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European Journal of Pharmacology 519 (2005) 43 – 47

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Novel parent structures for inhibitors of the murine GABA transporters mGAT3 and mGAT4

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Received 27 June 2005; accepted 30 June 2005
Available online 19 August 2005

Abstract

Searching for potent and subtype selective parent structures of the murine γ-aminobutyric acid (GABA) transporter subtypes mGAT3 and mGAT4 a series of amino acids was characterised in a uniform [^{3}H]GABA uptake test system based on transiently expressed mGAT1-4. From several potent inhibitors showing IC_{50} values at mGAT3 and mGAT4 in the low μM range *cis*-4-aminocrotonic acid and (*RS*)-2,3-diaminopropionic acid turned out to be most subtype selective for these transporters. With (*RS*)-isoserine — a compound unknown as GAT inhibitor until now — one of the most potent amino acids selectively inhibiting mGAT3 and mGAT4 was found. Furthermore, (2-amino-1,3-thiazol-4-yl)acetic acid was identified as the first parent structure exhibiting a clear, though still moderate, selective inhibition of GABA uptake at mGAT3.

Keywords: Murine GABA transporter (mGAT1-4); GABA (γ-aminobutyric acid) uptake; Structure activity relationship; Subtype selectivity

1. Introduction

Four distinct subtypes of sodium-dependent transporters mediating the transport of γ-aminobutyric acid (GABA) as the major inhibitory neurotransmitter in the mammalian brain are known (Palacín et al., 1998). When cloned from murine brain these GABA transporters, are generally termed mGAT1, mGAT2, mGAT3, and mGAT4 (Liu et al., 1993; for the differing nomenclature among various species see Dalby, 2003). In the 1970s guvacine and (*RS*)-nipecotic acid were found to be inhibitors of the GABA uptake in neuronal and astroglial cell cultures (reviewed in Krogsgaard-Larsen et al., 1987). From these parent structures upon addition of lipophilic side chains to the ring nitrogen a series of potent and subtype selective mGAT1 inhibitors were developed including 1-{2-[(diphenylmethylene)amino]oxyethyl}-1,2,5,6-tetrahydropyridine-3-carboxylic acid hydrochloride (NO711, Suzdak et al., 1992; Borden et al., 1994) and (*R*)-1-[4,4-bis(3-methylthien-2-yl)but-3-en-1-yl]piperidine-3-carboxylic acid ((*R*)-tiagabine, Braestrup et al., 1990; Andersen et al., 1993; Borden et al., 1994) as well as (*S*)-1-{2-[tris(4-methoxyphenyl)methoxy]ethyl}piperidine-3-carboxylic acid ((*S*)-SNAP5114) with moderate potency and selectivity for mGAT4 (Dhar et al., 1994). In case of mGAT2 and mGAT3 subtype selective inhibitors are still rare. 1-[3-(9*H*-Carbazol-9-yl)propyl]-4-(2-methoxyphenyl)-piperidin-4-ol (NNC05-2090) lacking the carboxylic acid function of nipecotic acid or guvacine is one of the few compounds identified by Thomsen et al. (1997) as inhibitors of mGAT2 possessing a reasonable potency and selectivity for this subtype of GABA transporters. The nipecotic acid derivative (±)-1-(2-{[9-(4-methoxyphenyl)fluoren-9-yl]methoxy}ethyl)piperidine-3-carboxylic acid (SNAP5294) synthesized by Dhar et al. (1994) is the only mGAT3 selective inhibitor available at present. However, the compound's potency and subtype selectivity are still low (Dhar et al., 1994).

Whereas mGAT1 is an approved target in the treatment of epilepsy (Meldrum and Chapman, 1999), the therapeutic potential mediated by mGAT4 is less clear so far. (*S*)-

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doi:10.1016/j.ejphar.2005.06.053

SNAP5114 as a moderately selective inhibitor for mGAT4 has been shown to enhance GABA levels in the rat thalamus and to protect against tonic hind limb extension after maximal electroshock and against sound induced convulsions in mice (Dalby, 2000). In contrast, the pharmacological role of mGAT3 remains completely unclear at the moment. After having found this transporter distributed much more extensively in rat cerebral cortex than had been supposed before, Conti et al. (1999) suggested it may play a decisive role in GABAergic neurotransmission with respect to the regulation of GABA levels in the extracellular space (Conti et al., 1999).

As there is still a need for a better understanding of the pharmacological role of mGAT3 and mGAT4, in general, as well as regarding their potential as drug targets, it seems worthwhile to develop new inhibitors displaying high potency and subtype selectivity for these transporters. In this paper we report our efforts directed towards the identification of new GABA uptake inhibitors. Following the general lines of drug development we thought it most suitable to first search for basic structures of subtype selective mGAT3 and mGAT4 inhibitors. In a second step, they might be further modified with respect to their potency and subtype selectivity by addition of lipophilic side chains.

2. Materials and methods

2.1. Materials

[^{3}H]GABA (3 TBq/mmol) was purchased at Amersham Biosciences (Freiburg, Germany) scintillation cocktail at Roth (Rotiscint eco plus, Karlsruhe, Germany). The test compounds were from the following commercial sources: (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid, (*RS*)-2-amino-3-methylaminopropionic acid and GABA from ICN; guvacine, (*RS*)-nipecotic acid, NO711, *cis*-4-aminocrotonic acid, (*S*)-(−)-4-amino-2-hydroxybutyric acid, (*RS*)-4-amino-3-hydroxybutyric acid, (*RS*)-isoserine ((*RS*)-3-amino-2-hydroxypropionic acid), (*RS*)-2,3-diaminopropionic acid, 3-guanidinopropionic acid, (2-amino-1,3-thiazol-4-yl)acetic acid, SKF89976A, (*S*)-2,4-diaminobutyric acid, hypotaurine, taurine, 4-pyrazolecarboxylic acid, compounds 2, 3, 5 and 9 (for structures see Fig. 1) as well as L-β-homoglutamine, L-β-homoglutaminic acid, DL-homoleucine, L-β-homolysine, L-β-homomethionine and L-β-homothreonine from Sigma-Aldrich-RBI-Fluka (Taufkirchen, Germany); the enantiomers of nipecotic acid from Digital Specialty Chemicals (Dublin, NH, USA); *trans*-4-aminocrotonic acid and CI966 from Tocris-Biotrend (Köln, Germany), β-alanine from Merck-VWR (Darmstadt, Germany), compounds 1 and 6 (for structures see Fig. 1) from Acros (Schwerte, Germany); compounds 4, 7, and 8 (for structures see Fig. 1) from Maybridge (Tintagel, England). (*R*)-Tiagabine, (*S*)-SNAP5114, (*R*)-homoproline, and (*S*)-homoproline were synthesized by published methods. Restriction enzymes were from NEB (Frankfurt, Germany). Cell culture media, sera and additives were obtained from PAA (Cölbe, Germany).

2.2. Cell culture and transient transfection

The mammalian expression vector pRC-CMV2 containing the cDNA for the murine GABA transporter mGAT1 was kindly provided by Prof. H. Lüddens. The cDNAs encoding the murine GABA transporters mGAT2, mGAT3, and mGAT4 (kindly provided by Prof. N. Nelson) were subcloned from pGEM-HJ (mGAT2) or pBluescript (mGAT3, mGAT4) into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany, mGAT2: BamH I and *Xho*I; mGAT3: Nhe I and BamH I; mGAT4: EcoR I and *Xba*I). COS-7 and HEK cells were cultured in Dulbeccos modified Eagles medium (DMEM) supplemented with

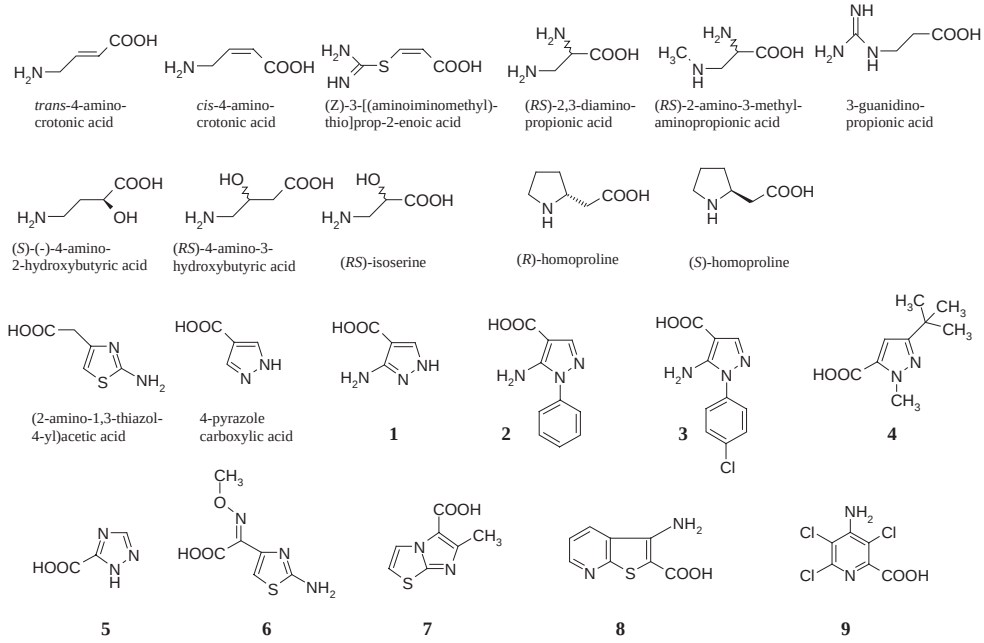

Fig. 1. Structures of investigated GAT inhibitors.

10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (92% air, 8% CO_2) at 37 °C. Transfections were performed using FuGENE6 (Roche, Mannheim, Germany) according to manufacturers protocol: Cells were plated in DMEM the night before transfection (3×10^6 COS-7 cells for mGAT1, mGAT3, mGAT4, 3.5×10^6 HEK cells for mGAT2 per 182 cm^2 flask). On the next day cells were treated with a mixture of 40 μl FuGENE6 and 13.3 μg cDNA (mGAT1, mGAT3, mGAT4) or 60 μl FuGENE6 and 10.0 μg cDNA (mGAT2).

2.3. [^{3}H]GABA uptake

Two days after transfection the medium was aspirated and the remaining cells were treated with 4 ml trypsin-EDTA. After 5 min at 37 °C for COS-7 or 30 s at room temperature for HEK cells, 8 ml of medium was added. The cells were collected and washed three times with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4) and finally resuspended in Krebs buffer (2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 11 mM glucose, 25 mM Tris, 119 mM NaCl, pH 7.2). The uptake assays were performed with aliquots of these cell suspensions in polystyrene tubes as described recently (Höfner and Wanner, 2004), except for the following modifications: the samples containing Krebs buffer, test compounds and about 6×10^4 cells (mGAT1, mGAT3, mGAT4) or about 4×10^5 cells (mGAT2) were incubated for 10 min in the presence of 13.3 nM [^{3}H]GABA and 26.7 nM GABA (mGAT1, mGAT3 and mGAT4) or 26.7 nM [^{3}H]GABA and 13.3 nM GABA (mGAT2). Non-specific uptake was defined as uptake in the presence of 10 μM NO711 (mGAT1) or 1 mM GABA (mGAT2, mGAT3, mGAT4).

3. Results

For a reliable estimation of the subtype selectivity of the test compounds with respect to each of the four cloned GABA transporters a uniform test system was needed. In order to avoid species differences the murine GATs (mGAT1-4) representing the only complete series of all four transporters from one species was selected. As to flexibility and efficiency a transient expression of mGAT1-4 in mammalian cells in combination with uptake assays employing these cells in suspension appeared to be most promising. While such "suspension assays" with heterologously expressed transporters have been recently described for other neurotransmitter transporters, e.g., the human serotonin transporter (Roman et al., 2003), they are still unknown for GABA transporters. Therefore, we first examined a representative series of GAT inhibitors already characterized at cloned GABA transporters by other groups (see Table 1) in order to check the feasibility of this approach. We obtained results, i.e., the rank order of potency and subtype selectivities, reasonably in line with the data presented by Borden et al. (1994, 1995) and Dhar et al. (1994) for the rat and human analogues of the four mGAT transporters indicating the validity of our test system.

As we were interested in parent structures exhibiting mGAT3 or mGAT4 subtype selectivity, we started to test several amino acids already known as GAT inhibitors but insufficiently characterized as to their potencies at the individual GABA transporter subtypes (see Table 2).

The *cis*- and *trans*-diastereomer of 4-aminocrotonic acid, respectively, are known to inhibit GABA uptake in cultured neurons as well as in rat brain membrane preparations, but have not been examined as to their subtype selectivity yet (Falch et al., 1986; Vale et al., 1999). We found *trans*-4-aminocrotonic acid to display a similar high potency for the three transporters mGAT1, mGAT3 and mGAT4 (IC_{50}~5 μM). Here the compound is about ten times more potent than at mGAT2 (IC_{50}=64 μM). The subtype selectivity of *cis*-4-aminocrotonic acid appeared to be noticeably different, exhibiting a marked and almost identical potency at mGAT3 and mGAT4 (IC_{50}~9 μM) and a far lower activity at mGAT1 and mGAT2 (IC_{50}=280 μM in each case). The isothiourea derivative (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid, structurally related to *cis*-4-aminocrotonic acid and known to inhibit [^{3}H]GABA uptake in rat brain slices (Allan et al., 1986), turned out to be most potent at mGAT1 (IC_{50}=9.5 μM) and distinctly less — by a factor of about 5 to 20 — at mGAT2-mGAT4.

GABA derivatives with additional hydroxy functions were reported to inhibit GABA uptake in rat homogenates (Falch et al.,

Table 1
IC_{50} values [μM] of GABA uptake inhibitors at mGAT1-mGAT4 compared with data from Borden et al. (1994, 1995) and Dhar et al. (1994)

	mGAT1[a,b]	hGAT-1[c]	rGAT-1[c]	mGAT2[a,b]	hBGT-1[c]	mGAT3[a,b]	rGAT-2[c]	mGAT4[a,b]	hGAT-3[c]	rGAT-3[c]
GABA	2.8±0.3	5±1	30±8	14±2	36±3	3.9±0.4	5±2	3.4±0.5	7±1	33±10
Guvacine	3.4±0.9	14±3	39± 6	220±20	1870±387	27±6	58±5	14±1	119±24	378±175
(*RS*)-nipecotic acid	2.6±0.3	8±0.4	24±6	310±40	2370±617	29±7	38±4	16±2	106±13	159±30
(*R*)-nipecotic acid	2.2±0.4	5.9±0.8	n.d.	140±40	2310±114	14±1	19±2	11±2	51±6	n.d.
(*S*)-nipecotic acid	24±5	116±8	n.d.	>1000	6410±213	350±30	763±55	190±40	333±76	n.d.
(*S*)-DABA[d]	24±5	n.d.	128±12	590±60	528±62	230±20	300±51	150±50	n.d.	710±237
β-alanine	660±120	5690±1890	2920±197	350±50	1320±224	12±2	19±7	22±3	58±3	110±40
Hypotaurine	170±50	n.d.	1010±135	240±100	536±41	4.9±0.6	52±6	8.1±0.7	n.d.	73±7
Taurine	>1000	n.d.	>10000	1800±500	>10,000	170±30	1270±40	220±80	n.d.	2860±358
CI966	0.66±0.18	0.26±0.05	1.2±0.2	>100	300±10	140±40	297±34	86±18	333±76	1140±337
NO711	0.14±0.02	0.04±0.01	0.38±0.07	350±40	622±265	260±40	171±53	570±50	2320±86	349±151
(*R*)-tiagabine	0.22±0.01	0.07±0.01	0.64±0.15	>100	1670±722	>100	1410±250	>100	917±193	2040±107
SKF89976A	0.80±0.03	0.13±0.01	0.64±0.19	190±50	7210±3630	200±20	550±225	410±70	4390±1420	944±259
(*S*)-SNAP5114	85±14	388±92	n.d.	35±13	140±30	22±4	21±3	3.0±0.3	5±1	n.d.

[a]Results from the present study (mean±S.E.M., n=3), [b] for published potencies at mGAT1-4 of a few compounds of the series tested in the present study see Thomsen et al. (1997) and Clausen et al. (2005); [c] data for the human and rat analogues of mGAT1-4 from Borden et al. (1994, 1995) and Dhar et al. (1994); [d] (*S*)-2,4-diaminobutyric acid; n.d.: non-determined.

Table 2
IC_{50} values ([μM], mean±S.E.M., $n=3$) of test compounds at mGAT1-mGAT4

Compound	mGAT1	mGAT2	mGAT3	mGAT4
trans-4-aminocrotonic acid	3.3±0.4	64±10	7.2±1.3	5.1±0.8
cis-4-aminocrotonic acid	280±40	280±50	9.5±1.6	9.2±1.4
ZAPA[a]	9.5±2.2	190±30	51±14	50±6
(*S*)-(−)-4-amino-2-hydroxybutyric acid	4.0±0.4	50±3	20±4	12±2
(*RS*)-4-amino-3-hydroxybutyric acid	15±5	170±40	21±3	23±0.4
(*RS*)-isoserine	2500±600	230±50	4.3±0.7	5.9±1.6
(*RS*)-2,3-diaminopropionic acid	320±70	190±30	11±2	5.6±0.4
(*RS*)-2-amino-3-methylaminoprop. a.[b]	260±50	160±40	35±11	20±4
3-guanidinopropionic acid	26±3	24±5	3.6±0.3	3.7±1.0
(*R*)-homoproline	60±7	>1000	200±30	400±60
(*S*)-homoproline	110±10	>1000	75±9	53± 7
(2-amino-1,3-thiazol-4-yl)acetic acid	1 mM/72%[c]	1 mM/62%[c]	190±10	1 mM/72%[c]
4-pyrazolecarboxylic acid	1 mM/81%[c]	640±90	1 mM/56%[c]	1 mM/81%[c]

[a](*Z*)-3-[(aminoiminomethyl)thio]prop-2-eonic acid, [b] (*RS*)-2-amino-3-methylaminopropionic acid, [c] remaining [^{3}H]GABA uptake in the presence of 1 mM test compound as compared to a control without inhibitor.

1986). (*S*)-(−)-4-Amino-2-hydroxybutyric acid and (*RS*)-4-amino-3-hydroxybutyric acid proved to be potent inhibitors at mGAT3 and mGAT4, too, but did not exhibit a substantial subtype selectivity for these transporters.

Having in mind the high potency and subtype selectivity of β-alanine for mGAT3 and mGAT4 as compared to mGAT1 and mGAT2 (Liu et al., 1993), we thought it worthwhile to examine (*RS*)-isoserine ((*RS*)-3-amino-2-hydroxypropionic acid) as a compound structurally related to β-alanine but so far not known as a GAT inhibitor. (*RS*)-Isoserine turned out to be one of the most potent parent structures at mGAT3 (IC_{50}=4.3 μM) and mGAT4 (IC_{50}=5.9 μM) that have been described until now. In addition, it shows a clear preference for these two transporters (e.g., IC_{50} mGAT3 : IC_{50} mGAT1 = 580 : 1; IC_{50} mGAT4 : IC_{50} mGAT1 = 420 : 1).

So far, the β-amino acid derivatives, (*RS*)-2,3-diaminopropionic acid and 3-guanidinopropionic acid, had been characterized as inhibitors of murine GABA transporters in Xenopus oocytes only at a single concentration (Liu et al., 1993). In accordance with the data published by Liu et al., these compounds were found to be distinctly more potent at mGAT3 and mGAT4 than at mGAT1 and mGAT2, their IC_{50} values for mGAT3 and mGAT4 being in the low μM range. The *N*-methyl derivative of (*RS*)-2,3-diaminopropionic acid, the neurotoxic, excitatory amino acid (*RS*)-2-amino-3-methylaminopropionic acid (Smith and Meldrum, 1990; Duncan et al., 1991), yet unknown as a GABA uptake inhibitor, showed a slightly lower potency and subtype selectivity than the parent compound.

It seemed reasonable to assume that other β-amino acid derivatives might be potent and subtype selective mGAT3/mGAT4 inhibitors, too. However, none of the following compounds, L-β-homoglutamine, L-β-homoglutaminic acid, DL-homoleucine, L-β-homolysine, L-β-homomethionine, and L-β-homothreonine, caused an inhibition of GABA uptake at any of the four murine GABA transporters above 50% in a concentration of 1 mM.

(*R*)- and (*S*)-homoproline, are known from bovine GABA transporter assays to be inhibitors of GAT-1 (corresponding to mGAT1) and GAT-3 (corresponding to mGAT4), respectively (Fülep, 1998). The present study showed this to be the case also for murine GABA transporters. (*R*)-Homoproline was found to display a reasonable potency and moderate subtype selectivity for mGAT1, whereas (*S*)-homoproline showed its highest potency at mGAT4, though its preference for this transporter is low.

Since both 3-guanidinopropionic acid and (*RS*)-2,3-diaminopropionic as well as its *N*-methyl derivative were found to be potent and subtype selective inhibitors of mGAT3/mGAT4 we examined several nitrogen containing heterocyclic carboxylic acids loosely related to the compounds mentioned above with respect to the nitrogen functionality (Fig. 1). One of these compounds, (2-amino-1,3-thiazol-4-yl)acetic acid turned out to be a selective inhibitor at mGAT3. Actually, this inhibitor represents the first parent structure selective for mGAT3, though its potency (IC_{50} mGAT3: 190±10 μM) and selectivity for this GABA transporter are only moderate (5 fold selective for mGAT3 compared to mGAT1, mGAT2, and mGAT4). Apart from 4-pyrazolecarboxylic acid which was found to be a weak inhibitor at mGAT2 (IC_{50} mGAT2: 640±90 μM) none of the other heterocyclic compounds (see Fig. 1, 1–9) showed an IC_{50}-value<1 mM at any of the four murine GABA transporters.

4. Discussion

Based on transiently expressed mGAT1-mGAT4 a uniform [^{3}H]GABA uptake test system has been established that allows to dependably characterize the subtype selectivities of test compounds with respect to all four GAT transporters. It was used to search for parent structures being subtype selective mGAT3 or mGAT4 inhibitors.

(*RS*)-Isoserine was identified as a new GABA uptake inhibitor displaying a high potency at mGAT3 and mGAT4 associated with a high preference for these two transporters. Representing a new parent structure for GABA uptake inhibitors (*RS*)-isoserine might serve as a promising starting point for the development of distinctly more potent derivatives. (*RS*)-2-Amino-3-methylaminopropionic acid was found to be an inhibitor of mGAT3 and mGAT4, too, though its potency and subtype selectivity are far less pronounced. Interestingly, its potency and subtype selectivity are only slightly reduced by the additional *N*-methyl group as compared to (*RS*)-2,3-diaminopropionic acid. Accordingly, the terminal amino function of (*RS*)-2,3-diaminopropionic acid seems reasonably tolerant of substitution which might

allow the addition of appropriate lipophilic residues to significantly improve the potency of this compound at mGAT3 and mGAT4. (2-Amino-1,3-thiazol-4-yl)acetic acid has to be regarded as the first parent structure selective for mGAT3 albeit its potency and selectivity are low. Nevertheless it is likely to be useful as a lead in the development of potent and selective mGAT3 inhibitors.

Acknowledgements

For technical support we thank Silke Duensing-Kropp and Ljiljana Galogaza. We thank Monika Simon for assistance with editing and proofreading. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged. We also thank Prof. Dr. H. Lüddens for providing the cDNA of mGAT1 and Prof. Dr. N. Nelson for providing the cDNAs of mGAT2, mGAT3 and mGAT4.

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