Stereospecific antibodies to propranolol

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The β-adrenergic antagonist propranolol was activated through its side chain, coupled to bovine serum albumin, and injected into BALB/c mice. After fusion of the splenocytes from these immunized mice with the NS-1 myeloma cell line, two hybridomas, producing monoclonal anti-propranolol antibodies, were isolated. Clone P-49 was monospecific for propranolol, with a significant preference for the l-stereoisomer, as compared to the d form. On the other hand, clone P-28 cross-reacted with alprenolol as well as some other β-antagonists. Both classes of antibodies competed with A431 epidermoid carcinoma β_2-adrenoceptors for the binding of [^{3}H]propranolol. When ascites cells from clone P-28 were fixed with glutaraldehyde, the anti-propranolol monoclonal antibody became cell bound. These cell-bound P-28 antibodies bind propranolol and other β-adrenergic ligands with a similar ranking order to the soluble monoclonal antibody. The cell-bound antibody displayed a 5-fold higher affinity towards l-propranolol than the soluble monoclonal antibody. The practical implications of these findings are discussed.

Propranolol *Monoclonal antibody* *Stereospecificity* *β-Adrenergic receptor* *β-Adrenergic antagonist*

1. INTRODUCTION

Catecholamines synthesized in nerve terminals or in the chromaphine granules specifically interact with membrane-bound β- as well as α_1- and α_2-adrenergic receptors. The interaction of β-adrenergic agonists with β-adrenergic receptors triggers the activation of adenylate cyclase, which induces a cascade of biochemical events leading to the final physiological response [1,2]. In contrast, binding of the β-adrenergic antagonists to these receptors mediates a reverse effect by competing with the β-adrenergic agonists.

Elucidation of the interaction properties between β-agonists and/or β-antagonists with β-receptors is an important goal in understanding the mechanism of catecholamines and β-antagonist drugs at a molecular level. The widespread use of β-blockers in the control of blood hypertension [3] led to an increased medical interest in adrenergic receptors. However, one of the major obstacles in studying this interaction is associated with the necessity to purify large quantities of functionally active β-receptors. Hence, it is not surprising that a new line of investigation, based on immunological methods, has been initiated in recent years [4–6]. Development of specific antibodies to β-agonists, β-antagonists and β-receptors should provide new tools to study this important biological system [7–9].

Special attention should be paid to the stereospecificity of the β-adrenergic receptors towards both agonists and antagonists [10–13]. The present study was undertaken to (i) develop a chemically active propranolol molecule, (ii) use it for the development of an appropriate immunogen, (iii) develop monoclonal antibodies (McAb) with stereospecificity for the biologically active isomer l-propranolol and (iv) use these McAbs for the production of an artificial cell-bound receptor.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Propranolol, alprenolol, pindolol and catecholamines were from Sigma. The l- and d-stereoisomers of propranolol were obtained from ICI, England. All other chemicals were of analytical grade.

2.2. *Synthesis of 'propranolol-amine'*

The propranolol-amine, i.e. 2-[2-hydroxy-3-(1-naphthoxy)propylamino]-1-*tert*-butyloxycarbonylamino-2-methylpropane (compound III) was synthesized according to the scheme shown in fig.1. Compound I (1-naphthoxy-2,3-epoxypropane) [14] was prepared by mixing α-naphthol (14.4 g, 0.1 mol) and NaOH (4 g, 0.1 mol) in H_2O (4 ml), heating for 1–2 min, cooling and adding epichlorohydrin (10.1 g, 0.11 mol). After stirring overnight at room temperature ether (100 ml) was added, after which the mixture was filtered. The filtrate was dried and solvent removed under reduced pressure. Part of the crude product (1.6 g) was dissolved in dichloromethane (2 ml) and loaded onto a silica gel column equilibrated with petroleum ether (40–60°C b.p.). The same solvent was used to elute the product which appeared as the first peak, as monitored at 254 nm. Removal of the solvent from the pooled fractions containing the relevant peak yielded 0.78 g (40%) clear oily epoxide, which was identified by TLC (silica) with an R_f 0.74, using dichloromethane as solvent.

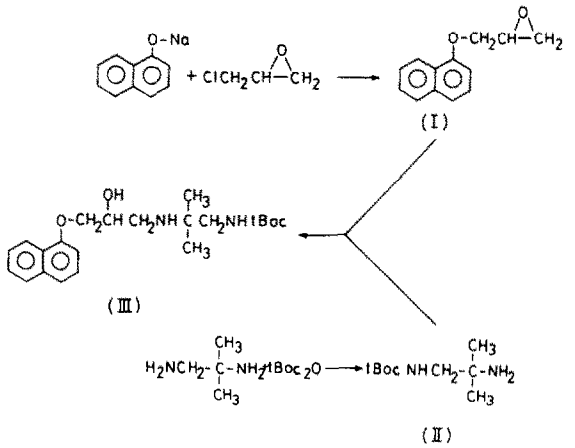

Fig.1. Synthesis of the protected propranolol-amine. *t*Boc = *tert*-butyloxycarboxyl.

For the synthesis of compound II (1-*N*-*t*-butyloxycarboxylamino-2-amino-2-methylpropane) a solution of di-*t*-butyldicarbonate (3.65 g, 0.0165 mol) in dichloromethane (100 ml) was slowly added (20 h) to a stirring solution of 1.2-diamino-2-methylpropane (5 ml, 0.05 mol) in dichloromethane (100 ml). Addition of 1 N NaOH (60 ml, saturated with NaCl) was followed by phase separation. The aqueous phase was washed with dichloromethane (3 × 50 ml) and the combined organic washings were extracted with brine until neutral pH. The organic phase was dried on anhydrous Na_2SO_4, filtered and stripped to obtain a clear oil (4 g) which was crystallized from cold hexane to yield 2.06 g (66%) of a white solid, m.p. 88°C. Characterization by TLC (silica) revealed an R_f value of 0.35 (*n*-BuOH/AcOH/H_2O, 4:1:1). For the final synthesis of compound III, a mixture of compound I (780 mg, 0.0039 mol) and compound II (1.02 g, 0.0054 mol) was dissolved in absolute ethanol (3 ml). A sequence of freezing, evacuating and thawing steps was repeated 3 times. The vacuum-sealed mixture was heated at 70°C for 3 days. Solvent was removed under reduced pressure and the residue obtained was taken up in ethyl acetate (30 ml) and extracted with 0.3 N $KHSO_4$ (2 × 5 ml), followed by brine. The organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent removed under reduced pressure. The final product obtained was a white solid (1.35 g, 89%) with a melting point of 77°C and an R_f value of 0.47, from TLC (silica) Ch_2Cl_2/MeOH/AcOH (18:1:0.5).

2.3. *Conjugation of propranolol to bovine serum albumin*

Deprotection of compound III by removing the *t*-butyloxycarbonyl group was performed as follows. Compound III (100 mg) was taken in a $CaCl_2$ protected flask and treated with thioanisol (100 μl), followed by EtOAc (5 ml) saturated with HCl gas. After 20 min, the solvent was removed under reduced pressure and the residue kept under vacuum, over KOH pellets, overnight. Addition of dry ether, followed by decantation, gave a residue which was used without further manipulation for coupling to the bovine serum albumin (BSA). The above-mentioned 'deprotected' compound III (21 mg) was conjugated to BSA by mixing it with the coupling reagent dimethyladipimidate (50 mg)

in the presence of triethylamine (50 μl) in methanol (750 μl) for 20 min. After removal of the solvent, the residue was dissolved in methanol (250 μl) and the methanolic solution (60 μl) was added to a solution of 100 mg BSA/ml, 0.1 M pyrophosphate buffer, pH 8.5. The mixture was stirred at room temperature for 2.5 h and the conjugate was passed through a Sephadex G-25 column. The final product has a molar ratio of propranolol to BSA of 8.

2.4. *Immunization and cell hybridizations*

The propranolol-BSA conjugate was used for immunization of BALB/c mice. The procedure for immunization and fusion was essentially as described [15]. The NS-1 myeloma cell line was obtained from Flow Laboratories. After 14 days in the selective medium, cultures secreting monoclonal antibodies to propranolol were isolated, cloned and finally propagated in vivo as ascites tumor in the peritoneal cavity of BALB/c mice. The ascites fluid obtained as well as the culture supernatant were used for characterization.

2.5. *Enzyme-linked immunosorbant assay*

An enzyme-linked immunosorbant assay (ELISA) was developed using propranolol-BSA conjugate as the antigen for screening purposes. The detailed procedures were essentially as described in [16]. The coating buffer used was 0.05 M $NaHCO_3$, pH 9, and antigen concentration used was 1 μg/ml.

2.6. *Radioimmunoassay for propranolol*

Propranolol as well as other β-adrenergic agonists and antagonists were freshly dissolved and diluted in drug-free human plasma converted serum. A properly diluted McAb (100 μl) was incubated with 100 μl of the propranolol standard or cross-reactants of different concentrations, as indicated in fig.2, at 37°C for 1 h. dl-[4-^{3}H]Propranolol hydrochloride (Amersham, spec. act. 20 Ci/mmol) was added (0.5 pmol/100 μl per reaction mixture, 5 nM) and incubation continued for an additional hour at room temperature. The following were added in order: 100 μl of 2% normal mouse serum; 100 μl of 0.1 M NaEDTA, pH 7.8, and 100 μl rabbit anti-mouse IgG (1 mg/ml). Polyethyleneglycol (M_r 6000) was added to a final concentration of 10% (w/v). All solutions were mixed well by vortex. After centrifugation at 3000 rpm for 20 min at 4°C (Sorvall RT6000), the supernatants were removed by aspiration and the precipitates washed once with 1 ml cold phosphate-buffered saline (PBS). The residue remaining after centrifugation was solubilized in a total of 300 μl of 0.1 N NaOH and transferred to 3 ml Pico-Fluor 30 (Packard) liquid scintillation cocktail. Radioactivity was measured with a Tricarb liquid scintillation counter.

2.7. *Competition between A431 β_2-adrenoceptors and McAbs for [^{3}H]propranolol*

A human epidermoid carcinoma cell line A431 (kindly provided by Dr Schlessinger of the Weizmann Institute of Science) enriched with β_2-adrenoceptors (40000/cell, Kashles and Levitzki, submitted) was used for binding assays. Confluent cultures of A431 in 2 cm^2 wells were incubated with 1 pmol [^{3}H]propranolol and different concentrations of ascites fluid, containing either monoclonal anti-propranolol antibodies, or a McAb from an irrelevant hybridoma, or unlabeled propranolol, in a total volume of 600 μl of PBS per reaction well. After an incubation of 1 h at 37°C, the reaction mixture was measured, the layers of A431 cells washed 4 times with ice-cold PBS, dissolved in 0.5 ml of 0.1 N NaOH and transferred to 3 ml Pico-Fluor 30 liquid scintillation cocktail and radioactivity measured.

2.8. *Fixation of the mAbs to hybridoma cells*

Hybridoma cells, bearing monoclonal anti-propranolol antibodies, were fixed by glutaraldehyde at a final concentration of 2.5%, as described elsewhere (Wang et al., in preparation). A total of 2×10^5 fixed hybridoma cells were incubated with propranolol or its analogs, as described in the soluble McAb assay. After the addition of [^{3}H]propranolol and incubation, the entire reaction mixture was centrifuged, washed once with cold saline and the remaining residue dissolved in 0.5 ml of 0.1 N NaOH. Pico-Fluor 30 (3 ml) was added and radioactivity measured.

3. RESULTS AND DISCUSSION

ELISA was used as the method for screening of culture supernatants for anti-propranolol activities. A number of hybridomas were isolated

and two clones (P-49 and P-28) were selected for characterization. McAb of clone P-49 bound propranolol with high affinity – as demonstrated by radioimmunoassay (RIA) – possessing a preference for the l-stereoisomer of the hapten, as opposed to the d form (fig.2). These results are reflected in their calculated dissociation constants, obtained from Scatchard plots: l-propranolol, 3.3 nM, as compared to d-propranolol, 38.6 nM (fig.3). It was mono-specific for this antagonist only, with minimal cross-reaction to alprenolol (4%) and with practically no recognition to pindolol, practotol or sotalol (fig.4). Moreover, it did not react with any of the catecholamines tested such as isoprenaline, epinephrine (both l- and d-isomers), norepinephrine and phenylephrine (not shown).

McAb from clone P-28 can also distinguish between the stereospecific forms of propranolol (table 1). In addition, unlike clone P-49, it recognized alprenolol to a large extent (78%) but with practically no cross-reaction to other antagonists or agonists. When the McAb were fixed to the hybridoma cells and these cell-bound antibodies were examined for ligand specificity, it was found that the specificity remained almost identical to the soluble native antibodies (not shown). As shown in table 2, both McAbs from clone P-28 and P-49 compete with the surface β_2-adrenoceptors of A431 cells for the binding of [^{3}H]propranolol.

McAb from clone P-49 belongs to mouse IgG1 subclass and that from clone P-28 to IgG2a, as determined by Ouchterlony immunodiffusion.

β-Adrenergic receptors only interact with the l-isomer of both agonists and antagonists [10–13]. In fact, most, if not all, biological systems

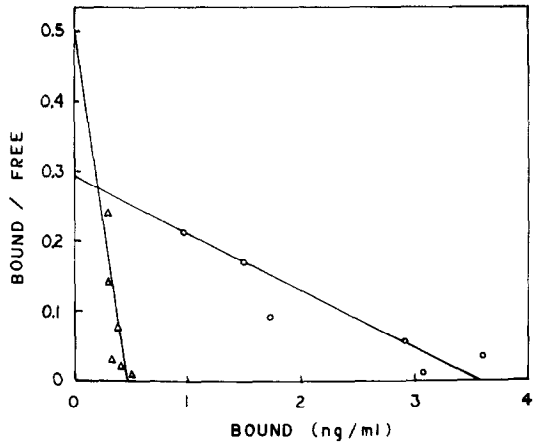

Fig.3. Scatchard plot of propranolol binding to McAb from clone P-49. (△) l-Propranolol, (○) d-propranolol.

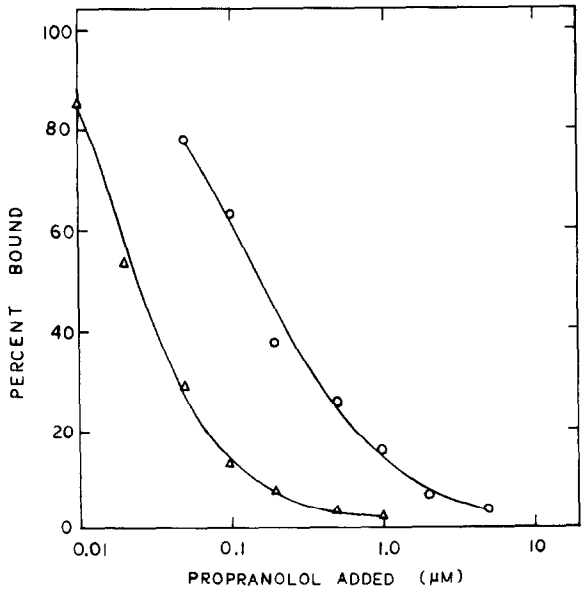

Fig.2. Reaction of McAb from clone P-49 with the isomers of propranolol. (△) l-Propranolol, (○) d-propranolol.

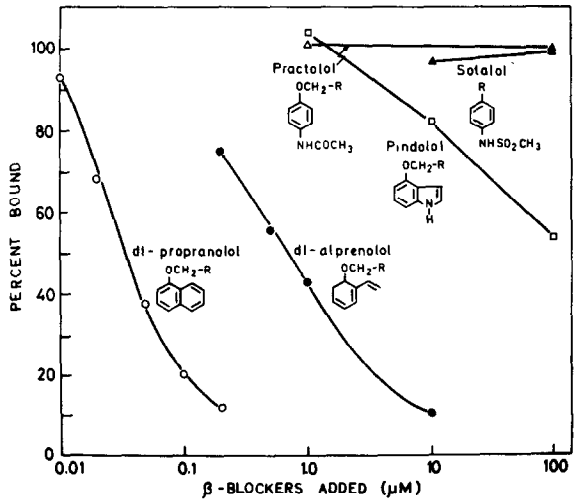

Fig.4. Specificity of monoclonal anti-propranolol antibodies from clone P-49 with some β-adrenergic blockers. R = $CH(OH)CH_2NHCH(CH_3)_2$.

Table 1

Binding properties of P-28 anti-propranolol antibodies

β-Antagonists or agonists	McAb P-28 (% cross-reaction)
Antagonists	
dl-Propranolol	100
l-Propranolol	169
d-Propranolol	35
Practolol	<0.1
Sotalol	<0.1
Pindolol	<0.2
Agonists	
l-Epinephrine	<0.1
d-Epinephrine	<0.1
Norepinephrine	<0.1
Phenylephrine	<0.1
Isoprenaline	<0.1

RIA were performed as described in the text. The cross-reactivity reported here was calculated as the amount of dl-propranolol used relative to that needed for each of the analogs to cause a 50% inhibition in a standard dose-response curve

Table 2

Competition of monoclonal anti-propranolol antibodies with β-adrenoceptors in the binding of the ligand [^{3}H]propranolol

Competing agent	Monoclonal anti-propranolol		Irrelevant McAb	Propranolol
	P-28	P-49		
	(% of maximum)			
McAb (μl/reaction)				
0	100	100	100	–
2	10	25	96	–
10	3	15	94	–
Propranolol (nM/reaction)				
0	–	–	–	100
1	–	–	–	80
10	–	–	–	50
100	–	–	–	24
1000	–	–	–	5

Different concentrations of either McAb to propranolol or unlabeled dl-propranolol were added to confluent cultures of A431 cells, together with a constant amount of [^{3}H]propranolol (5 nM). The radioactivity bound to the cells was measured. The data presented here are reported as the percent [^{3}H]propranolol bound to the cells relative to the control (only [^{3}H]propranolol added). 100% is 1500 ± 200 cpm/well, where each well has 5×10^5 cells. Non-specific binding was 360 ± 100 cpm/well. Total β_2-adrenoceptor number was calculated as $6.1 \pm 2.1 \times 10^4$ receptors/cell. This value is in good agreement with ^{125}I-cyanopindolol binding (Keshles and Levitzki, submitted)

associated with recognition of chiral molecules show specificity to one isomeric form only. The specific 'arrangement' of the receptor to 'accept' only the l-isomer of both the agonists and the antagonists could possibly be detected immunologically via an anti-idiotype to stereospecific anti-antagonist antibodies. Indeed, l-propranolol specific antibodies have been described using the l-propranolol-BSA as antigen [17]. In another report, a population of l-isomer-specific anti-propranolol antibodies was isolated from a pool of anti-propranolol antibodies [4]. The same group of investigators has recently isolated specific monoclonal anti-l-alprenolol antibodies [9].

Here we observed a preference of the McAb towards the l-isomer, as compared to d-propranolol, although the immunogen used was the dl-mixture. This type of preferential stereospecificity towards the l-isomer has also been reported in the case of monoclonal anti-alprenolol antibodies [8]. In fact, our results from clone P-49 were quite similar to that of the McAb from clone 37A4 [8] and clones 5D9 and 5B7 [9]. However, all the three monoclonal antibodies described recognized propranolol and alprenolol (the immunogen) equally well, while our McAb from clone P-49 cross-reacted with alprenolol only to the extent of 4% (fig.4).

Using clone P-28, an artificial 'receptor' was constructed in the form of fixed hybridoma cells containing McAb to propranolol. This receptor showed similar recognition characteristics towards various β-antagonists as the native soluble antibodies, confirming its being another form of the original McAb. It is of interest to note that the affinity of l-propranolol to this artificial receptor form of the McAb (K_d = 4.1 nM) is of the same order of magnitude as that of the 'natural' β-adrenergic receptor (approximately 1.4 nM) [10] and higher than the affinity of the soluble McAb

(20.4 nM). Monoclonal antibodies from both clones P-28 and P-49 compete effectively for the binding of [^{3}H]propranolol to A431 β_2-adrenoceptors (table 2), suggesting the ability of these McAbs to compete for the drug in vivo.

In conclusion, our results confirm the notion that such monoclonal antibodies, with the same stereospecificity as the receptor, could possibly serve as the 'internal image' of the receptor and be used as the first step toward the development of anti-idiotype antibodies [18]. Furthermore, monoclonal antibodies with stereospecificity may introduce a new concept for the separation of racemic mixtures.

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