Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 18, pp. 2237-2242, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00406-4

RECOGNITION IN MOLECULARLY IMPRINTED POLYMER α_2-ADRENORECEPTOR MIMICS

Johanna Berglund, Ian A. Nicholls[a], Christer Lindbladh and Klaus Mosbach*

Department of Pure and Applied Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden, Fax:+46 46 2224611, klaus.mosbach@tbiokem.lth.se

[a]*Department of Natural Sciences, University of Kalmar, P.O. Box 905, S-391 29 Kalmar, Sweden*

Abstract Molecularly imprinted polymers (MIPs) selective for the α_2-adrenoreceptor antagonist yohimbine (**1**) have been prepared and studied as α_2-adrenoreceptor mimics. Marked ligand stereoselectivity was demonstrated in radioligand binding and HPLC studies upon comparison to blank and corynanthine (**2**) MIPs. K_D values in the nanomolar range have been shown for *anti*-**1** MIP prepared in chloroform solutions upon rebinding in organic media.

The receptor subtype diversity evident within the α_2-adrenoreceptor class makes these receptors of great interest for studying the subtle structural and electronic effects underlying ligand recognition.[1] The clinical significance of this class of central nervous system receptors lies in their moderation of a range of autonomous functions spanning from blood pressure control to mood.[2] Numerous attempts have previously been made to find new improved ligands for this receptor class in the hope that they may be useful for studying the various central nervous system disorders stemming from dysfunction in these areas.[3] Quite notable amongst the compounds active at this class of receptors are the prototypical α_2-antagonists, yohimbine (**1**), and its stereoisomer corynanthine (**2**), Figure 1. Both compounds demonstrate significant affinities and selectivities for this receptor population.[4] These *rauwolfia* alkaloids comprise fused ring structures differing only in the configuration at the carboxyl bearing stereogenic center.

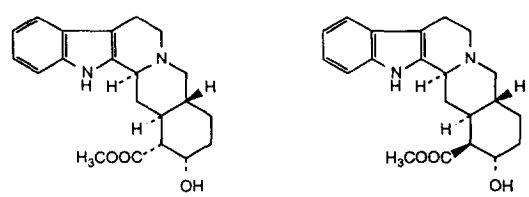

Figure 1 Yohimbine (**1**) and corynanthine (**2**)

Molecular imprinting is a technique that has been used for the development of recognition sites with predetermined selectivity in synthetic polymers.[5] The technique involves polymerization of functional monomers around a molecular template which is subsequently removed. In recent reports molecular imprinting has been used for the preparation of synthetic polymeric antibody mimics demonstrating affinities and selectivities comparable to their biological counterparts.[6]

The *rauwolfia* alkaloids present themselves as ideal candidates for molecularly imprinted polymer (MIP) production. Their inherent rigidity limits the number of accessible solution conformations, which will ensure a more homogeneous population of imprint species-monomer adducts in solution. This will, in turn, be manifested as a narrower distribution of polymer recognition sites.[7] Secondly, these structures are rich in the types of functionality which have been found to be necessary for interaction with the types of monomers employed in non-covalent molecular imprinting. Methacrylic acid, the functional monomer used in this study, has been shown to engage in hydrogen bonding and ionic interactions with template molecules containing the types of functionality present in **1** and **2**.[8] Furthermore, the development of synthetic receptor mimics may be of interest to avoid the use of receptors derived from biological sourses (animal tissues) in clinical diagnostics and research.

Anti-yohimbine MIP, **P(1)**, *anti*-corynanthine MIP, **P(2)**, and non-imprinted polymer, **P(Blank)**, were prepared by the thermally initiated polymerization of methacrylic acid and ethylene glycol dimethacrylate (cross linking agent) in the presence of either **1**, **2** or no template species.[9] The optimal ratio of template to functional monomer to cross linking agent (molar ratio 1:4.6:35) was determined in a systematic study.[10] The polymers were rendered as fine powders and washed exhaustively under acidic conditions to remove residual template material.[11] The role of polymerization solvent was examined using chloroform containing *N*,*N*-dimethylformamide (15% v/v), sufficient to maintain template solubility, or methanol.

Studies were performed using the polymers as chiral stationary phases in HPLC. All of the polymers prepared in chloroform were found to have higher affinities for **1** and **2** than all of the polymers prepared in methanol, Table1 legend. This may be due to the larger surface areas of the polymers prepared in chloroform.[12] Studies using **P(1)**, prepared in methanol or chloroform, revealed that the polymers were selective for yohimbine, Table1. The selectivity observed for the chloroform imprinted polymer was superior to that of its methanol counterpart. This can be attributed to the stronger hydrogen bonding interactions between template and functional monomer during polymerization in the more non-polar solvent. The non-imprinted polymers, **P(Blank)** prepared in methanol or chloroform, showed essentially no preference for **1** or **2**. Analysis of **P(2)**, prepared in chloroform, showed distinct preference for the template species. The methanol imprinted version, however, could not distinguish **2** from **1**.

Radioligand binding assays provide a more sensitive means of evaluating recognition in MIP systems.[6] The IC_{50} values and the dissociation constants (K_D) with corresponding numbers of binding sites present per gram polymer (B_{max}) were estimated using the programs EBDA and LIGAND (Elsevier-Biosoft). K_D values were calculated using a two site model which can be used to approximate the continuum of receptor site affinities present in a molecularly imprinted polymer.[6c] Experiments were conducted in non-aqueous media using

Table 1 HPLC recognition data

	P(1) (MeOH)	**P(2)** (MeOH)	**P(Blank)** (MeOH)	**P(1)** ($CHCl_3$)	**P(2)** ($CHCl_3$)	**P(Blank)** ($CHCl_3$)
α	1.20	1.01	0.97	2.27	0.67	0.96

Polymer particles were packed in columns (100x4.6mm) at 300 bar using acetone as solvent. The mobile phase for the polymers imprinted in methanol was acetonitrile/formic acid, 99.98:0.02 (v/v). For the polymers imprinted in chloroform, **1** and **2** were retained more strongly. Thus, acetonitrile/formic acid, 99.9:0.1 (v/v) was used in order to give k' values in a convenient range. The flow rate was 0.5 mL min^{-1}, the concentrations of injected samples were 0.2 mg mL^{-1} and the injection volume was 20 μL. Detection was at 280nm. Stereo-separation factors (α) were calculated from the relationship: $\alpha = k'_1/k'_2$, where k'_1 and k'_2 are the capacity factors of yohimbine (**1**) and corynanthine (**2**) respectively. Capacity factors were determined from: $k' = (t-t_0)/t_0$, where t is the retention time of a given stereoisomer and t_0 that of the void (determined by injection of acetone).

Table 2 Radiolabelled binding assays

competing ligand	**P(1)** (MeOH)	**P(1)** ($CHCl_3$)
1[a]	195	0.17
2[a]	1038	211
1[b]	398	1.80
2[b]	871	340

IC_{50} values (μM) for the displacement of [^{3}H] yohimbine. Assays in: a, acetonitrile/acetic acid, 99.9:0.1 (v/v); b, 50mM phosphate buffer pH5.0/Tween 20, 99.95:0.05 (v/v). The binding analyses with [^{3}H] yohimbine and one of the competing ligands yohimbine (**1**) or corynanthine (**2**), were run using polymer concentrations whereby 40-60% of the added radioligand was bound to the polymer in the absence of a competing ligand.[13] All experiments were carried out in duplicate and the results were derived from a minimum of two experiments.

conditions similar to those employed for the HPLC experiments described above. The two yohimbine imprinted polymers showed a distinct preference for **1**, relative to the stereoisomer **2**, Table 2. As expected, this effect was most pronounced in the case of the polymer prepared in chloroform. For a small number of high affinity sites comparison of K_D values showed a three orders of magnitude selectivity for **1** relative to **2** by **P(1)** imprinted in chloroform, Table 3. Differences in the relative selectivities between radiolabelled and chromatographic studies reflect the kinetics of ligand receptor binding under equilibrium and non-equilibrium conditions.[6c]

Table 3 Dissociation constants and site populations for various assay systems

polymer	competing ligand	High affinity sites		Low affinity sites	
		K_D (μM)	B_{max}(μmol/g)	K_D (μM)	B_{max}(μmol/g)
P(1) (MeOH)	**1**[a]	47	0.96	5400	59
	2[a]	330	11	2500	64
	1[b]	2.3	0.11	150	7.4
	2[b]	25	3.9	640	57
P(1) ($CHCl_3$)	**1**[a]	0.06	0.12	4.8	1.1
	2[a]	37	70	1300	840
	1[b]	0.12	0.06	62	11
	2[b]	5.6	2.5	170	28

Dissociation constants (K_D) and associated site populations (B_{max}) for yohimbine (**1**) and corynanthine (**2**) at low and high affinity sites in yohimbine MIPs prepared in either chloroform or methanol. a = binding in acetonitrile/acetic acid, 99.9:0.1 (v/v); b = binding in 50mM phosphate buffer pH5.0/Tween20, 99.95:0.05 (v/v).

Ligand recognition by these polymers in aqueous media was similarly investigated.[13] The selectivity of the MIP prepared in chloroform was attenuated by the use of aqueous binding conditions, a three order of magnitude difference in IC_{50} between **1** and **2** binding to **P(1)** in organic solvents being reduced to two orders of magnitude, Table 2. Thus, the binding of **1** was reduced more upon use of an aqueous binding environment than was the binding of **2**. This implies that the presence of water perturbs the selective hydrogen bonding arrangements involved in recognition of the template structure, **1**, whilst recognition of the stereoisomeric **2** relies more upon interactions other than hydrogen bonding to motivate binding to the polymers.[14] The affinities of the ligands for the polymers varied with the solvent of polymerization. In agreement with the HPLC studies **1** and **2** bind more strongly to the polymers prepared in chloroform.

In the presence of polymers imprinted with corynanthine, **P(2)**, the IC_{50} values were lower for **1** than for **2** (data not shown). By using radiolabelled yohimbine, sites highly selective for **2** will not be studied. The displacement of the radioligand by **2** will involve competition at sites accessible to both **1** and **2**. The IC_{50} values were lower when the assays were performed in organic media than in aqueous buffer, implying that binding to the polymer is reduced by an aqueous environment. This difference was most pronounced for **P(2)** imprinted in chloroform.

Synthetic receptors highly selective for the α_2-antagonist yohimbine (**1**) have been prepared by molecular imprinting. Pronounced stereoselectivity has been observed. The affinity of **P(1)** imprinted in chloroform for **1**, a K_D value of 6.0×10^{-8}M associated with a site population of 0.12 μmol/g, compares favourably with the binding of **1** to endogenous receptors, 8.0×10^{-9}M.[15] These synthetic polymer systems are currently being investigated for use in a range of diagnostic and screening applications.

Acknowledgement The authors are most thankful to Dr. Lars I. Andersson for helpful discussions and advice in the interpretation of ligand binding data, and to Dr. Richard Ansell for technical assistance with the chromatographic studies and helpful criticism.

References and Notes

1. (a) Berlan, M.; Montastruc, J.-L.; Lafontan, M. *Trends Pharmacol. Sci.* **1992**, *13*, 277-282. (b) McGrath, J. C, Brown, C. M., Wilson, V. G. *Med. Res. Rev.* **1989**, *9*, 407-533. Langer, S.Z. In *Psychopharmacology. The Third Generation of Progress*, Meltzer, H. Y. Ed.; Raven Press: New York, **1988**; 151-157.
2. Hoffman, B. B.; Lefkowitz, R. J. Adrenergic receptor antagonists In Goodman and Gilman's Phamacological Basis of Therapeutics 8th ed.; Goodman Gilman, A.; Rall, T. W.; Nies, A. S.; Taylor, P. Eds., McGraw-Hill, New York, **1990**, 221-229.
3. (a) Regunathan, G.-L. S.; Barrow, C. J.; Eshraghi, J.; Cooper, R.; Reis, D. J. *Science* **1994**, 966-969. (b) Nicholls, I. A.; Morrison, S. F.; Brinkworth, R. I.; Alewood, P. F.; Andrews, P. R. *Life Sci. Pharmacol. Lett.* **1993**, *53*, 343-347.
4. Obitz, P.; Stöckigt, J.; Mendonza, L. A.; Aimi, N.; Sakai, S.-I. Alkaloids from cell cultures of *Aspidosperma Quebracho-Blanco* In *Alkaloids: Chemical and Biological Perspectives*, Pelletier, S.W., Ed.; Pergamon: Oxford,**1995**; pp. 235-246.
5. (a) Flam, F. *Science* **1994**, *263*, 1221-1222. For recent reviews see: (b) Mosbach, K; Ramström, O. Bio/Technology **1996**, 14, 163-170. (c) Wulff, G. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1812-1232. (d) Nicholls, I. A.; Andersson, L. I.; Mosbach, K.; Ekberg, B. *Trends Biotechnol.* **1995**, *13*, 47-51. (e) Mosbach, K. *Trends Biochem. Sci.* **1994**, *19*, 9-14. (f) Shea, K. J. *Trends Polym. Sci.* **1994**, *2*, 166-173. (g) Whitcombe, M.J., Rodriguez, M.E., Villar, P., Vulfson, E.N., *J. Am. Chem. Soc.* **1995**, 117, 7105-7111.
6. (a) Andersson, L. I.; Nicholls, I. A.; Mosbach, K. Antibody mimics obtained by non-covalent molecular imprinting. In *Immunological Analysis of Agrochemicals: Emerging Technologies - ACS Symposium Series*; Nelson, J. O.; Karo, A. E.; Wong, R. B. Eds.; American Chemical Society: Washington DC, vol. 586, **1995**, pp. 89-97. (b) Vlatakis, G.; Andersson, L. I.; Müller, R.; Mosbach, K. *Nature* **1993**, *361*, 645-647. (c) Andersson, L. I.; Vlatakis, G.; Müller, R.; Mosbach, K. *Proc. Natl. Acad. Sci. (USA)* **1995**, *92*, 4788-4792.
7. Nicholls, I. A. *Chem. Lett.* **1995**, 1035-1036.
8. Sellergren, B.; Lepistö. M.; Mosbach, K. *J. Am. Chem. Soc.* **1988**, 110, 5853-5860.
9. Typically; a yohimbine imprinted polymer was made by dissolving yohimbine (0.56 mmol), methacrylic

acid (4.6 eq.), ethylene glycol dimethacrylate (35 eq.) and 2,2´-azobis(2,4-dimethylvaleronitrile) (111 mg) in methanol (10 mL), or chloroform (8 mL) containing *N*,*N*-dimethylformamide (15% v/v). The solution was cooled on ice and flushed with dry nitrogen after which the polymerization was carried out in a darkened waterbath (45°C) for 18 h. Corynanthine imprinted polymers were prepared as described above, but with the substitution of corynanthine (**2**) for yohimbine. Blank polymers were prepared as above but in the absence of template. Bulk polymers were ground (2x15min) in a mechanical mortar (Retsch, FRG) and sieved (Retsch, 25 μm) with acetone. Particles of less than 2μm diameter were removed by sedimentation (4x20min) in 200 mL acetone. The polymer particles were washed on a filter funnel, to remove the residual template, with methanol/acetic acid (500 mL, 7:3, v/v), methanol/acetic acid (200 mL, 3:7, v/v) and methanol (200 mL), and were then dried under vaccuum after a final wash with methyl *tert*-butyl ether (50 mL). All organic solvents and reagents were of analytical or HPLC grade, and the monomer was purified prior to use.

10. Berglund, J.; Lindbladh, C.; Nicholls, I. A.; Mosbach, K., unpublished results.
11. Polymer combustion analysis data (Mikro Kemi; Uppsala, Sweden): Found: N ≤ 0.02 %.
12. Polymer surface areas were determined by single point surface area measurement using a Micromeritics Flowsorb II 2400 instrument (30% N_2 in He), samples were degassed at 150 °C for 3h prior to determination. Polymers imprinted in methanol: **P(1)**, surface area 71.0 m^2g^{-1}, average pore diameter 32.5 Å; **P(2)**, 51.75 m^2g^{-1}, 30.3 Å; **P(Blank)**, 74.9 m^2g^{-1}, 29.0 Å. Polymers imprinted in chloroform: **P(1)**, surface area 304.6 m^2g^{-1}, average pore diameter 63.0 Å; **P(2)**, 304.2 m^2g^{-1}, 70.6 Å; **P(Blank)**, 299.5 m^2g^{-1}, 75.5 Å.
13. Polymer, [^{3}H] yohimbine (0.54 ng, specific activity 81.00 Ci/mmol, 3.0T Bq/mmol) and a competing ligand (yohimbine or corynanthine) over the concentration range 0.1 ng mL^{-1} - 2.0 mg mL^{-1} (10-14 concentrations per experiment), were mixed in 1 mL acetonitrile/acetic acid, 99.9:0.1 (v/v), and incubated for 20-24 h at room temperature. The polymer particles were separated by centrifugation (11535 x g, 5 min, room temperature) and the radioactivity in 200 μL of the supernatant was measured by liquid scintillation counting (LKB 1219 Rackbeta). 0.5 mg chloroform imprinted polymers or 10 mg methanol imprinted polymers were used in each assay. In the aqueous assays (50mM phosphate buffer pH5.0/Tween20, 99.95:0.05 (v/v)) 10 mg of methanol imprinted polymers per mL buffer were used, and 1.5 mg of chloroform imprinted polymers. Otherwise, analyses were performed as described for the nonaqueous assays.
14. Nicholls, I. A.; Ramström, O.; Mosbach, K. *J. Chromatogr. A* **1995**, *691*, 349-353.
15. Lanza, F.; Cazenave, J. P. *Thromb. Haemostasis* **1985**, *54*, 402-408.

(*Received in Belgium* 28 *May* 1996; *accepted* 26 *August* 1996)