SYNTHESIS OF A PHOTOAFFINITY PROBE FOR THE BETA-ADRENERGIC RECEPTOR

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SUMMARY: The synthesis and characterization of a beta-adrenergic photoaffinity label, N-(-2-hydroxy-3-naphthoxypropyl)-N'(-2-nitro-5-azidophenyl ethylenediamine, (NAP-propranolol) is described. The inhibition constants (Ki) for the NAP-propranolol inhibition of ^{3}H-dihydroalprenolol binding and the inhibition of (-)-isoproterenol-stimulated adenylate cyclase in turkey erythrocytes are 100 nM and 19 nM respectively.

INTRODUCTION: Radioactive agonists (1-2) and antagonists (3-5) have been used to measure beta-adrenergic receptors in several tissues. An important phase of research in this area is the isolation and purification of the beta-receptor. Its isolation has been delayed, in large part, by the unavilability of specific covalent binding ligands. Recently a non-photoactive covalent affinity probe has been synthesized (6).

Photoaffinity labeling has the advantage over covalent affinity labeling in that kinetic analysis of the photoaffinity label for its receptor can be determined by equilibrium studies prior to photolysis. This is not possible with a covalent affinity probe since it binds irreversibly to the receptor. Moreover, prior to photolysis, equilibrium can be attained and maximal occupancy of the receptors can be insured. Recent studies have shown that radioactive photoaffinity probes have been used successfully to isolate and characterize receptor molecules (7-10).

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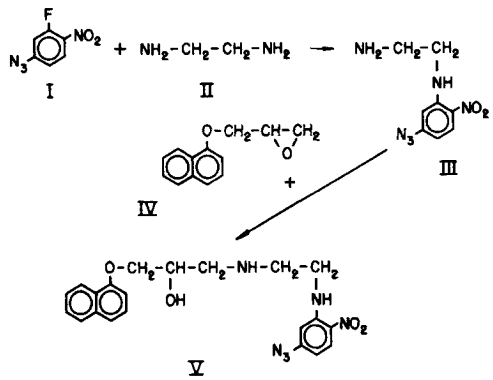

Fig. 1. Reaction scheme for the synthesis of NAP-propranolol.

In this report we present the synthesis and characterization of a propranolol photoaffinity probe, N-(-2-hydroxy-3-naphthoxypropyl)-N'-(-2-nitro-5-azidophenyl) ethylenediamine, which we shall name "NAP-propranolol". This probe is an effective inhibitor of the isoproterenol stimulated adenylate cyclase and the specific binding of dihydroalprenolol in intact turkey erythrocytes and ghosts.

MATERIALS AND METHODS: cAMP radioimmunoassay kit was obtained from Collaborative Research. [^{3}H-2,3 propyl]-(-)-Dihydroalprenol (spec. act 32.6 Ci/mmole) was obtained from New England Nuclear. Ultraviolet spectroscopy was performed on a Mikrotek Unicam Model SP800 Spectrophotometer. Nuclear magnetic resonance spectroscopy was performed at 100 MHz using a Jeol MH 100 instrument. Infrared spectroscopy was done in KBr discs using a Perkin-Elmer Model 257 Spectrophotometer. Thin layer chromatography was performed on Merck-Darmstadt silica gel G, 0.25 mm coated glass plates.

Male turkey blood was obtained from a local slaughterhouse. The cells were washed with 0.154 M NaCl and stored in Krebs-Ringer bicarbonate buffer pH 7.4 containing 11 mM glucose. Erythrocyte ghosts were prepared as previously described (11) and stored in Kreb's Ringer bicarbonate buffer, pH 7.4, at -20°C until used for binding studies. Only freshly prepared erythrocytes or erythrocyte ghosts were used for the assay of adenylate cyclase.

Synthesis of NAP-propranolol. The synthetic scheme is shown in Figure 1. Recrystallized α-naphthol (10 mmoles) was added to freshly distilled epichlorohydrin (10 mmoles) in 6.0 ml of 2 N NaOH (12 mmoles). This solution was refrigerated overnight, extracted into ether and dried to yield the epoxy derivative (IV). 0.36 mmoles of freshly redistilled ethylenediamine (II) was added to 0.14 mmoles of 5-fluoro-4-nitrophenylazide (I) in 4.0 ml of 95% ethanol. This latter step and all subsequent steps were performed in dim fluorescent light. 3.0 ml of 233 mM Na_2CO_3 were added to the latter solution

TABLE 1

THE INFRARED AND NMR ABSORPTION BANDS OF NAP-ED, NAP-PROPRANOLOL, and PROPRANOLOL

Compound	Infrared Bands (cm^{-1})	NMR Bands (δ)
NAP-ED	1030w, 1110w, 1165w, 1270w, 1310m, 1360m, 1525vs, 1560s, 2120vs, 3610w	
NAP-Propranolol	805m, 1030w, 1075w, 1110s, 1050w, 1165w, 1185w, 1250s, 1275s, 1310s, 1350m, 1410m, 1465m, 1525vs, 1560m, 1585m, 1635m, 2120vs, 2870w, 2940m, 3065w, 3220-3600sb, 3660w	3.02, 3.40, 4.32 6.80, 7.00, 7.40, 7.80, 8.15
L-Propranolol	1030w, 1075w, 1110s, 1275s, 1410s, 1465m, 1510m, 1585s, 1600m, 2700-2880mb, 3250-3420wb	

Infrared spectra were performed in $CHCl_3$ except for NAP-propranolol which was in $CDCl_3$. The spectra were corrected by subtracting peaks due to solvent. NMR of NAP-propranolol was carried out in $CDCl_3$ following deuterium exchange using D_2O. The absorption bands are listed as follows: w = weak, m = moderate, s = strong, vs = very strong, and b = broad.

and the mixture was allowed to react for 20 hrs at 70°C. The reaction mixture was applied to a silica gel plate and developed in heptane/chloroform/emthanol/pyridine 25:65:10:3 (v/v). NAP-ED (III) moved as an orange band with R_f value 0.08. NAP-ED was eluted with methanol. Removal of the methanol gave 16 mg of orange solid (mp 64-68°C). NAP-ED was observed to be photosensitive and had an ultraviolet absorption maximum at 256 nm in methanol. Infrared spectroscopy showed the characteristic azide absorption band at 2120 cm^{-1}.

0.07 mmole of NAP-ED (III) was added to 0.70 mmole of epoxide (IV) in 10 ml of 95% ethanol and allowed to react overnight in the dark at 70°C. The reaction mixture was evaporated to dryness, dissolved in chloroform, applied to a silica gel plate, and developed in heptane/chloroform/methanol/pyridine. The product, NAP-propranolol (V) migrated as a major yellow-orange band, R_f 0.32. It was eluted with methanol and centrifuged to remove silica gel. The solvent was evaporated to dryness to yield 5 mg of orange product (V)

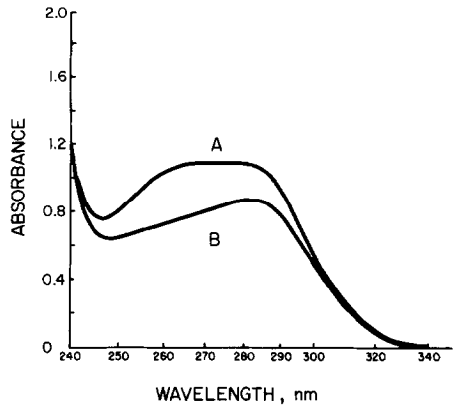

Fig. 2. Ultraviolet spectroscopy of 75 μM NAP-propranolol in ethanol. The photolyzed spectrum was taken following three hours of photolysis from a Spectroline XX-15 ultraviolet lamp (360 nm) at approximately 30 cm distance from the light source. Bleaching of NAP-propranolol was evident to the naked eye. The control spectrum is marked A and the spectrum after photolysis is marked B.

(mp 124-125°C). It is presumed that the synthesis of V reported here yields the racemic mixture.

A second photoactive probe was prepared exactly as described for NAP-propranolol except ethylenediamine was substituted by putrescine. This derivative had an R_f value of 0.15 in the same solvent system given above.

RESULTS AND DISCUSSION: NAP-propranolol was characterized by infrared, nmr and uv spectroscopy. Infrared spectral bands for NAP-ED, NAP-propranolol, and propranolol are shown in Table 1. Noteworthy is the characteristic azide band at 2120 cm^{-1} for NAP-ED and NAP-propranolol. NAP-propranolol has certain bands common to propranolol and NAP-ED. NMR spectroscopy of NAP-propranolol (Table 1) following deuterium exchange was consistent with the proposed structure. NAP-propranolol migrated as a single band, R_f .32, using thin layer chromatography on silica gel developed in heptane/chloroform/methanol/pyridine 25:65:10:3.

The photosensitivity of NAP-propranolol is shown in Figure 2. After three hours of mild photolysis the ultraviolet absorption at 265-285 nm was reduced in a manner consistent with the decomposition of the azide group.

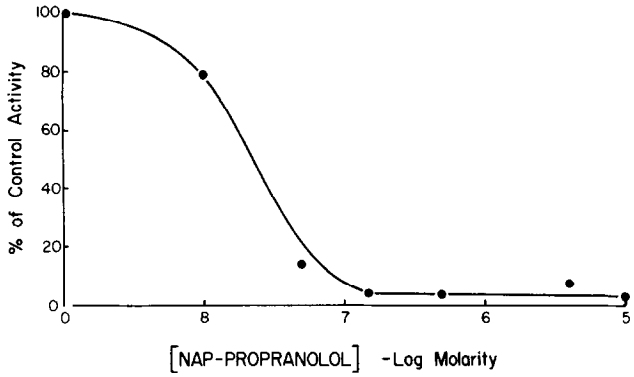

Fig. 3. NAP-propranolol inhibition of (-)-isoproterenol-stimualated adenylate cyclase activity in intact turkey erythrocytes. 1.0 ml of packed red cells were suspended in Kreb's Ringer bicarbonate buffer pH 7.4 containing 25 μM phenoxybenzamine and 11 mM glucose and were preincubated at 37°C for 15 min. Appropriate amounts of NAP-propranolol were added and then (-)-isoproterenol was added to make a final concentration of 100 nM. The samples were incubated for 17 min. at 37°C. 0.2 ml aliquots were removed and treated as described by Malchoff and Marinetti (11). Adenylate cyclase activity was determined using the acetylated cAMP radioimmunoassay method (12). Results represent the average values of one experiment performed in duplicate. The 100% value was 1.75 pmoles of cAMP produced/10^9 cells/min.

When intact turkey erythrocytes were incubated with 100nM (-)-isoproterenol, stimulation of adenylate cyclase was observed (1.75 pmole of cAMP/10^9 cells/min. compared to basal levels of 0.35 pmole/10^9 cells/min.). When NAP-propranolol was added to the incubation medium a dose-dependent inhibition of (-)-isoproterenol-stimulated adenylate cyclase was observed (Fig. 3). 50% inhibition was seen at 23 nM which gave a K_i of NAP-propranolol of approximately 19 nM. Similar results were obtained using turkey erythrocyte ghosts. The K_i was determined by the method of Cheng and Prusoff (12).

NAP-propranolol inhibited the specific binding of (-)-^{3}H-dihydroalprenolol to intact turkey erythrocytes (Fig. 4). Fifty percent inhibition occurred at 530 nM. This gives an inhibition constant (K_i) or approximately 100 nM for NAP-propranolol. Similar binding studies using erythrocyte ghosts yielded comparable results.

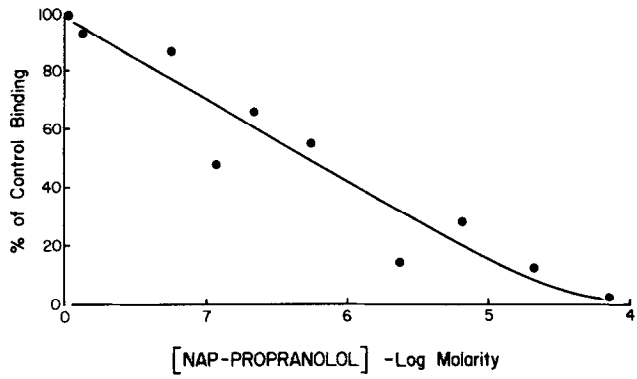

Fig. 4. NAP-propranolol inhibition from the specific binding of (-)-^{3}H-dihydroalprenolol to intact turkey erythrocytes.
0.25 ml of packed red cells were added to 0.5 ml of iced Kreb's Ringer bicarbonate vuffer pH 7.4 containing various amounts of NAP-propranolol and 8.5 nM ^{3}H-dihydroalprenolol. The samples were incubated at 4°C for five min. Stereospecific binding of ^{3}H-dihydroalprenolol was carried out by the method of Malchoff and Marinetti (11). Specific binding is defined as the difference in ^{3}H-dihydroalprenolol bound in the presence and absence of 8.5 μM (-)-propranolol. The results are the averages of two experiments performed in triplicate.

The K_i values reported here are for the probe which is assumed to be a racemic mixture. If the (-)-isomer is the predominant active form, the K_i values would then be half the reported values.

This report describes the synthesis of a photoaffinity azide probe for the beta-adrenergic receptor. This probe is effective in inhibiting the stereospecific binding of the beta-adrenergic antagonist dihydroalprenolol to intact turkey erythrocytes and also inhibits the (-)-isoproterenol-stimulated adenylate cyclase in this cell.

We have also synthesized a NAP-propranolol derivative in which putrescine replaces ethylenediamine and currently are attempting to make the radioactive NAP-propranolol using ^{3}H-putrescine with the hope of using this to label the beta-adrenergic receptor in turkey erythrocytes.

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