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Synthesis and Biological Evaluation of GABA Derivatives Able to Cross the Blood–Brain Barrier in Rats

Vincenzo Carelli,[a,*] Felice Liberatore,[a] Luigi Scipione,[a] Gianfabio Giorgioni,[b] Antonio Di Stefano,[b] Mariannina Impicciatore,[c] Vigilio Ballabeni,[c] Francesco Calcina,[c] Francesca Magnanini[c] and Elisabetta Barocelli[c]

[a]*Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy*

[b]*Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino, Italy*

[c]*Dipartimento di Scienze Farmacologiche, Biologiche e Chimiche Applicate, Università di Parma, Parco Area delle Scienze 27/A, 43100 Parma, Italy*

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Abstract—Two new GABA derivatives, **1** and **2**, were synthesized and tested for their capacity to display CNS activity, which was assessed by determining the effects on the duration of pentobarbital-induced hypnosis in rats. Compound **1**, peripherally injected, significantly prolonged the hypnosis time, a typical GABA-mimetic effect, while both intracerebroventricular and intravenous administration of compound **2** surprisingly shortened the hypnotic effect in an atropine-sensitive way. The study was extended also to compounds **1a**, **1b** and **2a**, putative oxidative/hydrolytic metabolites of **1** and **2**.

Deficiency in GABAergic transmission is involved in several neurological and psychiatric disorders, such as epilepsy, parkinsonism, anxiety and pain.[1] The peripheral administration of gamma-aminobutyric acid (GABA) cannot be usefully performed since this neurotransmitter is able to cross the blood–brain barrier only at extremely high doses which produce severe adverse side effects.[2] Therefore, various strategies have been proposed to deliver GABA to the brain.[3,4] To this purpose, many researches have been devoted to the synthesis of GABA-prodrugs, such as GABA esters and amides,[5] more lipophilic than the parent molecule and able to cross the blood–brain barrier and deliver and release into the brain the neurotransmitter. In this context, a useful GABA brain-specific delivery system, which showed a significant anxiolytic activity, has been developed by Anderson et al.[6] using a lipophilic 1,4-dihydronicotinamide derivative of the bioactive molecule (structure **3**, Fig. 1). This chemical system was able to penetrate the blood–brain barrier and reach the brain where it was enzymatically oxidized to the corresponding pyridinium derivative, from which GABA was released by enzymatic hydrolysis of the amide bond.

In this paper we describe the preparation and the biological properties of the two new GABA derivatives **1** and **2** (Fig. 1). To this end, GABA or GABA benzylester were linked to an interconvertible tetrahydrodinicotinamide/quaternary nicotinamide salt structure, able to participate to a redox-type reaction. Tetrahydrodinicotinamides are easily formed by irreversible dimerization of two radicals arising from one-electron chemical or electrochemical reduction of quaternary nicotinamide salts.[7–10] The tetrahydrodinicotinamides are lipophilic stable compounds, readily oxidized back to precursor quaternary salts by oxidase and peroxidase enzymes.[11–13] Therefore, the lipophilic tetrahydrodinicotinamide GABA derivatives could easily enter into the brain and here could be enzymatically oxidized to the quaternary form, from which the drug could be released by enzymatic hydrolysis. In this regard, we have already shown that a tetrahydrodinicotinamide derivative was used to successfully perform the brain-specific delivery of dopamine.[14] The CNS activity of **1** and **2** was studied evaluating their effects on duration of pentobarbital induced

*Corresponding author. Tel.: +39-06-445-5349; fax: +39-06-499-13888; e-mail: vincenzo.carelli@uniroma1.it

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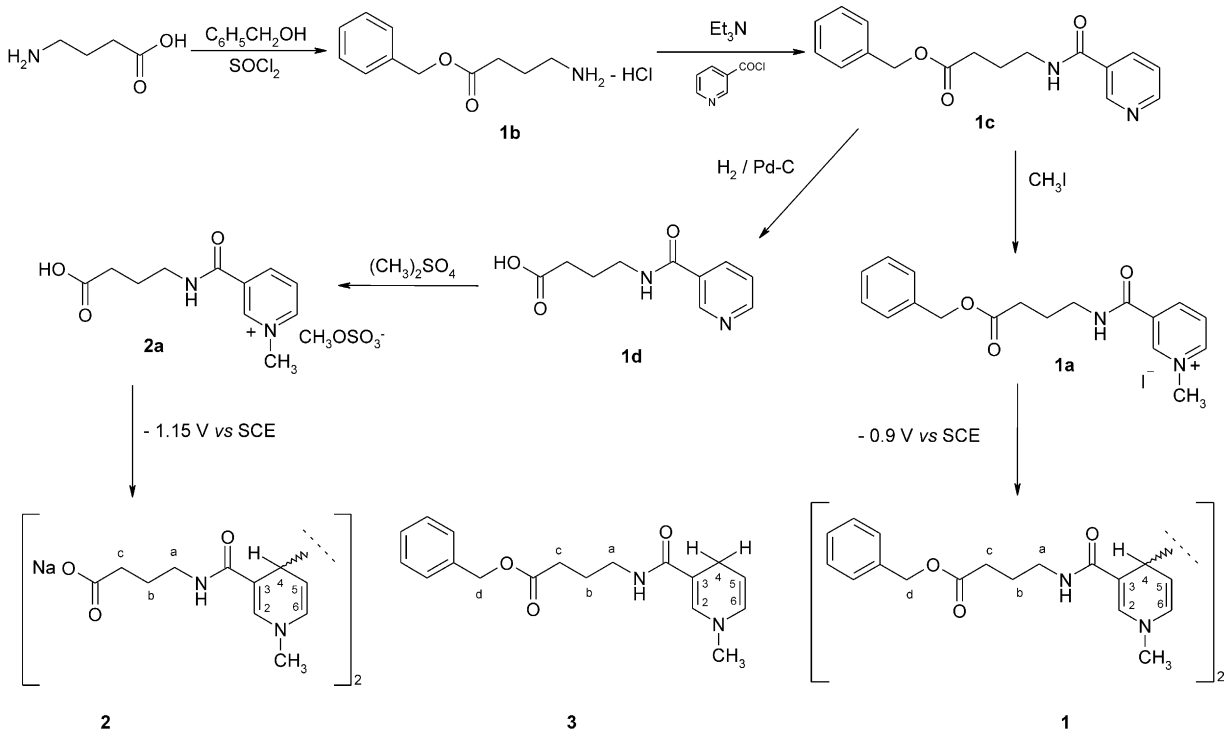

Figure 1.

hypnosis and this investigation was extended also to compounds **1a**, **1b** and **2a** (Fig. 1), their putative enzymatic and/or hydrolytic metabolites.

Furthermore, all the above compounds were examined in additional in vitro tests, in order to assess their ability to directly interfere with GABAergic subtype receptors.

Chemistry

Compound **1** and **2** were obtained as shown in Figure 1. Reaction of the GABA ester **1b**[15] with nicotinoyl chloride gave the corresponding nicotinamide derivative **1c**,[15] which afforded the quaternary salt **1a**[15] by treatment with methyl iodide. Benzyl ester **1c**, by hydrogenation on Pd/C catalyst, yielded the GABA derivative **1d**[16] which was transformed to the quaternary salt **2a**[17] by treatment with dimethyl-sulfate. Electrolysis of both quaternary salts **1a** and **2a**, carried out according to the procedure previously reported,[18] consumed one electron per mole of salt and gave crude mixtures from which **1** was isolated by preparative HPLC, while **2** was obtained as the sodium salt by exhaustive extraction with cold ethanol. Mass spectrometry and ^{1}H NMR allowed the identification of both **1** and **2** as symmetric dimers formed by coupling of the radicals resulting from the one electron reduction of **1a** and **2a** respectively. In particular the ^{1}H NMR data were consistent with 1,4-dihydronicotinamide structures, as confirmed also by comparison with ^{1}H NMR spectrum of **3**,[6] and, furthermore, the presence of methine hydrogen signals at $\delta = 3.36$ for **1** and $\delta = 3.26$ for **2**, unambiguously indicated carbons 4 as coupling sites of the dihydronicotinamide moieties.[9,10,18,19] From the dimerization of the pyridinyl radicals which gives rise to **1** and **2**, two equivalent centers of asymmetry are formed at the junction carbons; therefore, two diastereoisomers, a *meso* form (*R*,*S*) and a racemate (*RR* and *SS*) are possible in both cases. The corresponding protons of each of the diastereoisomers are stereochemically non equivalent and, therefore, have different chemical shifts. The ^{1}H NMR data[17] of **1** and **2** clearly show that from the crude reduction mixtures either the *meso* compound or the racemate have been obtained but not both, as two sets of peaks would be present in their ^{1}H NMR spectra in this case. We did not deem it necessary to ascertain which diastereoisomeric form have been isolated, because, as we previously reported,[11–13] oxidase and peroxidase enzymes do not discriminate among diastereoisomers of 4,4′bipyridinyl dimers, in account of the free-radical mechanism of the oxidation.

Biological Results

In vivo experiments

All the tests were carried out according to ethical standard guidelines approved by Italian Ministero della Salute. The ability of **1** and **2** to penetrate the blood–brain barrier and display CNS activity was assessed evaluating their effects on duration of pentobarbital-induced hypnosis, produced in rats by intracerebroventricular (icv) or peripheral (iv) administration, compared with GABA as the control compound. In these assays, 10 – 12 adult female Wistar rats (200–250 g body weight) were used in each group. Test compounds were dissolved in saline vehicle or dimethylsulphoxide (DMSO) (Tables 1 and 2) and were iv injected through

the caudal vein. Three min before, each animal received an intraperitoneal (ip) injection of sodium pentobarbital (30 mg/kg). The icv administration of test compounds was performed through a guide cannula stereotaxically implanted into the lateral cerebral ventricle of anaesthetized animals, according to Pellegrino's coordinates.[20] The hypnotic effects was assessed by measuring the time elapsed from loss to recovery of righting reflex.

GABA, icv administered at a dose of 3.9 μmol/rat, significantly prolonged pentobarbital induced hypnosis, while it was ineffective when iv injected at doses ranging from 92 to 184 μmol/rat (Tables 1 and 2).

Peripheral administration of **1** and its putative oxidative metabolite **1a**, produced dose-dependent significant prolongation of hypnosis time (Table 1). Such a response was associated with a moderate adverse side effect (dyspnea) in about 30% of treated rats.

In contrast, their putative hydrolytic metabolite, GABAbenzyl ester **1b**, did not affect hypnosis, but displayed a severe toxic effect (death consequent to a pronounced dyspnea in 30–40% of treated rats) (Table 1).

Unexpectedly, compound **2** significantly shortened the hypnosis duration by both central and peripheral administration. Its putative metabolite **2a**, when centrally administered, displayed the same arousing activity as **2**, but failed to affect hypnosis time when iv injected showing to be unable to enter the brain in significant amount (Tables 1 and 2). These surprising effects prompted us to search for a possible interaction of **2** on cholinergic neurotransmission. In this regard it was previously reported that some GABA derivatives were able to enhance high affinity choline uptake in rat brain,[21] a marker of cholinergic function, whose increase has been proved to produce arousing effects in pentobarbital-treated rats.[22,23] Actually, the shortening of hypnosis time, produced by **2** and **2a** by icv administration, was antagonized by peripheral pretreatment with atropine (Table 2).

In vitro tests

In the assays on the interactions at $GABA_A$ and $GABA_B$ receptor subtypes, agonist potency was indicated by $-\log IC_{50}$ or $-\log EC_{50}$, determined through linear regression analysis of the concentration–response curves. Antagonist potency was indicated by $-\log K_B$ values, according to Furchgott's equation.[24] When no surmountable antagonism was detected, the antagonist potency was expressed by pD'_2 values, according to van Rossum's equation.[25] Cumulative addition of GABA (1–100 μM) to electrically stimulated guinea-pig isolated ileum, produced partial inhibition of twitch response (%

Table 1. Effects produced by GABA derivatives **1** and **2** and their probable metabolites **1a**, **1b** and **2a** on pentobarbital-induced[a] hypnosis after intravenous injection in rats

Treatment by iv route	Dose μmol/rat	Hypnosis duration[b] (min)	Adverse effect
Vehicle = saline (*S*)	2 mL/kg	153 (±8)	
Vehicle = DMSO (D)	2 mL/kg	195 (±11)	
GABA (*S*)	92	155 (±9)	
	184	128 (±8)	
1 (D)	23	206 (±16)	
	46	257 (±14)**	Moderate dyspnea
1a (*S*)	23	156 (±14)	
	46	207 (±6)*	
	92	194 (±15)	Moderate dyspnea
2 (*S*)	92	149 (±13)	
	184	80 (±20)*	
2a (*S*)	368	153 (±18)	

[a]Pentobarbital (30 mg/kg ip).
[b]Values are means of 10–12 experiments, standard error is given in parentheses. Student's *t*-test was performed in statistical analysis: * $p < 0.05$, ** $p < 0.01$ versus vehicle treated rats.

Table 2. Effects produced by GABA derivatives **2** and its probable metabolites **2a** on pentobarbital-induced[a] hypnosis after intracerebroventricular injection in rats and effects produced by pretreatment with atropine[b]

Treatment by icv route	Dose μmol/rat	Hypnosis duration[c] (min)
Vehicle = saline (*S*)	10 μL/rat	166 (±5)
GABA (*S*)	3.90	188 (±7)*
2 (*S*)	1.95	147 (±19)
	3.90	142 (±25)
	7.80	60 (±19)*
2 (*S*) + Atropine (1.0 mg/kg iv)	7.80	184 (±15)
2a (*S*)	15.6	100 (±18)*
2a (*S*) + Atropine (1.0 mg/kg iv)	15.6	158 (±12)

[a]Pentobarbital (30 mg/kg ip).
[b]Atropine (1 mg/kg, iv) was injected 5 min before **2** and **2a**.
[c]Values are means of 10–12 experiments, standard error is given in parentheses. Student's *t*-test was performed in statistical analysis: * $p < 0.01$ versus vehicle treated rats.

maximal inhibition = 49 ± 8%; −log IC_{50} = 5.65 ± 0.14), as previously reported.[26] This effect on $GABA_B$ receptors subtypes was competitively antagonized by the selective $GABA_B$ blocker CGP-52432 (pK_B = 6.56 ± 0.10) and not competitively by compound **1** pD'_2 = 5.59 ± 0.16. Compounds **2** and **2a** were completely ineffective in this test at concentrations up to 100 μM. GABA dose-dependently contracted the guinea-pig ileal smooth muscle (1–100 μM) (−log EC_{50} = 5.11 ± 0.11), and this $GABA_A$ receptor subtype mediated response was competitively antagonized by bicuculline (pK_B = 5.34 ± 0.50). In contrast, compounds **1**, **2** and **2a** did not affect ileal tone at concentrations up to 100 μM. Furthermore, compounds **2** and **2a** (0.01–10 mM) were not able to inhibit L-glutamic acid decarboxylase activity in the specific enzymatic test.[27]

Discussion

The findings reported in the present paper show that both the compounds **1** and **2**, after peripheral administration, entered the brain differently from GABA.[28] Indeed, the activities on CNS produced by peripheral administration of **1** and **2**, could occur only if they were able to cross the blood–brain barrier. Compound **1** was capable to produce prolongation of the barbiturate induced hypnosis (Table 1), a typical GABAmimetic effect, which is indeed associated with an enhancement in central GABAergic transmission.[29]

Prolongation of the duration of hypnosis time was also exhibited from **1a**, the putative oxidative metabolite of **1** (Table 1).

In this regard, should be noted that **1a**, the corresponding brain oxidative metabolite of the dihydronicotinamide **3**, is likely to be responsible for the anxiolytic effects described by Anderson et al.[6]

The GABA derivative **2** gave rise to a CNS effect opposite to **1** and **1a**, because it produced in rats reduction of hypnosis time, both by icv or iv administration. The quaternary salt **2a** evoked the same arousing effects only by icv injection; therefore, it is likely that this compound, resulting from metabolic oxidation of **2** in the brain, could be the true active principle. The excitatory properties of **2** and **2a** were confirmed by the convulsive effects displayed after their central administration in awake rats (data not shown). These GABA opposing responses could be ascribed either to attenuation of the GABAergic function through the inhibition of GABA receptors or the reduction of GABA availability.[30] A $GABA_A$ or $GABA_B$ antagonist activity of **2** and **2a** can be ruled out owing to their total inactivity in guinea-pig isolated ileum tests earlier described. On the other hand, interference with GABA cerebral biosynthesis can be excluded because of the inability of **2** and **2a** in blocking L-glutamic acid decarboxylase (GAD) activity in the in vitro specific enzymatic test. Most likely, the inhibition exerted by atropine on arousal effect of compounds **2** and **2a**, allows us to hypothesize that **2** enhances the central cholinergic neurotransmission through its metabolite **2a**.

Further studies on the pharmacokinetic profile of the compounds as well as on the paradoxical activity of **2** and **2a** are currently in progress.

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17. Compound **1**: yield 56%; MS (ESI) *m/z* 649 $(M+Na)^+$. ^1H NMR 500 MHz ($CDCl_3$): 6.86 (1H, d, H2, $J_{2,6}$ = 1.3 Hz); 5.88 (1H, dd, H6, $J_{5,6}$ 7.8); 6.98 (1H. bs, NH); 3.36 (1H, d, H4, $J_{4,5}$ = 4.7 Hz); 4.57 (1H, dd, H5); 7.34 (5H, s, Ar); 5.11 (2H, s, (d) O-CH_2); 2.39 (3H, s, N–CH_3); 3.36 (2H, t, (a) N–CH_2, $J_{a,b}$ = 6.7 Hz); 2.46 (2H, t, (c) CH_2–CO, $J_{b,c}$ = 7.4Hz); 1.91 (2H, m, (b), CH_2-C). Anal. calcd for C_{36} H_{42} N_4 O_6: C, 68.99; H, 6.75; N, 8.94. Found C, 68.61; H, 6.99; N, 8.63. Compound **2**: yield 72%; MS (ESI) *m/z* 491 $(M+H)^+$. ^1H NMR 500 MHz (D_2O): 6.86 (1H, s, H2); 5.03 (1H, d, H6, $J_{5,6}$ = 8.0 Hz); 3.26 (1H, d, H4, $J_{4,5}$ = 4.3 Hz); 4.51 (1H, dd, H5); 2.88 (3H, s, N–CH_3); 3.35 (2H, t, (a) N–CH_2, $J_{a,b}$ = 7.0 Hz); 2.37 (2H, t, (c) CH_2–CO, $J_{b,c}$ = 7.6 Hz); 1.96 (2H, m, (b) CH_2–C). Compound **2a**: yield 1.2 g (80%), mp 95–96 °C. ^1H NMR 500 MHz (D_2O): 9.21 (1H, s, H2); 8.95 (1H, d, H6, $J_{5,6}$ = 7.8 Hz); 8.83 (1H, d, H4, $J_{4,5}$ = 5.4 Hz); 8.17 (1H, dd, H5); 4.47 (3H, s, N–CH_3);

3.35 (2H, t, (a) N-CH_2, $J_{a,b}$ = 5.6 Hz); 2.50 (2H, t, (c) CH_2–CO, $J_{b,c}$ = 7.6 Hz); 1.84 (2H, m, (b) CH_2-C).
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