lacking GABA antagonistic activity. Further development of the GABAergic potency of furosemide derivatives can now focus on the modification of the carboxyl group, replaceable by tetrazole but not by sulfonic or phosphinic acids and the furanyl moiety which could be substituted by thienyl and benzyl groups. © 1998 Elsevier Science B.V.

Keywords: $GABA_A$ receptor, subtypes; Diuretic; Furosemide; Cerebellum; [^{35}S]TBPS ([^{35}S]-*t*-butylbicyclophosphorothionate)

1. Introduction

Allosteric, highly receptor subtype-specific modulation of γ-aminobutyric acid (GABA) mediated inhibition provokes enormous therapeutic interest, as all hitherto clinically used compounds acting on GABA neurotransmission, such as anxiolytics, hypnotics, anticonvulsants and anaesthetics, produce multifaceted effects. The diverse actions are most easily explained by their receptor subtype-unselective interaction with GABA type A receptors ($GABA_A$ receptors). Barbiturates serve as prime examples as, at low concentrations, they allosterically increase GABA-induced Cl^--flux similarly in all tested binary and ternary $GABA_A$ receptor subunit combinations (Schofield et al., 1987; Sigel et al., 1990; Saxena and Macdonald, 1994). Though slightly better in their selectivity, benzodiazepines such as diazepam and flunitrazepam, still recognise all ternary $\alpha i \beta j \gamma 2/3$ $GABA_A$ receptors, except those containing the $\alpha 4$ or $\alpha 6$ variants. Even zolpidem, the $GABA_A$ receptor benzodiazepine site ligand with the hitherto highest subtype selectivity acts on all ternary $\alpha 1/2/3 \beta 1/2/3 \gamma 2$ receptors, i.e. possibly on 9 structurally different receptor subtypes (Pritchett and Seeburg, 1990; Puia et al., 1991; Ducic et al., 1993; Lüddens et al., 1994). Loreclezole, like the barbiturates, acts on binary and ternary receptors, but does not modulate $\beta 1$-containing $GABA_A$ receptors. In contrast to these compounds, furosemide only blocks $\alpha 4/6 \beta 2/3$ receptors, independent of the presence of the $\gamma 2$ or δ subunits (Korpi et al., 1995a; Knoflach et al., 1996; Korpi and Lüddens, 1997). There are no indications for the existence of selectively furosemide-sensitive $GABA_A$ receptors outside the cerebellar granule cells indicating that native $\alpha 4$ subunit-containing receptors, if they exist, should be rare (Pearce, 1993; Knoflach et al., 1996; Korpi and Lüddens, 1997). This leaves the cerebellar granule cell $GABA_A$ receptor as the prime site for furosemide action in the brain.

Furosemide belongs to the therapeutic group of high-ceiling diuretics exerting its action on the cortical thick

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ascending limb of Henle's loop in the kidney by interfering with the Na^+–K^+–$2Cl^-$-cotransport system at the lumen membrane (Greger et al., 1983). It is known to inhibit the binding of [^{3}H]Ro 5-4864 ([^{3}H]4′-chlorodiazepam) to peripheral benzodiazepine receptors (Basile et al., 1988), at which its inhibitory potency is tenfold lower than its antagonistic potency at $GABA_A$ receptor channels (Korpi et al., 1995a) or its inhibition of the Na^+–K^+–$2Cl^-$-cotransporter (Schlatter et al., 1983).

A large number of structural analogues of furosemide have been screened for their effectiveness as diuretics (Schlatter et al., 1983; Masereel et al., 1992). Furosemide and a few of these compounds, e.g. azosemide (5-(4′-chloro-5′-sulfamyl-2′-thenyl-aminophenyl)-tetrazole) and bumetanide ((3-butylamino)-4-phenoxy-5-sulfamoylbenzoic acid) and its congeners, are in clinical use in various countries as diuretics. In our initial study on furosemide as a $GABA_A$ receptor subtype-selective compound (Korpi et al., 1995a), we demonstrated that bumetanide is ineffective in this respect. This indicated the possibility to develop furosemide-derived compounds lacking diuretic properties. In a biochemical test for $GABA_A$ receptor antagonistic activity which is sensitive to competitive as well as allosteric GABAergic interaction (Squires and Saederup, 1987; Korpi et al., 1995b) we examined a number of analogues for reversal of the inhibition of [^{35}S]-*t*-butylbicyclophosphorothionate [^{35}S]TBPS binding by 5 μM GABA in rat brain cortical and cerebellar membranes. Our results indicate that the overall structure of the parental compound furosemide needs to be retained in order to preserve the inhibitory properties on cerebellum-specific $GABA_A$ receptors, leaving the replacement of the furanyl-moiety and the carboxyl as routes to novel compounds.

2. Material and methods

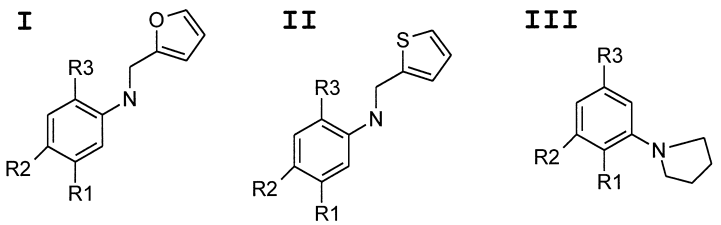
2.1. Membrane preparation

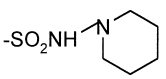
Male Wistar rats were decapitated at the age of 3–6 months and cerebral cortices and cerebella dissected. Tissues from three animals were pooled, homogenised with a Ultraturrax or a Polytron in 50 volumes of ice-cold 50 mM Tris–citrate buffer (pH 7.4) supplemented with 1 mM Na_2–EDTA and centrifuged at $20\,000 \times g$ for 20 min. Pellets were resuspended in the same buffer and recentrifuged five times. The final suspension was prepared in 50 mM Tris–citrate buffer and aliquots were stored frozen at −80°C.

2.2. Binding assays

Resuspended cell membranes (50–100 μg protein per tube) were incubated in a final volume of 0.5 ml of 50 mM

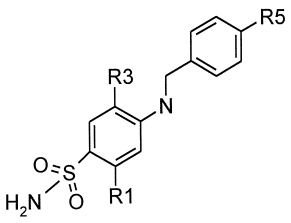
Table 1
Furosemide analogues and tetrazolo-piretanide

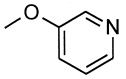
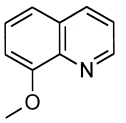


		compound	R1	R2	R3	Source	IC_{50} Cl^- current [a]	diuresis in dogs [b]	figure/ table
I	a	furosemide	-Cl	$-SO_2NH_2$	-COOH	SIGMA	$3\cdot10^{-6}$	+	fig. 2
	b	OT2097	-Cl	$-SO_2NH_2$	-CONH-Phe	Leo	n.d.	n.d.	---[c]
	c	OT5121	-Cl	$-SO_2NH_2$	$-CONH-CH_2$-Phe	Leo	n.d.	n.d.	fig. 1
	d	OT5122	-Cl	$-SO_2NH_2$	$-CONH-(CH_2)_3CH_3$	Leo	n.d.	n.d.	---[c]
	e	sulfofurosemide	-Cl	$-SO_2NH_2$	$-SO_3K$	Hoe	$5.2\cdot10^{-6}$	n.d.	---[c]
	f	N-methyl-furosemide	-Cl	$-SO_2NH-CH_3$	-COOH	Hoe	$1.5\cdot10^{-5}$	n.d.	---[c]
	g	N-butyl-furosemide	-Cl	$-SO_2NH-(CH_2)_3-CH_3$	-COOH	Hoe	n.d.	n.d.	table 4
	h	PF1543	-Cl	-SO$_2$NH-Phe	-COOH	Leo	n.d.	+	table 5
	i	PF1885 (hydrazinosulfonyl-furosemide)	-Cl		-COOH	Leo	n.d.	+	fig. 1

[a] Schlatter et al., 1983.
[b] Data provided by Björkling.
[c] See text.
n.d. = not determined.

Table 2
Benzyl-furosemide analogues



		compound	R1	R3	R5	Source	IC_{50} Cl^- current [a]	diuresis in dogs [b]	figure/ table
IV	a	benzyl-furosemide	-Cl	-COOH	-H	Hoe	$7.9 \cdot 10^{-7}$	n.d.	fig. 2
	b	OT 1443	-F	-COOH	-H	Leo	n.d.	n.d.	---[c]
	c	OT 1252	-F	$-COOCH_2CH_3$	-H	Leo	n.d.	n.d.	table 5
	d	4-azido-2-benzylamino-5-sulfamoylbenzoic acid	$-N_3$	-COOH	-H	Hoe	n.d.	n.d.	table 4
	e	OT 1169	-S-Phe	-COOH	-H	Leo	n.d.	+	table 5
	f	OT 1222	$-S-CH_2-CH_2-Phe$	-COOH	-H	Leo	n.d.	0	fig. 1
	g	OT 5132		-COOH	-H	Leo	n.d.	n.d.	---[c]
	h	OT 5130		-COOH	-H	Leo	n.d.	n.d.	table 5
	i	2-(4'-chloro-benzyl)-furosemide	-Cl	-COOH	-Cl	Hoe	n.d.	n.d.	table 4

[a] Schlatter et al., 1983.
[b] Data provided by Björkling.
[c] See text.
n.d. = not determined.

Tris/citrate buffer, pH 7.3, for [^{3}H]muscimol, or in 50 mM Tris/citrate buffer, pH 7.3, supplement with 0.2 M NaCl for [^{35}S]TBPS. All radioligands were from Du Pont-New England Nuclear. Nonspecific binding was determined by 100 μM GABA and 20 μM picrotoxinin (both from Sigma, St. Louis, MO) for the two radioligands, respectively. [^{35}S]TBPS binding was performed in the presence and absence of furosemide or its analogues in concentrations ranging from 1 μM to 1 mM in the presence or absence of 5 μM GABA. After 1 h at 4°C ([^{3}H]muscimol) or 90 min at room temperature (24°C, [^{35}S]TBPS), the assay mixtures were rapidly diluted to 5 ml with 10 mM Tris/HCl, pH 7.5 and filtered through glass fibre filters (Schleicher and Schuell, No. 52). Filters were immersed in 4 ml of scintillation fluid and the radioactivity determined in a liquid scintillation counter using external standardisation (Korpi et al., 1995b).

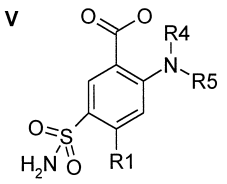
2.3. Material and chemical syntheses

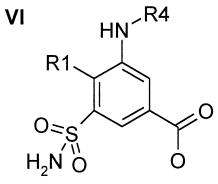
Furosemide and bumetanide were purchased from Sigma (St. Louis, MO). Compounds marked by Leo were provided by Fredrik Björkling (Leo Pharmaceutical Products A/S, Ballerup, Denmark) and azosemide by Boehringer-Mannheim (Mannheim, Germany), respectively. All other compounds were synthesised by Hoechst AG (Frankfurt, Germany) (Sturm et al., 1983).

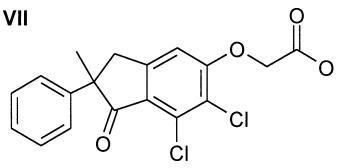
Preparation of compound I e and I o was as reported (Sturm et al., 1983). The synthesis of compounds I q and IV k is described in Eur. Pat. Appl.: EP 51816 (1982) (C.A. 97: 162991). The synthesis of tetrazolo-piretanide (compound III) was performed analogously (C.A. 97: 162991) by reacting the corresponding nitrile of piretanide with HN_3 which led to colourless crystals (m.p. 168–172°C).

4-Chloro-2-(2-furylmethylamino)-5-methylsulfamoylbenzoic acid ('*N*-methyl-furosemide' = compound I f) was obtained by heating 1.4 g 2,4-dichloro-5-methylsulfamoylbenzoic acid in 6.9 ml 2-furylmethylamine at 118°C under argon atmosphere for 8 h, then pouring the reaction mixture into ice cold aqueous acetic acid (10%). The resulting crystals were colourless to light brown (m.p. 204–207°C). 5-Butylsulfamoyl-4-chloro-2-(2-furylmethy-

Table 3
2-Amino-5-sulfamoylbenzoic acid derivatives, bumetanide analogues and indacrinone







		compound	R1	R4	R5	Source	IC_{50} Cl^- current [a]	diuresis in dogs [b]	figure/ table
V	a	PF 1497	-Cl	$-(CH_2)_3-CH_3$	-H	Leo	n.d.	+	table 5
	b	HH600	-Cl	$-CH_2CH_3$	$-CH_2CH_3$	Leo	n.d.	n.d.	---
	c	OT1189	-F	-H	-H	Leo	n.d.	n.d.	---
	d	OT1187	-F	$-COCH_3$	-H	Leo	n.d.	n.d.	---
	e	OT 1214	-F	$-CH_2$-Phe	$-CH_2$-Phe	Leo	n.d.	n.d.	---
	f	OT 5113	-S-Phe	$-COCH_3$	-H	Leo	n.d.	n.d.	---
	g	OT 1165	-S-Phe	$-CH_2$-(N-pyridyl)	-H	Leo	n.d.	0	---
VI	a	OT 1816	-Phe	$-CH_2$-(furyl)	---	Leo	n.d.	+	---
	b	OT 1537	$-CH_2$-Phe	$-CH_2$-(furyl)	---	Leo	n.d.	+	---
	c	PF 1666	-O-Phe	$-CH_2$-(furyl)	---	Leo	n.d.	+	---

[a] Schlatter et al., 1983.
[b] Data provided by Björkling.
n.d. = not determined.
Data to compounds other than Va (PF 1497) and VII (indacrinone) are referenced in the text.

Table 4
Diuretic analogues decreasing the binding of [^{35}S]TBPS in the absence of GABA

	Cerebellum				Cortex			
	w/o GABA		5 μM GABA		w/o GABA		5 μM GABA	
	100 μM	1000 μM	100 μM	1000 μM	100 μM	1000 μM	100 μM	1000 μM
Tetrazolo-piretanide	87 ± 7	6 ± 6	1 ± 11	-1 ± 8	47 ± 6	2 ± 4	7 ± 2	1 ± 4
2-(4′-Chlorobenzyl)-furosemide	72 ± 3	29 ± 2	13 ± 5	28 ± 7	40 ± 30	18 ± 3	12 ± 3	14 ± 4
N-Butyl-furosemide	102 ± 22	35 ± 0	16 ± 6	25 ± 4	64 ± 24	49 ± 1	17 ± 1	46 ± 32
Hoe 374	59 ± 29	32 ± 4	28 ± 25	22 ± 30	72 ± 15	39 ± 7	18 ± 8	7 ± 10
Indacrinone	37 ± 3	15 ± 14	15 ± 3	31 ± 27	50 ± 6	5 ± 4	18 ± 17	15 ± 16
4-Azido-benzyl-furosemide	82 ± 8	9 ± 9	16 ± 17	6 ± 14	93 ± 11	23 ± 26	15 ± 5	13 ± 13
Azo-374	13 ± 5	8 ± 13	0 ± 4	-4 ± 5	4 ± 4	2 ± 2	-3 ± 3	1 ± 2
Hoe 19	12 ± 8	36 ± 24	0 ± 10	10 ± 8	-5 ± 2	5 ± 2	-2 ± 4	13 ± 4

The binding of [^{35}S]TBPS to cortical and cerebellar membranes was evaluated in the presence and absence of 5 μM GABA and 100 or 1000 μM of the diuretic analogues. All analogues failed at 1 and 10 μM to interfere with the [^{35}S]TBPS binding in the presence of 5 μM GABA. 10 μM Hoe 19 decreased the binding of [^{35}S]TBPS to $62 \pm 5\%$ and $74 \pm 7\%$ in cortex and cerebellum, respectively. The corresponding value for 10 μM azo-374 were $45 \pm 8\%$ and $36 \pm 15\%$. All other compounds were ineffective at this concentration as well as at 1 μM. Shown are the means $\pm$ S.E.M. of 3–4 independent experiments in % of the control value obtained in the absence of GABA or diuretic. 5 μM GABA alone decreased the control value to $23 \pm 6\%$ and $30 \pm 10\%$ in cerebellum and cortex, respectively.

Table 5
Diuretic analogues decreasing [^{35}S]TBPS binding in the presence of GABA

	Cerebellum		Cortex	
	100 μM	1000 μM	100 μM	1000 μM
CB 560	6±3	n.d.	6±4	12±3
OT 1159	18±2	1±4	4±2	−1±5
OT 1169	7±1	1±1	10± [a]	1[a]
OT 1190	10±3	1±1	17[a]	4[a]
OT 1288	15±8	7±5	26[a]	3[a]
OT 1450	4±1	n.d.	3[a]	n.d.
PF 1543	7±2	1±1	6[a]	0[a]
OT 5130	16±8	7±5	5±4	4±0.3
OT 1252	8±3	n.d.	6±4	n.d.
PF 1497	13±1	−2±1	13±1	0±2

The binding of [^{35}S]TBPS to cortical and cerebellar membranes was evaluated in the presence of 5 μM GABA and 100 or 1000 μM of the diuretic analogues. 1 and 10 μM of the compounds failed to reverse the GABA effect. Shown are the means±S.E.M. of 3–4 independent experiments as percent of control in the absence of GABA or diuretics.
[a]Values were determined with duplicates in a single experiment. 5 μM GABA alone decreased the control value to 23±6% and 30±10% in cerebellum and cortex, respectively.

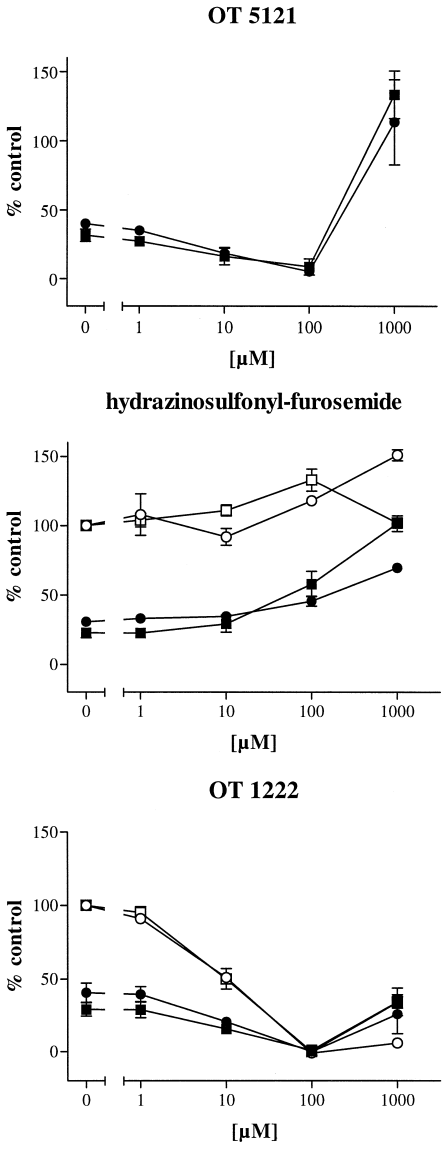


Fig. 1. Diuretic analogues increasing [^{35}S]TBPS binding in cortical and cerebellar membranes. The binding of [^{35}S]TBPS to cortical (●, ○) and cerebellar (■, □) membranes was evaluated in the presence (●, ■) and absence (○, □) of 5 μM GABA and increasing concentrations of the diuretic analogues. Shown are the means±S.E.M. of 3–4 independent experiments.

lamino)benzoic acid ('*N*-butyl-furosemide' = compound I g) was obtained analogously to *N*-methyl-furosemide by reacting 1.6 g 5-butylsulfamoyl-2,4-dichlorobenzoic acid in 6.9 ml 2-furylmethylamine. The crystalline compound was colourless to yellowish (m.p. 166–168°C). The structures were verified by infrared, mass and nuclear resonance spectroscopy.

4-Chloro-2-(4-chlorobenzylamino)-5-sulfamoylbenzoic acid ['2-(4′-chlorobenzyl)furosemide' = compound IV i] was obtained by reacting 2.25 g 2,4-dichloro-5-methylsulfamoylbenzoic acid and 4-chlorobenzylamine at 140°C and crystallisation from 1 M HCl. The yellowish crystalline compound had a melting point of 260–263°C. The structure was verified by infrared, mass and nuclear resonance spectroscopy.

4-Chloro-5-sulfamoyl-2-(2-thienylamino)benzoic acid ('thienyl-furosemide' = compound II b) was synthesized by heating a mixture of 1.26 g 4-chloro-2-fluoro-5-sulfamoylbenzoic acid and 1.55 ml 2-aminomethylthiophene in 40 ml *N*-methyl-2-pyrrolidone at 95°C for 4 h. After pouring the reaction mixture into 150 ml aqueous HCl (pH 1) the colourless crystals were precipitated and filtrated (m.p. 203–204°C). The structure was verified by infrared, mass and nuclear resonance spectroscopy.

4-Azido-2-benzylamino-5-sulfamoylbenzoic acid (compound IV d) was obtained by dissolution of 1.7 g 2-benzylamino-4-hydrazino-5-sulfamoylbenzoic acid in a mixture of 10 ml 1 M NaOH and 0.35 g $NaNO_2$ which was poured into a solution of 3 ml acetic acid in 10 ml water at 10°C. The precipitated colourless crystals which decomposed at 195°C were collected by filtration. The structure was verified by infrared, mass and nuclear resonance spectroscopy.

Table 6
Modulation of [^{3}H]muscimol binding to rat cortical membranes by furosemide analogues

	OT 1222	CB 560	OT 1159
100 μM	53±4	81±3	119±15
1000 μM	33±6	54±3	155±19

Shown are the percentages from control values (mean±S.E.M., $n=3$) of [^{3}H]muscimol binding (6 nM) for three diuretic analogues at 100 μM and 1 mM concentrations in rat cortical membranes. Specific binding was 483±50 fmol per mg protein. None of the other compounds affected [^{3}H]muscimol binding.

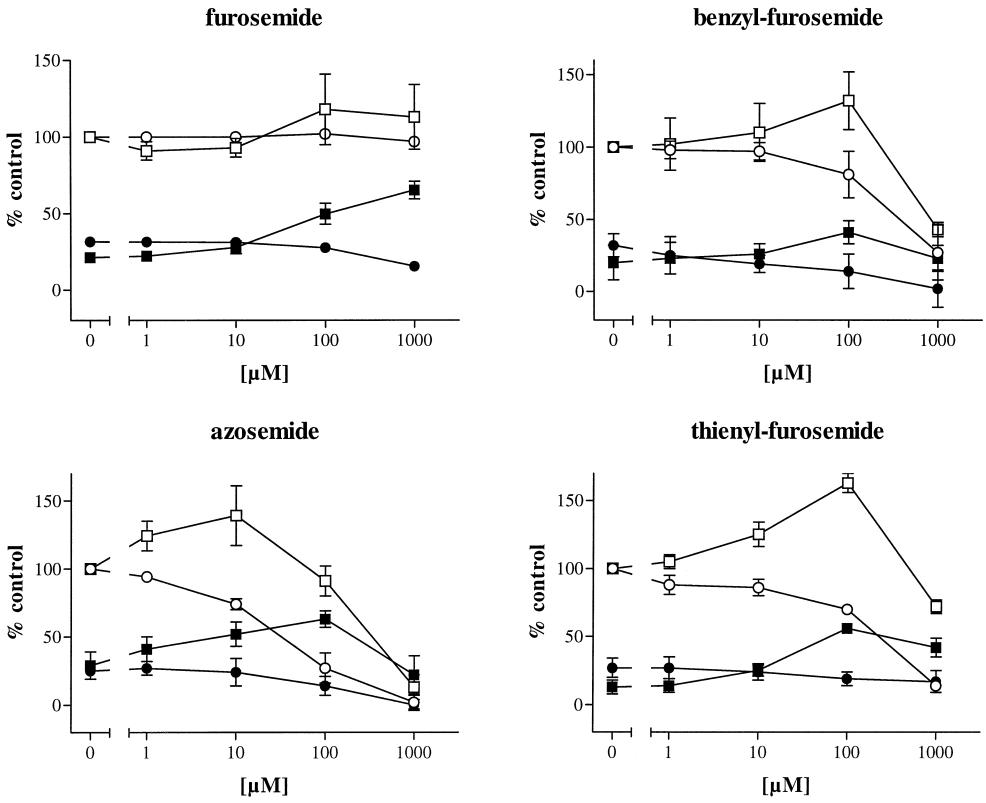


Fig. 2. Diuretic analogues counteracting GABA action on [^{35}S]TBPS binding in cerebellar membranes. The binding of [^{35}S]TBPS to cortical (●, ○) and cerebellar (■, □) membranes was evaluated in the presence (●, ■) and absence (○, □) of 5 μM GABA and increasing concentrations of the diuretic analogues. Shown are the means ± S.E.M. of 3–4 independent experiments.

Leo Pharmaceutical Products classified the Leo compounds as positive or negative in inducing diuresis in dogs, other data pertaining to diuretic action of the compounds were derived from the literature (Schlatter et al., 1983).

3. Results

We investigated the compounds listed in Tables 1–3 (grouped according to the basic structures I–VII together with their previously defined diuretic properties (Schlatter et al., 1983)), for their interaction with native $GABA_A$ receptors. We used $GABA_A$ receptors derived from rat cerebral cortex and rat cerebellum, and a specific ligand, [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]TBPS), to label the $GABA_A$ receptor convulsant sites (Squires and Saederup, 1987). As currently no high-affinity ligand specific for the furosemide-recognition site on $GABA_A$ receptors is available, the indirect measure of interference with the [^{35}S]TBPS binding site had to be used to screen the activity of these compounds. The first paradigm was to assess the cerebellum-specificity of the reversal of GABA-induced inhibition of [^{35}S]TBPS binding by furosemide and related compounds. The reversal of GABA inhibition of [^{35}S]TBPS binding is diagnostic for competitive as well as allosteric $GABA_A$ receptor antagonistic activity (Squires and Saederup, 1987; Korpi et al., 1995b). The second measure was the GABA-independent receptor non-selective inhibition of [^{35}S]TBPS binding, a feature seen at high furosemide concentrations in cortical as well as cerebellar membranes (Korpi et al., 1995a; Korpi and Lüddens, 1997), which does not directly predict functional activity.

3.1. Diuretic analogues not interfering with [^{35}S]TBPS binding

As we mainly aimed at identifying compounds with allosteric GABA antagonistic action most compounds were only screened for their effect on [^{35}S]TBPS binding in the presence of exogenous 5 μM GABA. Furosemide phosphinic acid, isofurosemide and sulfofurosemide and the furosemide precursors 4-chloro-3-sulfamoylbenzoate and 3-sulfamoylbenzoate were ineffective in cortical and cerebellar membrane preparations, in the presence or absence of external GABA irrespective of their properties to induce diuresis (data not shown). Compounds OT 2097, OT 5122, OT 1214, OT 5132, PF 1666, OT 1443, OT 1537, OT 5113, OT 1816, HH 600, OT 1165, OT 1187, OT 1189 and *N*-methylfurosemide did not reverse the GABA-inhibition of [^{35}S]TBPS binding and were not studied further (data not shown).

3.2. Compounds reducing [^{35}S]TBPS binding in a GABA-independent manner

Up to the highest concentration, some tested compounds were unable to significantly ($P < 0.01$, paired *t*-test) interfere with the GABA-reduced binding, but potently decreased [^{35}S]TBPS binding in cerebellar and cortical membranes in the absence of added GABA. The compounds belonging to this class of derivatives are tetrazolo-piretanide, azo-374, 4-azido-benzylfurosemide, indacrinone, Hoe 374, *N*-butyl-furosemide, 2-(4′-chlorobenzyl)-furosemide and Hoe 19 (Table 4).

We observed a significant further reduction of GABA-inhibited [^{35}S]TBPS binding by compounds OT 1159, OT 1288, CB 560, PF 1543, OT 1252, OT 1169, PF 1497, OT 1190, OT 1450 and OT 5130 (Table 5).

3.3. Diuretic analogues reversing the GABA-induced inhibition

A few compounds reversed the GABA-inhibition of the binding in both brain regions (OT 5121, OT 1222, PF 1885; Fig. 1). The most potent of these compounds, hydrazinosulfonyl-furosemide, started to significantly antagonise the GABA effect at 100 μM, whereas compounds OT 1222 and OT 5121 were only effective at 1 mM, the highest concentration we were able to test.

The lead compound furosemide started to reverse the action of 5 μM GABA on [^{35}S]TBPS binding at 100 μM, but only in cerebellar membranes, whereas the binding to cortical membranes was further decreased (Fig. 2). We observed a similar mode of activity for benzyl-furosemide, azosemide and the thienyl analogue of furosemide (Fig. 2). However, direct inhibition of [^{35}S]TBPS binding lead to a decrease of basal as well as GABA-inhibited binding in both tissues at 1 mM. Azosemide and thienyl-furosemide were more potent and more efficacious than furosemide and benzyl-furosemide in reversing the GABA-inhibition of [^{35}S]TBPS binding.

3.4. Effects on [^{3}H]muscimol binding

A number of furosemide derivatives can be regarded as rigid GABA analogues. We therefore determined the inhibition of [^{3}H]muscimol binding in cortical membranes in the presence of 100 μM and 1 mM (0.5 mM for OT 5121 and OT 1288) of the ligands listed in Tables 1–3. Only three of the compounds concentration dependently interfered with the [^{3}H]muscimol binding (Table 6). OT 1222 and CB 560 both decreased the binding, whereas OT 1159 increased it to 155% at 1 mM. No obvious correlation of their effect on [^{3}H]muscimol and their effect on [^{35}S]TBPS binding can be drawn as CB 560 and OT 1159 both decreased [^{35}S]TBPS binding in cortical membranes, but OT 1222 increased this binding at 1 mM.

4. Discussion

The high-ceiling diuretics form a structurally heterogeneous group of compounds. Therefore our initial data that furosemide but not bumetanide is a non-competitive $GABA_A$ receptor antagonist (Korpi et al., 1995a) could be taken as a hint for the possible existence of a non-diuretic GABAergic furosemide derivative but it was not giving any clue as to the structure–activity relationship on $GABA_A$ receptors, since these two molecules differ substantially in structure. Now our data indicate that the action of furosemide on the cerebellum-specific $GABA_A$ receptors requires the fulfillment of strict structural properties which deviate from those needed for its diuretic action.

4.1. Specific requirements for the acidic group

One striking example is sulfofurosemide (I e). It possesses nearly the same diuretic potency as furosemide, when judged by the IC_{50} values for the inhibition of the short circuit currents, indicative of the Cl^--flux across renal tubules (Schlatter et al., 1983). Though the substitution of the carboxylic acid by sulfonic acid only slightly altered the potency to inhibit the short circuit currents, sulfofurosemide does not affect $GABA_A$ receptor function as measured by [^{35}S]TBPS binding. In addition, furosemide phosphinic acid (compound I p), lacking diuretic efficacy, does not interact with [^{35}S]TBPS binding either. From our data we can postulate that the $GABA_A$ receptor subtype-specific action of furosemide requires a defined acidic group, i.e. a carboxylate or a tetrazole in position 3 of structures I, II or IV. This position cannot be replaced by other acidic groups like sulfonate (compound I e) or phosphinate (compound I p) irrespective of their diuretic potency, stressing the more restricted structure–activity requirements of furosemide on $GABA_A$ receptors than on the Na^+–K^+–$2Cl^-$-cotransporter.

4.2. The Cl^- position and the sulfamoyl-group of fursosemide are essential

The necessity of the chloride position (R1 in structures I, II and IV) in the furanyl or thienyl compounds cannot be fully assessed, as substitutions by more reactive groups are chemically unstable. However, the benzyl-furosemide derivatives carrying a fluoro- and azido substitution (IV b, IV d) do not interfere with [^{35}S]TBPS binding. Larger substituents at position R1 in structure I (I l, I m, I n, I o) fully abolished the GABAergic properties of these compounds regardless of their diuretic properties, again arguing for a strict requirement of the chloro-substituent in this position.

The same holds true for the sulfamoyl group. It is essential for the $GABA_A$ receptor subtype-specific action of furosemide as any substitution (*N*-butylfurosemide,

Table 4; PF 1885, Fig. 1) or replacement by -O–N^+–O^- (CB 560, Table 5) led to a change of selectivity and activity, respectively. The bumetanide derivatives VI a–VI c were ineffective in interfering with [^{35}S]TBPS binding, indicating that the absence of GABAergic activity seen for bumetanide itself (Korpi et al., 1995a) is a general feature of this class of compounds.

4.3. The furanyl-group can be substituted

The furanyl-moiety could be replaced by a thienyl ring without a change in specificity, i.e. thienyl-furosemide and azosemide were both selective in their $GABA_A$ receptor activity in that they only reversed the action of GABA on [^{35}S]TBPS binding on cerebellar but not on cortical membranes (Fig. 2). As well, a benzyl ring could substitute the furanyl ring in furosemide (Fig. 2). The resulting benzyl-furosemide was a potent, selective and non-competitive GABA antagonist as indicated by the increase in [^{35}S]TBPS binding in the presence of this compound. The action of 2-(4′-chloro-benzyl)-furosemide, however, was not as clear-cut as it decreased the binding of [^{35}S]TBPS in both membrane preparations (Table 4). Azosemide, the tetrazole derivative of thienyl-furosemide seemed to be a more potent subtype-selective $GABA_A$ receptor antagonist than furosemide, reaching its optimal concentration in the 100 μM range as compared to 1 mM for furosemide (Fig. 2). The compounds exhibiting furosemide-like action, i.e. benzyl-furosemide, thienyl-furosemide and azosemide, shared the lead compound's GABA-independent, receptor-subtype non-selective inhibition of [^{35}S]TBPS binding at high concentrations (Fig. 2). Furthermore, they were more potent in this respect than furosemide itself, a property that might have obscured a higher subtype-selective antagonistic efficacy of thienyl-furosemide and azosemide. Furosemide and the other three receptor-subtype selective compounds can be regarded as rigid GABA analogues. Still, their allosteric antagonistic interaction did not seem to occur via the GABA recognition site, as none of the four analogues displaced the GABA-mimetic [^{3}H]muscimol from its recognition site (Table 6 and see Korpi et al., 1995a).

4.4. PF 1885 increases [^{35}S]TBPS binding in cortical and cerebellar membranes

As apparent from the structural requirements described so far, any further modification of these four basic structures led to a total loss of selectivity for the cerebellum-specific $GABA_A$ receptor subtype. But three compounds derived from furosemide and substituted at the Cl^--(OT 1222; IV f), the carboxyl-(OT 5121; I c) or the sulfamoyl group (PF 1885; I i) reversed the GABA-inhibition of the [^{35}S]TBPS binding, apparently in a $GABA_A$ receptor-subtype non-selective manner. With PF 1885, a continuous increase was observed over the full concentration range in the presence of 5 μM exogenous GABA (Fig. 1). The other two compounds decreased the [^{35}S]TBPS binding at concentrations up to 100 μM, but increased it at 1 mM, irrespective of the GABA concentration. No obvious structure–activity relationship could be deduced for these compounds. The low solubility in aqueous solutions precluded any further testing of these two compounds and for OT 5121 this might even be the reason for its abnormal effect. The possibility of PF 1885 acting at the same site as furosemide but being $GABA_A$ receptor subtype non-selective is currently under investigation. In this case the action of PF 1885 in the cortex should be antagonised by furosemide. Of these compounds, only OT 1222 potently interacted with [^{3}H]muscimol binding, displacing 47% of the label at 100 μM, i.e. OT 1222 could be a GABA site antagonist, a hypothesis which, as well, is currently being followed. Though CB 560 decreased and OT 1159 increased [^{3}H]muscimol binding, both compounds further inhibited concentration dependently the GABA-reduced [^{35}S]TBPS binding. A positive allosteric interaction with the GABA-binding site can be postulated for OT 1159, with a similar but presumably not identical mode of action to that of benzodiazepines. On the other hand, CB 560 might act as a direct GABA site agonist.

Of the more than 40 compounds tested, thienyl-furosemide comes closest to the properties of the parental compound furosemide as a $GABA_A$ receptor subtype-selective substance. Its potency seems to be higher than that furosemide in spite of its lower diuretic potency. Our present results further stress the feasibility of obtaining a non-diuretic derivative of furosemide with highly specific GABAergic activity, a task which now can be based on the four lead compounds furosemide, thienyl-furosemide, azosemide and benzyl-furosemide.

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