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Ligands for expression cloning and isolation of $GABA_B$ receptors

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Abstract

Outlined is the rationale behind the syntheses of radioligands [^{125}I]CGP64213 and [^{125}I]CGP71872, which led to the identification of cloned $GABA_B$ receptors 1a and 1b 17 years after the first pharmacological characterisation of native $GABA_B$ receptors by Bowery et al. [Nature 283 (1980) 92–94]. More recently it was shown that the *N*-terminal extracellular domains of $GABA_B$ receptors 1a and 1b contain the binding sites for agonists and antagonists [B. Malitschek et al., Mol. Pharmacol. 56 (1999) 448–454]. In order to isolate the extracellular domain(s) of $GABA_B$ receptors 1a (or 1b) and to purify and crystallise these proteins a third ligand [^{125}I]CGP84963 was designed, which combines, in one molecule, a $GABA_B$ receptor binding part, an azidosalicylic acid as photoaffinity moiety and 2-iminobiotin, which binds to avidin in a reversible, pH-dependent fashion [W. Froestl et al., Neuropharmacology 38 (1999) 1641–1646].

Keywords: $GABA_B$ receptors 1a and 1b; Radioligands [^{125}I]CGP64213, [^{125}I]CGP71872 and [^{125}I]CGP84963

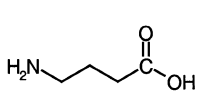
1. Introduction

At the XIIth International Symposium on Medicinal Chemistry in Basel in September 1992 we presented a novel class of selective $GABA_B$ receptor ligands, which were discovered when we replaced the carboxylic acid group of the endogenous neurotransmitter γ-aminobutyric acid (GABA) by phosphinic acid residues [1]. We found that the phosphinic acid analogue CGP27492 and the methyl-phosphinic acid derivative CGP35024 (Fig. 1) are highly potent $GABA_B$ receptor agonists [2], whereas phosphinic acid analogues with substituents larger than methyl displayed properties of $GABA_B$ receptor antagonists, e.g. the CGPs 36216, 35348 and 36742 [3]. The phosphinic acid derivative CGP47656 substituted with a group R between methyl and ethyl, such as difluoromethyl, turned out to be a partial agonist [2].

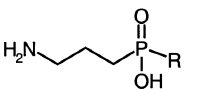
We invested years of optimisation work in order to improve the affinity of the weak $GABA_B$ receptor antagonists, e.g. CGP36742 ($IC_{50} = 32\ \mu M$) to obtain compounds with single-digit nanomolar affinity, for example CGP54626A ($IC_{50} = 7$ nM) or CGP62349 ($IC_{50} = 2$ nM; Fig. 2; IC_{50}s: inhibition of binding of [^{3}H]CGP27492) [1,4–7].

2. Ligands for expression cloning of $GABA_B$ receptors

Since the first pharmacological characterisation by Bowery et al. in 1980 [8] several groups have attempted the isolation of $GABA_B$ receptors. Kuriyama and coworkers [9] and Facklam and Bowery [10] tried to purify $GABA_B$ receptors from bovine and pig brains, respectively, via affinity chromatography. The groups of Sekiguchi [11], Tanaka [12], Woodward [13], and others, attempted expression cloning in *Xenopus* oocytes. Ultimately, all attempts failed.



GABA



R = H	CGP27492
R = Me	CGP35024
R = CHF_2	CGP47656
R = Et	CGP36216
R = $CH(OEt)_2$	CGP35348
R = *n*-Bu	CGP36742

Fig. 1.

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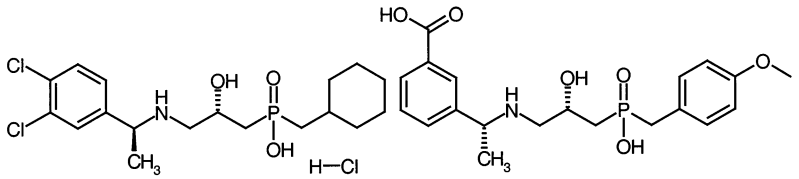


Fig. 2.

A useful ligand has to fulfil at least three important requirements: it must

- be an antagonist labelling all affinity states of the receptor;
- have a very high affinity to the receptor;
- contain a radioisotope with very high specific radioactivity, e.g. ^{125}I or ^{32}P.

We identified a useful ligand, i.e. CGP54748 (IC_{50} = 5 nM; Fig. 3), which was sent to Amersham for the exchange reaction of non-radioactive iodine with the ^{125}I isotope.

This reaction, however, resulted in decomposition of the desired product. We first rationalised that the strong radiation of the ^{125}I isotope caused a radical cleavage of the oxygen–hydrogen bond, triggering a fragmentation reaction as outlined in Fig. 4.

In order to test this hypothesis we prepared the corresponding 2-deoxy compound CGP57076A (IC_{50} = 19 nM), a pure enantiomer with the α-methyl substituent in (*S*)-configuration, and submitted this compound to the isotope exchange reaction. Decomposition also occurred in this case. We assume the fragmentation reaction proceeds via the benzylic radical (Fig. 5).

We came to the conclusion that we should place the radioactive iodine isotope at a position in the molecule as remote as possible from this fragile tertiary benzylic C–H bond. The α-methyl substituent cannot be omitted, as it is indispensable for the single-digit nanomolar affinity of our ligands to $GABA_B$ receptors [1,5]. For this reason we prepared ligands with amino-alkyl side chains at the phosphinic acid moiety of the molecule (Fig. 6), to which we attached the iodine-bearing aromatic ring via an amide bond. Amides, unlike the primary amines, showed high affinity to $GABA_B$ receptors.

This idea proved to be feasible and led, finally, to the desired antagonist ligand [^{125}I]CGP64213 (Fig. 7) with an even higher affinity to $GABA_B$ receptors than expected, having the required very high specific radioactivity. This ligand is stable for one half-life of the ^{125}I isotope, i.e. 60 days.

The synthesis of the photoaffinity ligand [^{125}I]CGP71872 (Fig. 8) was carried out in a similar way. On irradiation at a wavelength of 365 nm for

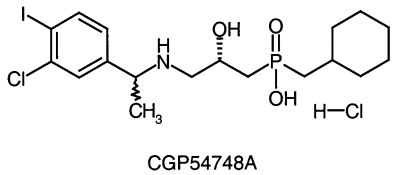


Fig. 3.

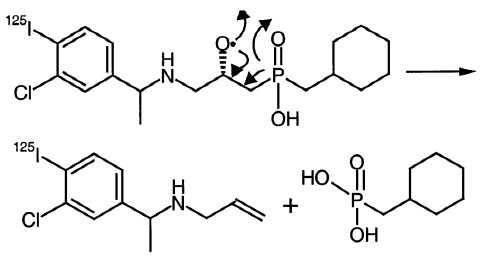


Fig. 4.

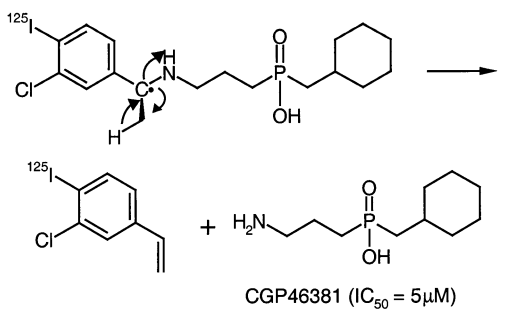


Fig. 5.

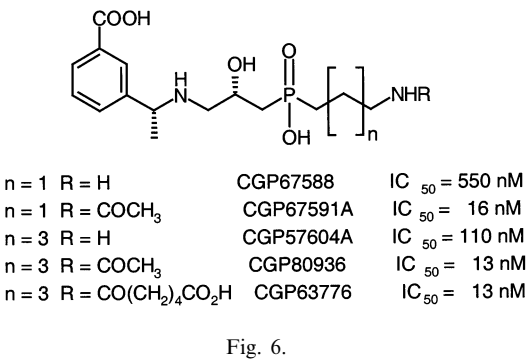
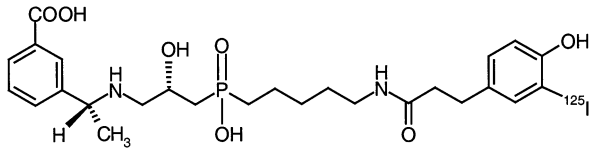
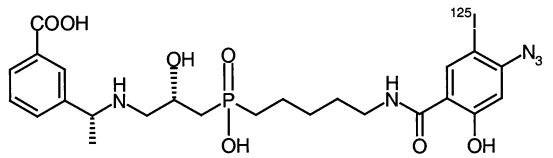


Fig. 6.



[125I]CGP64213 K_D = 1.2 nM, specific radioactivity >2000 Ci/mmol

Fig. 7.



[125I]CGP71872 K_D = 1 nM, specific radioactivity >2000 Ci/mmol

Fig. 8.

3 min with a 24 W lamp, [125I]CGP71872 bound irreversibly to native $GABA_B$ receptors in rat cortex, cerebellum and spinal cord membranes, revealing two bands of 130 K and 100 K in SDS gel electrophoresis, which were attributed to two $GABA_B$ receptor splice variants.

Our molecular biologists set up a screening procedure for [125I]CGP64213 binding at transfected COS cells. By screening a cDNA library of two million clones prepared from cerebella and cortices of 7-day-old rats they succeeded in identifying the clones encoding $GABA_B$ receptor 1a, a protein of 960 amino acids and $GABA_B$ receptor 1b, a protein of 844 amino acids, both with the typical seven transmembrane-spanning domains of G-protein coupled receptors [14]. The syntheses of both radioligands are described with full experimental details in our patent [15]; 2 years after the publication of our patent Merck scientists re-published the synthesis of [125I]CGP71872 [16].

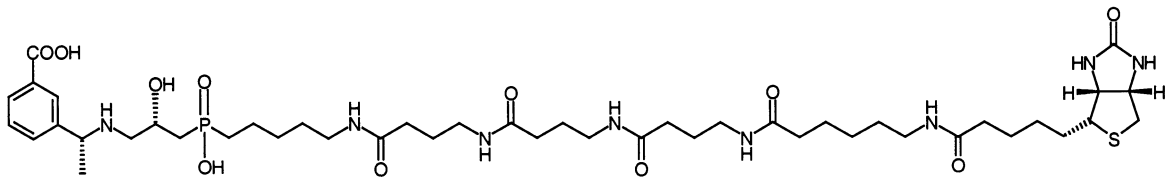
3. Ligands for isolation of $GABA_B$ receptor fragments

Malitschek et al. [17] succeeded in expressing the soluble *N*-terminal extracellular domains (ECDs) of both $GABA_B$ receptors 1a and 1b in insect cells using recombinant baculovirus. The photoaffinity ligand [125I]CGP71872 binds to the soluble ECDs, which can be blocked by prior exposure to CGP54626A. These experiments showed that the ECDs could fold correctly even when dissociated from the transmembrane domains to provide full ligand binding. Now we can attempt to isolate the ECDs, purify and crystallise these proteins. By determining their X-ray structures we will obtain precise information on the three-dimensional environment of the $GABA_B$ receptor 1 binding sites.

The method of choice for protein purification is affinity chromatography utilising ligands to which biotin is attached. Biotin has an extremely high affinity to the glycoprotein avidin ($K_D = 10^{-15}$ M). We prepared several $GABA_B$ receptor antagonist ligands containing biotin, e.g. CGP73415, with a spacer of three molecules of GABA and one of 6-amino-hexanoic acid between the $GABA_B$ receptors binding part and biotin (IC_{50} = 33 nM; Fig. 9) [18].

This ligand may be useful to retain the ECD of $GABA_B$ receptor 1 on an avidin column. Desorption can be achieved by competition with excess of a potent $GABA_B$ receptor antagonist, e.g. CGP54626A. Using this protocol we have to expect a mixture of three species, i.e. the receptor fragment with and without the antagonist plus unbound ligand due to the equilibrium between association and dissociation. A ligand, which is bound irreversibly to the receptor (fragment), may enhance our chances of crystallisation. Therefore, we require both a photoaffinity- and an affinity chromatography-ligand combined in one molecule. The most straightforward solution is to attach a spacer plus biotin as fifth substituent on the azidosalicylic acid moiety of [125I]CGP71872 (Fig. 8).

Theoretically, we can envisage four chemically feasible permutations of the pentasubstituted iodo-azidosalicylic acid, taking into account that the OH group directs the iodine substituent into para (**A** and **B**) or ortho positions (**C** and **D**) (Fig. 10). Reactive groups at the receptor fragment bind to the nitrene intermediate generated by the photoreaction from the azide. In structures **A**, **B** or **D** both ortho positions adjacent to the azido group are substituted, whereas in structure **C** one ortho position is free, thereby causing less steric hindrance for the reaction with the protein than structures **A**, **B**, or **D**.



CGP73415

Fig. 9.

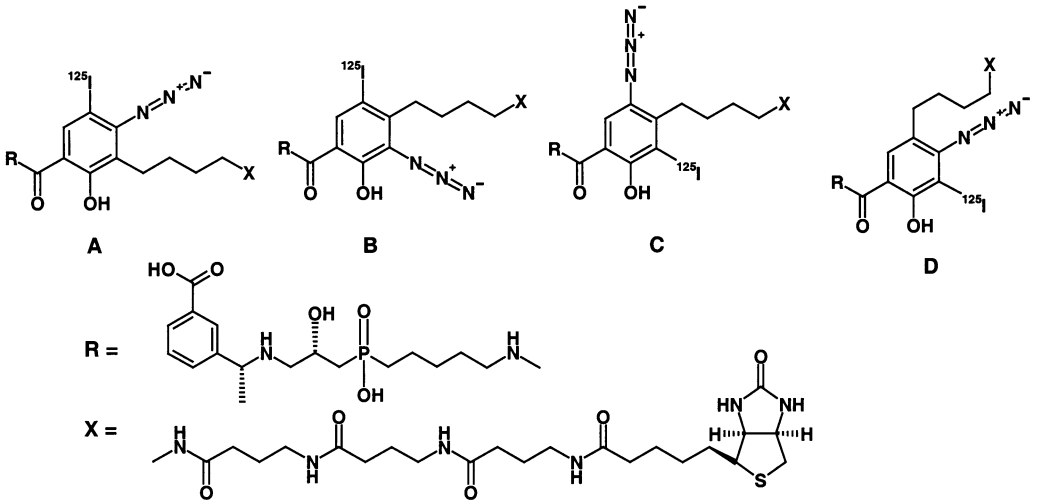


Fig. 10.

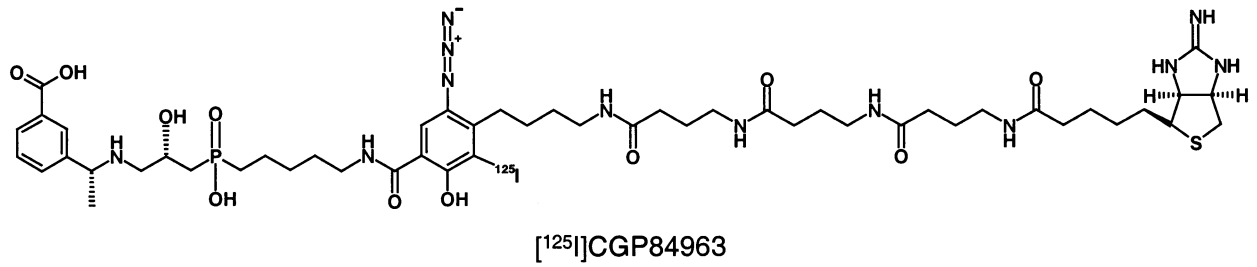


Fig. 11.

However, by attaching the ligand of structure **C** irreversibly to the $GABA_B$ receptor fragment we have to face another serious problem, i.e. to detach the receptor–ligand complex after the purification step from the avidin columns. One solution to this problem is the use of imino-biotin, whose affinity to avidin is pH-dependent [19]. Protonated 2-iminium-biotin binds to avidin with eight orders of magnitude weaker affinity than imino-biotin. Recovery of the protein–ligand complex from avidin columns can be achieved by washing with ammonium acetate solutions at pH 4.

All these considerations went into the 30-step synthesis of [^{125}I]CGP84963 (Fig. 11). A dissociation constant K_D of 2 nM was calculated from best-fit analyses of saturation curves. 30% of the total radioactivity was bound irreversibly to native $GABA_B$ receptors of rat cortex after the photoreaction (irradiation with a 24 W lamp, $\lambda = 365$ nm for 3 min) [18].

Experiments are currently under way to purify the extracellular domain of $GABA_B$ receptor 1a expressed in large quantities in *Escherichia coli* with the aim of obtaining a crystalline receptor fragment–ligand complex suitable for X-ray structure determination.

References

[1] W. Froestl, P. Furet, R.G. Hall, S.J. Mickel, D. Strub, G. von Sprecher, P.A. Baumann, R. Bernasconi, F. Brugger, A. Felner, C. Gentsch, K. Hauser, J. Jaekel, G. Karlsson, K. Klebs, L. Maître, C. Marescaux, P. Moser, M.F. Pozza, G. Rihs, M. Schmutz, M.W. Steinmann, H. van Riezen, A. Vassout, C. Mondadori, H.-R. Olpe, P.C. Waldmeier, H. Bittiger, $GABA_B$ antagonists: novel CNS-active compounds, in: B. Testa, E. Kyburz, W. Fuhrer, R. Giger (Eds.), Perspectives in Medicinal Chemistry, Verlag Helvetica Chimica Acta, Basel, 1993, pp. 259–272.

[2] W. Froestl, S.J. Mickel, R.G. Hall, G. von Sprecher, D. Strub, P.A. Baumann, F. Brugger, C. Gentsch, J. Jaekel, H.-R. Olpe, G. Rihs, A. Vassout, P.C. Waldmeier, H. Bittiger, Phosphinic acid analogues of GABA. 1. New potent and selective $GABA_B$ agonists, J. Med. Chem. 38 (1995) 3297–3312.

[3] W. Froestl, S.J. Mickel, R.G. Hall, G. von Sprecher, P.J. Diel, R.G. Hall, L. Maier, D. Strub, V. Melillo, P.A. Baumann, R. Bernasconi, C. Gentsch, K. Hauser, J. Jaekel, G. Karlsson, K. Klebs, L. Maître, C. Marescaux, M.F. Pozza, M. Schmutz, M.W. Steinmann, H. van Riezen, A. Vassout, C. Mondadori, H.-R. Olpe, P.C. Waldmeier, H. Bittiger, Phosphinic acid analogues of GABA. 2. Selective, orally active $GABA_B$ antagonists, J. Med. Chem. 38 (1995) 3313–3331.

[4] H. Bittiger, N. Reymann, W. Froestl, S.J. Mickel, ^{3}H-CGP54626: a potent antagonist ligand for $GABA_B$ receptors, Pharmacol. Commun. 2 (1992) 23.
[5] W. Froestl, S.J. Mickel, Chemistry of $GABA_B$ modulators, in: S.J. Enna, N.G. Bowery (Eds.), The GABA Receptors, Humana Press, Totowa, NJ, 1997, pp. 271–296.
[6] H. Bittiger, C. Bellouin, W. Froestl, J. Heid, M. Schmutz, P. Stampf, [^{3}H]CGP 62349: a new potent $GABA_B$ receptor antagonist radioligand, Pharmacol. Rev. Commun. 8 (1996) 97–98.
[7] W. Froestl, S.J. Mickel, M. Schmutz, H. Bittiger, Potent, orally active $GABA_B$ receptor antagonists, Pharmacol. Rev. Commun. 8 (1996) 127–133.
[8] N.G. Bowery, D.R. Hill, A.L. Hudson, A. Doble, D.N. Middlemiss, J. Shaw, M. Turnbull, (−)Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor, Nature 283 (1980) 92–94.
[9] H. Nakayasu, M. Nishikawa, H. Mizutani, H. Kimura, K. Kuriyama, Immunoaffinity purification and characterization of γ-aminobutyric acid $(GABA)_B$ receptor from bovine cerebral cortex, J. Biol. Chem. 268 (1993) 8658–8664.
[10] M. Facklam, N.G. Bowery, Solubilization and characterization of $GABA_B$ receptor binding sites from porcine brain synaptic membranes, Br. J. Pharmacol. 110 (1993) 1291–1296.
[11] M. Sekiguchi, H. Sakuta, K. Okamoto, Y. Sakai, $GABA_B$ receptors expressed in *Xenopus* oocytes by guinea pig cerebral mRNA are functionally coupled with Ca^{2+}-dependent Cl^- channels and with K^+ channels, through GTP-binding proteins, Mol. Brain Res. 8 (1990) 301–309.
[12] K. Taniyama, K. Takeda, H. Ando, T. Kuno, C. Tanaka, Expression of the $GABA_B$ receptor response in *Xenopus* oocytes and inhibition of the response by activation of protein kinase C, FEBS Lett. 278 (1991) 222–224.
[13] R.M. Woodward, R. Miledi, Sensitivity of *Xenopus* oocytes to changes in extracellular pH: possible relevance to proposed expression of atypical mammalian $GABA_B$ receptors, Mol. Brain Res. 16 (1992) 204–210.
[14] K. Kaupmann, K. Huggel, J. Heid, P.J. Flor, S. Bischoff, S.J. Mickel, G. McMaster, C. Angst, H. Bittiger, W. Froestl, B. Bettler, Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors, Nature 386 (1997) 239–246.
[15] K. Kaupmann, B. Bettler, H. Bittiger, W. Froestl, S.J. Mickel, Metabotropic $GABA_B$ receptors, receptor-specific ligands and their uses, WO 97/46675, Publication: 19971211 (Priority: 19960530).
[16] M. Belley, R. Sullivan, A. Reeves, J. Evans, G. O'Neill, G.Y.K. Ng, Synthesis of the nanomolar photoaffinity $GABA_B$ receptor ligand CGP71872 reveals diversity in the tissue distribution of $GABA_B$ receptor forms, Bioorg. Med. Chem. 7 (1999) 2697–2704.
[17] B. Malitschek, C. Schweizer, M. Keir, J. Heid, W. Froestl, J. Mosbacher, R. Kuhn, J. Henley, C. Joly, J.-P. Pin, K. Kaupmann, B. Bettler, The *N*-terminal domain of $GABA_B$ receptors is sufficient to specify agonist and antagonist binding, Mol. Pharmacol. 56 (1999) 448–454.
[18] W. Froestl, B. Bettler, H. Bittiger, J. Heid, K. Kaupmann, S.J. Mickel, D. Strub, Ligands for the isolation of $GABA_B$ receptors, Neuropharmacology 38 (1999) 1641–1646.
[19] B. Fudem-Goldin, G.A. Orr, 2-Iminobiotin-containing reagent and affinity columns, in: M. Wilchek, E.A. Bayer (Eds.), Avidin–Biotin Technology. In: Methods in Enzymology, vol. 184, Academic Press, New York, 1990, pp. 167–173.