J. Pharm. Pharmacol. 1989, 41: 191–193
Communicated September 5, 1988

In-vitro evaluation of filaricidal activity of GABA and 1,3-dipalmitoyl-2-(4-aminobutyryl)glycerol HCl: a diglyceride prodrug

JEAN R. DEVERRE, PHILIPPE LOISEAU*, PATRICK COUVREUR, YVES LETOURNEUX†, PHILIPPE GAYRAL*, JEAN P. BENOIT, *Laboratoire de Pharmacie Galénique et de Biopharmacie, UA CNRS 1218, Université de Paris XI, Chatenay-Malabry, * Laboratoire de Parasitologie, Université de Paris XI, Chatenay-Malabry, and † Laboratoire de Chimie Organique, Université de Nantes, UER des Sciences Pharmaceutiques, France*

Abstract—A diglyceride ester of γ-aminobutyric acid (GABA) has been synthesized and its filaricidal activity compared with GABA, and progabide in-vitro, on infective larvae and microfilariae of *Molinema dessetae*, a rodent filaria. GABA induced paralysis in infective larvae but was inactive on microfilariae. There were interactions between the culture medium and GABA. The ester drug at 0·1 mmol L^{-1} (1,3-dipalmitoyl-2-(4-aminobutyryl)glycerol HCl) was as active as progabide on infective larvae and hundredfold more potent than GABA. Its microfilaricidal activity at 1 mmol L^{-1} was lower than that progabide at 0·1 mmol L^{-1} but a delayed effect was observed. The data confirm filariae sensitivity to GABA derivatives.

γ-Aminobutyric acid (GABA) plays a major role as a neurotransmitter in the central nervous system. Recently, the discovery of high levels in macrofilariae (Jacquot et al 1986) suggested the existence of GABA receptors in such organisms, thereby creating a new field of interest in filariasis therapeutics. One of the modes of action of ivermectin, a microfilaricidal drug used in onchocerciasis treatment, could be its interference with putative GABA receptors on the worms (Aziz et al 1982; Gayral 1987; Bennett et al 1988).

Diglyceride prodrugs have received attention as potential carriers of active ingredients (Garzon-Aburbeh et al 1983; Vaizoglu & Speiser 1986). In-vivo studies have shown a reduction of gastrointestinal toxicity (Kumar & Billimoria 1978; Paris et al 1980) and high lymphatic concentrations (Garzon-Aburbeh et al 1983) when a drug was covalently bound into the 2-position of a diglyceride.

An orally administrable system, capable of increasing GABA lymphatic concentrations should therefore be of interest for the treatment of filariae in the lymphatic system.

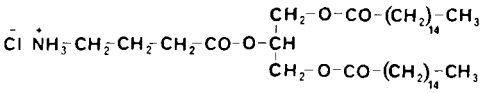
To explore this approach, we synthesized 1,3-dipalmitoyl-2-(4-aminobutyryl)glycerol HCl (I), a diglyceride derivative of GABA, with the aim of demonstrating the feasibility of such a preparation, keeping intact the pharmacological activity of GABA when linked to the diglyceride system.

Materials and methods

Materials. All chemicals were provided by Aldrich (Strasbourg, France) or Fluka (Strasbourg, France) and used without further purification unless otherwise indicated. Progabide was provided by Synthelabo (Paris, France). Culture media and foetal calf serum were from Gibco (Bioculture, Paris, France) and culture plates from Falcon (Grenoble, France). The parasite was *Molinema dessetae* (Gayral et al 1982).

Chemistry. 1,3-D-GABA HCl (I) was synthesized by a method similar to that described by Jacob et al (1985). The amino group of GABA was first protected by formation of its *t*-butoxycarbonyl derivative. This was converted to the symmetrical anhydride by using dicyclohexylcarbodiimide. 1,3-Dipalmitoyl-2-propanol was esterified by reaction with the anhydride. Treatment with hydrochloric acid (12M) in dioxane, to remove the *t*-butoxycarbonyl group gave the hydrochloride salt of 1,3-D-GABA.

Correspondence to: J. P. Benoit, Laboratoire de Pharmacie Galénique et de Biopharmacie, UA CNRS 1218, Université de Paris XI, 92296 Chatenay-Malabry Cedex, France.



(I): 1,3-Dipalmitoyl-2-(4-aminobutyryl) glycerol HCl

Preparation of the aqueous dispersion of 1,3-D-GABA HCl. An aqueous dispersion of the prodrug was obtained by the classical method for the preparation of liposomes (Bangham et al 1965). A thin film of 1,3-D-GABA HCl (58·7 mg) was deposited on the wall of a round-bottomed flask after evaporation of organic solvent (30 mL methylene chloride). The film was then dispersed under stirring in deionized water (6 mL) at 50°C for 1 h. The preparation was placed for 45 min in a bath-type sonicator (Bransonic 220, Paris, France). Thin-layer chromatography (TLC) was performed to study the degradation of the prodrug, on 200 μm thick TLC aluminium sheets precoated with silica gel 60F254 (Merck, Nogent-sur-Marne, France) with the following systems: n-hexane–methylene chloride–methanol (60:30:10); the spots were detected with sulphuric acid (95%)–absolute methanol (50:50) following calcination. TLC analysis showed no degradation of the prodrug.

In-vitro assay. Infective larvae and microfilariae of *Molinema dessetae*, a rodent filaria, were used as a model for in-vitro filaricidal assays (Gayral et al 1987) on which to test the compound.

The in-vitro assays were performed in plastic flat-bottomed 24 well trays with a well size of 17·8 × 16 mm. Each well contained 1·4 mL of a culture medium consisting of Hepes (25 mmol L^{-1}), buffered RPMI 1640 supplemented (or not) with 10% foetal calf serum and the usual antibiotics. The temperature was 37°C; the environment was 10% CO_2.

Infective larvae were isolated by dissection from the intermediate host, the mosquito *Aedes aegypti*, and decontaminated as described by Bories et al (1986). Two protocols were followed. *Protocol 1*: larvae (about 20) were added to each well, previously filled with the medium (1·4 mL). 100 μL of aqueous solution or dispersion of test compound was added. Control wells contained 100 μL of water.

Protocol 2: 100 μL of an aqueous solution of GABA (0·15 mol L^{-1}) was added to 1·4 mL of the medium 0, 10, 30, 60 min before the addition of the infective larvae. Larvae viability was estimated during the first hour at 2 min intervals, then on days 2 and 8 by direct observation of the motility of infective larvae using a microscope (×40). During the first hour, the infective larvae response was homogeneous and expressed as: comparable with untreated larvae, showing contracture or showing paralysis. After, the percentages of surviving larvae were calculated for each well compared with untreated wells. Results were expressed as shown in Table 1. The ratio of surviving to the total number of larvae was calculated for each well and corrected with the ratio of untreated parasites.

Microfilariae were isolated from *Proechimys oris* blood. When rodent microfilaremia reached about 15×10^3 microfilariae mL^{-1} (Gayral et al 1987) rodent blood (0·5 mL) was collected

Table 1. In-vitro sensitivity of infective larvae and microfilariae of *Molinema dessetae* to GABA, 1,3-D-GABA, and progabide. (The culture medium was supplemented with 10% foetal calf serum).

Compound	Dispersing phase	Well concn. (mmol L^{-1})	Infective larvae			Microfilariae		
			1 h	day 2	day 8	1 h	day 2	day 8
1,3-Dipalmitin	5% Tween 80(a)	1	–	–	–	–	–	–
	Aqueous solution	0·1	–	–	–	–	–	–
GABA	Aqueous solution	10	+(b)	–	–	–	–	–
		1	–	–	–	–	–	–
1,3-D-GABA (ester)	Colloidal suspension	1	–	–	++	–	–	++
		0·1	–	–	++	–	–	–
Progabide	Aqueous solution with 0·7% DMSO (c)	1	++	++	++	–	++	++
		0·1	++	++	++	–	–	++

(a) unpublished data show that Tween 80 was inactive below 0·05 g L^{-1} in a well.
(b) temporary paralysis.
(c) unpublished data show that DMSO was inactive below 0·7% (w/w) in a well.
++ 0 to 36% of surviving larvae; complete microfilariae paralysis.
+ 37 to 70% of surviving larvae; significant slowing down of microfilariae motility.
– 71 to 100% of surviving larvae; same motility as untreated microfilariae.

from the retro-orbital sinus using a Pasteur pipette and diluted with the medium (4·5 mL). Red cells were not discarded. 0·1 mL of this dilution (about 100 microfilariae) was then added to each well, which had previously been filled with the medium (1·3 mL). The protocol was then similar to the infective larvae screening (protocol 1), but microfilariae were not counted because of their homogeneous response; so viability was estimated globally, after addition of the prodrug, at hour 1, on days 2 and 8 using the reverse microscope (× 400). Results were expressed as in Table 1.

The stability of 1,3-D-GABA HCl in the medium was assessed as follows: 1 mL of aqueous dispersion (10^{-3} mol L^{-1}) of the prodrug was added to 2 mL of medium and left for 7 days at 37°C. Then the solution was dried under vacuum and the residue subjected to TLC as previously described.

Results

The in-vitro results are reported in Table 1 and Fig. 1. Infective larvae and microfilariae are two development stages of *Molinema dessetae*, the first one was the most sensitive to the compounds studied.

1,3-Dipalmitin, which was expected to result from the ester bond hydrolysis of the prodrug, was assayed in presence of 5% (w/w) Tween 80. No activity was detected, irrespective of the concentrations used (Table 1).

GABA, at a concentration (10 mmol L^{-1}), had no effect on microfilariae (Table 1) but induced a temporary paralysis on infective larvae. That decreased or disappeared, respectively, when GABA was incubated 10 min and 1 h in the culture medium before adding infective larvae to the wells (Fig. 1). To gain more insight into the nature of the GABA interactions with the culture medium, the foetal calf serum was discarded and the same experiments were repeated. The filaricidal activity of the neurotransmitter was further reduced. Fig. 1 shows GABA interaction with the RPMI 1640 buffer.

On the contrary, 1,3-D-GABA HCl and progabide showed a significant in-vitro activity on the microfilariae and infective

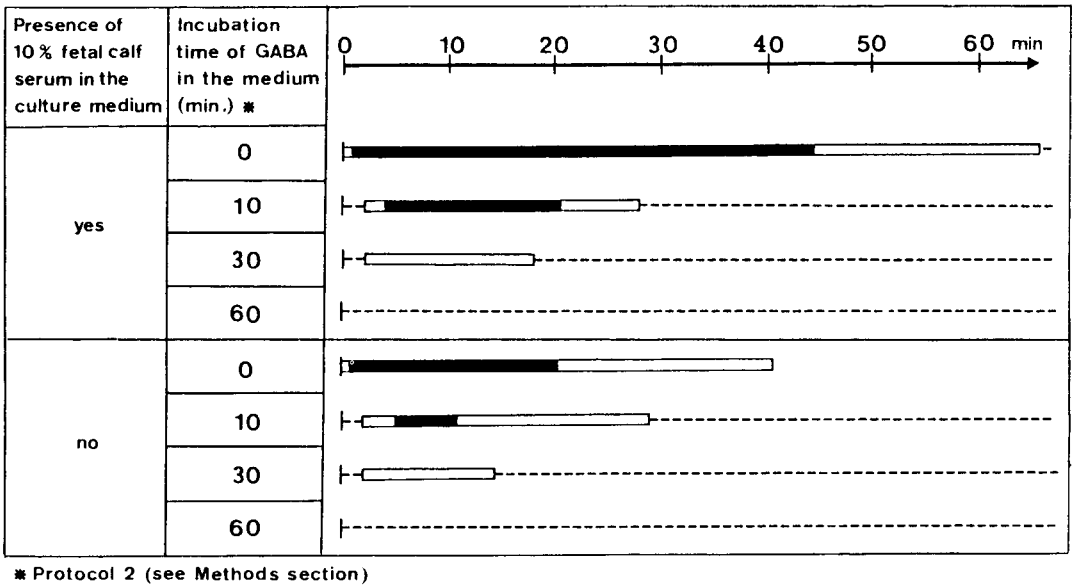


FIG. 1. Effect of GABA incubation time in the medium on its filaricidal activity on infective larvae (10^{-2} mol L^{-1} concentration). ---: motility as in untreated wells, □: contracture, ■: paralysis.

larvae (Table 1). The diglyceride prodrug was as potent (0·1 mmol L^{-1}) as progabide on infective larvae on day 8 but less effective (1 mmol L^{-1}) in microfilariae (0·1 mmol L^{-1} was inactive). Where the prodrug was active, the activity lasted longer than with progabide.

About 80% of 1,3-D-GABA HCl was hydrolysed in the medium after 8 days at 37°C, leading to 1,3-dipalmitoyl-2 propanol as a degradation product.

Discussion

The study has shown filariae sensitivity to the GABA derivatives: 1,3-D-GABA HCl and progabide.

GABA exhibits a transient effect on infective larvae at 10 mmol L^{-1} but the results in Fig. 1 show that the RPMI 1640 medium itself interferes with the neurotransmitter and leads to a decrease of the potentially resorbable fraction of the amino acid. Since no proteins (i.e. enzymes) are present in this type of medium, no degradation process can occur and the nature of the interaction between GABA and the medium is likely to be ionic. The fact that no effect was detected on the parasites at 1 mmol L^{-1}, even at the beginning of the experiment, tends to show that the passage of GABA through the parasite cuticle is poor. This is consistent with the physicochemical properties of GABA in water. Its low partition coefficient (0·042) between octanol and water (Yunger & Cramer 1981), the predominant zwitterionic structure (Tanaka et al 1978; Cabani et al 1981) associated with high molecular dipole moment, 13 Debye (Craven & Weber 1983), do not favour a passive diffusion of a molecule through a lipidic milieu such as the parasite cuticle. This can be related to previous observations showing that GABA's ability to cross the blood brain barrier is poor (Chase et al 1976). On the other hand, 1,3-D-GABA HCl and progabide exhibit a strong lipophilicity based on their chemical structure and their behaviour in common solvents. Consequently, the difference in activity seen with GABA and its two derivatives could be mainly due to the difference in their penetration of the cuticle of the two forms of the parasite.

Whatever the stage of development of the parasite, progabide shows a faster filaricidal effect than that observed with the glycerolipid prodrug. This suggests either that the hydrolysis generating GABA molecules is rapid or that the progabide acts as a specific agonist, as described earlier with mammalian cells (Bowery et al 1982). On the contrary, the glycerolipidic derivative exhibits a clear delayed effect since the activity of 1,3-D-GABA HCl on the infective larvae and microfilariae was only observed on day 8. It is believed that this derivative behaves as a genuine prodrug and not as a specific agonist. Therefore, if this delayed effect is confirmed in-vivo, anaphylactic shock, commonly observed with some microfilaricidal drugs (Gentilini et al 1986), could be avoided.

Furthermore, it was observed that 1,3-D-GABA HCl was progressively hydrolysed into GABA and 1,3-dipalmitoyl-2-propanol during the in-vitro experiment in the artificial medium. As GABA had no activity at the concentration of the prodrug activity, the sensitivity of the parasites to the prodrug was underestimated during the course of this study.

Conclusion. The diglyceride ester of γ-aminobutyric acid has an interesting in-vitro activity on infective larvae and microfilariae. This study suggests that this prodrug because of its lipophilicity penetrates the biological membranes much better than GABA itself. However, the filaricidal activity of the glycerolipidic derivative seems to be related to the progressive release of the original transmitter inside the parasite, as was suggested by the observed delayed effect.

References

Aziz, M.A., Diallo, S., Diol, I. N., Lariviere, M., Porta, M. (1982) Efficacy and tolerance of ivermectin in human onchocerciasis. Lancet 24: 171–173

Bangham, A. D., Standish, M. M., Watkins, J. C. (1965) Diffusion of swollen lipids. J. Mol. Biol. 13: 238–252

Bennett, J. L., Williams, J. F., Dave, V. (1988) Pharmacology of ivermectin. Parasitology Today 4: 226–228

Bories, C., Loiseau, P., Gueyouche, C., Gayral, P. (1986) Utilisation des larves infectantes de *Dipetalonema dessetae* en survie pour l'étude et la recherche des substances filaricides. J. Pharmacol. (Paris) 17: 301–307

Bowery, N. G., Hill, D. R., Hudson, A. L. (1982). Evidence that SL75102 is an agonist at $GABA_B$ as well as $GABA_A$ receptors. Neuropharmacology 21: 391–395

Cabani, S., Conti, G., Matteoli, E., Tine, M. R. (1981) Volumetric properties of amphionic molecules in water J. Chem. Soc., Faraday Trans. 1. 77: 2377–2384

Chase, T. N., Walters, J. R. (1976) In: Roberts, E., Chase, T. N., Tower, D. B. (eds) GABA in nervous system function. Raven Press, New York, pp 497

Craven, B. M., Weber, H. P. (1983) Charge density in the crystal structure of γ-aminobutyric acid at 122 K—An intramolecular methylene H bridge. Acta Cryst. B39: 743–748

Garzon-Aburbeh, A., Poupaert, J. H., Claesen, M., Dumont, P., Atassi, G. (1983) 1,3-Dipalmitoylglycerol ester of chlorambucil as a lymphotropic, orally administrable antineoplasic agent. J. Med. Chem. 26: 1200–1203

Gayral, P., Bories, C., Loiseau, P., Gueyouche, C. (1987) *Molinema* (ex *Dipetalonema*) *dessetae* for in vitro and in vivo evaluations of filaricidal activities. Trop. Med. Parasit. 38: 65

Gayral, P. (1987) Actualité des avermectines comme modèle pharmacologique. Actualités de Chimie Thérapeutique, 14 éme série: 31–36

Gayral, P., Dreyfuss, G., Gantier J. Ch. (1982) *Dipetalonema dessetae* chez *Proechimyoris*: cycle biologique de la filaire et proposition d'un modèle de filariose expérimentale de rongeur. Cah. O.R.S.T.O.M., ser Ent. Med. et Parasitol. 20: 81–94

Gentilini, M. (1986) Maladies parasitaires. In: Gentilini, M., Duflo, B., Danis, M., Lagardére, B., Richard-Lenoble, D., (eds) Médecine tropicale. Flammarion Médecine-Sciences, Paris, pp 186

Jacob, J. N., Shashoua, V. E., Campbell, A., Baldessarini, R. J. (1985) Aminobutyric acid esters.2.Synthesis, brain uptake, and pharmacological properties of lipid esters of aminobutyric acid. J. Med. Chem. 28: 106–110

Jacquot, C., Barreteau, H., Trouvin, J. H., Gayral, P., Leroy, J. P. (1986) Putative neurotransmitters in three experimental filariasis models. Life Sci. 39: 1539–1542

Kumar, R., Billimoria, J. D. (1978) Gastric ulceration and the concentration of salicylate in plasma in rats after administration of ^{14}C-labelled aspirin and its synthetic triglyceride, 1,3-dipalmitoyl-2(2'-acetoxy-[^{14}C] carboxybenzoyl] glycerol. J. Pharm. Pharmacol. 30: 754–758

Paris, G. Y., Garmaise, D. L., Cimon, D. G., Sweet, L., Carter, G. W., Young, P. (1980) Glycerides as prodrugs.3. Synthesis and antiinflammatory activity of [1-(p-chlorobenzoyl)-5-methoxy-2-methyl indole-3-acetyl)] glycerides (Indomethacin glycerides). J. Med. Chem. 23: 9–12

Tanaka, K., Akutsu, H., Ozaki, Y., Kyogoku, Y., Tomita, K. (1978) Molecular conformations of γ-aminobutyric acid and γ-aminobutyric-β-hydroxybutyric acid in aqueous solution. Bull. Chem. Soc. Jpn. 51: 2654–8

Vaizoglu, M.O., Speiser, P. (1986) Phase behavior of a diglyceride prodrug: spontaneous formation of unilamellar vesicles. Acta Pharm. Suec. 23: 163–172.

Yunger, L. M., Cramer, R. D. (1981) Measurement and correlation of partition coefficients of polar amino acids. Mol. Pharmacol. 20: 602–608