Beta-Receptor Blocking Potencies of the Three Newly Synthesized \( \beta \)-Adrenergic Antagonists (S-596, K-351, N-696) as Assessed with the Radioligand Binding Assay Method in Rat Cardiac Muscle Membrane Treated with Neuraminidase

Takafumi NAGATOMO, Hiroshi TSUCHIHASHI, Miyuki SASAKI, Yoshito NAKAGAWA*, Hajime NAKAHARA* and Shoichi IMAI*

Department of Pharmacology, Niigata College of Pharmacy, Niigata 950-21, Japan

*Department of Pharmacology, Niigata University School of Medicine, Niigata 951, Japan

Accepted October 29, 1983

Abstract—To assess the \( \beta \)-blocking potencies of three newly synthesized \( \beta \)-blockers, i.e., S-596: dl-2-(\( \beta' \)-t-butylamino-\( \beta' \)-hydroxypropylthio)-4-(\( \beta' \)-carbamoyl-\( \beta' \)-thienyl)-thiazole hydrochloride, K-351: 3,4-dihydro-8-(2-hydroxy-3-isopropylaminopropoxy)-3-nitroxy-2H-1-benzopyran, and N-696: 4,3-(\( \beta' \)-t-butylamino)-2-hydroxypropoxy)-N-methylisocarbostyril hydrochloride, values of \( K_i \) (inhibition constant) were obtained by the radioligand binding assay method using rat heart membrane treated with neuraminidase. The \( pA_2 \) values of these blockers as antagonists against the positive chronotropic and inotropic actions of isoproterenol were also determined by pharmacological methods using guinea pig atria. To assess the selectivity of these \( \beta \)-blockers, \( pA_2 \) values were also obtained in the isolated trachea using isoproterenol as an agonist. The order of potencies as assessed by \( K_i \) and \( pA_2 \) values were S-596 > K-351 > N-696. When the \( K_i \) values were plotted against the \( pA_2 \) values together with the \( K_i \) and \( pA_2 \) values of our previous study (Horii et al., 1974; Nakazawa et al., 1978; Nakagawa et al., 1980; Nakagawa et al., 1983; Nagatomo et al., 1983), straight lines were obtained with \( r \) values of 0.93 (\( P<0.001 \)) (positive chronotropic action) and 0.91 (\( P<0.001 \)) (positive inotropic action), respectively. Thus, the validity of using the \( \beta \)-adrenoceptor binding assay method developed in this laboratory using cardiac membrane treated with neuraminidase for assessment of the potencies of \( \beta \)-adrenoceptor blockers in cardiac muscle was further substantiated.

Our previous report suggested that the \( \beta \)-adrenoceptor binding assay method developed in our laboratory using cardiac membrane treated with neuraminidase could be used for assessment of newly synthesized \( \beta \)-adrenergic antagonists because a significant correlation (\( r=0.91 \)) was observed between the potencies of \( \beta \)-adrenoceptor antagonists as assessed by binding to the \( \beta \)-adrenergic receptor (\( K_i \)) and the potencies as assessed by pharmacological methods (\( pA_2 \)) by Nagatomo et al. (1, 2). In the present study, the \( K_i \) values of three newly-synthesized \( \beta \)-blockers were determined using the binding assay method just mentioned and were plotted against the \( pA_2 \) values obtained by a pharmacological method together with the \( K_i \) and \( pA_2 \) values of our previous reports (2) in order to further test the validity of using the method for assessment of \( \beta \)-blocking potencies of the new compounds.

Materials and Methods

Binding assay: Preparations of the membrane samples and \( ^3 \)H-dihydroalprenolol (\( ^3 \)HDHA) (New England Nuclear, Co. Ltd; 90.0 Ci/mmol) binding assay were carried out with the method described previously (1, 2).
using male Wistar rats weighing 200–300 g. After removal of the heart, ventricle muscles were minced with small scissors in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and then homogenized twice using a Polytron homogenizer at setting 8 for 10 sec. The suspension was filtered through 4 layers of cheese-cloth. This suspension was treated with 0.3 M KCl and 25 mM sodium pyrophosphate and centrifuged at 177,000 g for 45 min. The pellets obtained were then treated with neuraminidase (6.2 unit/mg protein) (Sigma, Type V) in a medium containing 100 mM KCl, 10 mM MgCl2, 20 mM Tris-maleate, pH 7.4, at 25°C for 30 min. The suspension was again centrifuged at 177,000 g for 45 min, and the pellets were suspended in an incubation buffer (75 mM Tris-HCl, pH 7.4, 25 mM MgCl2).

In the binding assays, membrane suspensions (0.4–0.6 mg protein) were incubated at 23°C with 3H-DHA (2.5 nM), and appropriate concentrations of the drugs in 60 mM Tris-HCl buffer, pH 8.0, 20 mM MgCl2 in a final volume of 1 ml. After 20 min, the incubation medium was filtered under reduced pressure through Whatman glass-fiber filters (GF/C), and the filters were washed with 3×5 ml of cold buffer. The radioactivity of 3H-DHA bound to the membrane remaining in the filters in the absence (total) and the presence (non-specific) of drugs was determined by liquid scintillation counting, and the specific binding was defined as the difference between the total and the non-specific binding. As described previously (1, 2), the specific binding of 2.5 nM 3H-DHA reached equilibrium after 15 min at 23°C and constituted 40–60% of the total binding. Values of K_i (inhibition constant) of each β-blocker were calculated according to the equation described in the previous paper (2). Protein concentration was determined using the method of Lowry et al. (3).

Pharmacological observations: Beta-blocking actions were studied using the method described previously (4). The right and left atria of guinea-pig were used for the assessment of the antagonistic potencies against the positive chronotropic and inotropic actions of isoproterenol (β_1-effect).

The rate of the spontaneous contraction of the right atria was recorded on an ink-writing oscillograph to test the positive chronotropic actions. The left atria stimulated electrically by a square-wave stimulator (Nihon Kohden MSE-40) at the frequency of 1 Hz with voltages 30% above the threshold were used to evaluate the inotropic effects. Their contractile tension was recorded on an ink-writing oscillograph with a strain-gauge transducer and a carrier amplifier. Isolated tracheal smooth muscle preparations of the guinea pig were used to assess the antagonistic effects towards the β_2-receptor. Seven tracheal rings removed from guinea pigs were sutured together and mounted vertically in a 5 ml organ bath. The contractile tension of the preparation was recorded on a potentiometric recorder (Hitachi QPD-74). Relaxation of the preparation was induced by isoproterenol. Drugs were administered in a cumulative fashion, and pA_2 values were calculated using the equation:

\[
pA_2 = -\log_{10} \left( \frac{C}{B} \right) \left( \frac{A}{C} \right)
\]

where A is a dose of drug at which the contraction was 50% of the maximum obtainable without the same compound, B is a dose contraction of drug at which was 50% of the maximum in the presence of the same compound and C is a dose of the drug.

The bathing solution used contained in mM: NaCl, 118; CaCl_2, 2.5; NaHCO_3 24.9; MgSO_4 1.2; KH_2PO_4 1.2; glucose 12. The temperature of the solution was maintained at 32±0.30°C. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2. After dissection, all preparations were allowed to equilibrate for one hour prior to addition of any drugs.

The β-blockers used were S-596: dl-2-(3′-1-butylamino-2′-hydroxypropylthio)-4-(5′-carbamoyl-2′-thienyl)-thiazole hydrochloride (5), K-351: 3,4-dihydro-8-(2-hydroxy-3-isopropylaminoproxy)-3-nitroxy-2H-1-benzopyran (6) and N-696: 4,3-(tret-butylamino)-2-hydroxypropoxy)-N-methyl-isocarbostyril hydrochloride (7). These chemicals were kindly donated by Sumitomo Chemical Co., Ltd (S-596), Kowa
Company, Ltd. (K-351) and Nisshin Flour Milling Co. (N-696), respectively. The chemical structures of these compounds are shown in Fig. 1.

**Results**

As shown in Table 1, the values of the $K_1$ for S-596, K-351 and N-696 were 1.52±0.88, 19.85±6.00 and 159.87±41.80 nM, respectively. The $K_1$ value of S-596 (1.52 nM), the lowest among the three, was lower than those of pindolol (9.83 nM) and propranolol (5.18 nM), while the $K_1$ value of K-351 (19.85 nM) was higher than those of pindolol and propranolol. The $K_1$ value of N-696 (159.87 nM) was almost the same as that of labetalol (127.08 nM).

The $pA_2$ values of S-596, K-351 and N-696 as antagonists against the positive inotropic and chronotropic effects in the heart and the relaxant effects in the trachea of isoproterenol are also listed in Table 1. The order of the $\beta$-blocking potency in the heart was S-596 > K-351 > N-696, while the potencies of K-351 and N-696 in the trachea were almost the same. Thus, as regards to the $\beta$-blocking activity of N-696, there existed a certain degree of selectivity towards the $\beta_2$-receptor.

Figure 2 depicts the relationship between the $K_1$ values of $\beta$-blockers, including the three newly synthesized ones, determined by the binding assay method and $pA_2$ values obtained by the pharmacological method as regards to the antagonistic effects towards the positive chronotropic (A) and the positive inotropic (B) actions of isoproterenol. The $K_1$ and $pA_2$ values of $\beta$-blockers other than the 3 newly-synthesized blockers were taken from the data reported in previous papers (2, 4, 7–9). There was a good correlation between $K_1$ values and $pA_2$ values, giving straight lines which could be expressed by equations, $y=0.69x+2.91$ ($r=0.93$) for the positive chronotropic action and $y=0.64x+3.38$ ($r=0.91$) for the positive inotropic action.

**Discussion**

Among the three new $\beta$-blockers, the highest potency was found with S-596, both in the radioligand binding assay and in the pharmacological evaluations. Potencies as assessed by the binding assay method ($K_1$) and those as assessed using the pharmacological method corresponded very closely with each other. The previous study with $\beta_2$-adrenergic antagonists showed a significant correlation ($r=0.91$) between $K_1$ values obtained by the radioligand binding assay method developed in this laboratory and $pA_2$ values (positive inotropic action) reported in the literature (2). In this paper, the values of the $K_1$ for the newly synthesized $\beta$-blockers were plotted against the $pA_2$

<p>| Table 1. $K_1$ and $pA_2$ values of $\beta$-blockers |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>$\beta$-Blockers used</th>
<th>$K_1$ (nM)</th>
<th>Positive chronotropic action ($pA_2$)</th>
<th>Positive inotropic action ($pA_2$)</th>
<th>Trachea ($pA_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-596</td>
<td>1.52±0.88 (3)</td>
<td>9.31±0.09 (5)</td>
<td>9.09±0.09 (5)</td>
<td>9.58±0.16 (5)</td>
</tr>
<tr>
<td>K-351</td>
<td>19.85±6.00 (4)</td>
<td>8.59±0.07 (5)</td>
<td>8.53±0.04 (5)</td>
<td>8.36±0.06 (4)</td>
</tr>
<tr>
<td>N-696</td>
<td>159.87±41.80 (4)</td>
<td>7.10±0.03 (5)</td>
<td>7.26±0.06 (5)</td>
<td>8.55±0.02 (6)</td>
</tr>
</tbody>
</table>

The values given in parentheses represent the number of experiments. Numbers are given as the mean ±S.E. The $pA_2$ values of N-696 were obtained from the previous report (7).
Fig. 2. Relationship between the potencies of β-adrenoceptor antagonists for inhibition of the ³H-DHA binding to receptor sites of the neuraminidase-treated rat heart membrane preparation (Kᵢ) and the potencies as regards to the inhibition of the positive chronotropic (A) and inotropic (B) effects of isoproterenol as assessed by the pharmacological method in guinea-pig atria (pA₂).

A significant correlation (P<0.001) was found with r value of 0.93 (positive chronotropic action) or 0.91 (positive inotropic action), further supporting the idea that the radioligand binding assay method developed in this laboratory using the values obtained in this laboratory together with the Kᵢ and pA₂ values of our previous reports. A significant correlation (P<0.001) was found with r value of 0.93 (positive chronotropic action) or 0.91 (positive inotropic action), further supporting the idea that the radioligand binding assay method developed in this laboratory using the
neuraminidase-treated membrane preparations could be used for screening of \( \beta \)-adrenergic antagonists yet to come. Bieth et al. (10) reported a good correlation between \( K_i \) and \( pA_2 \) values using cardiac membrane preparations without enzyme treatment in the guinea-pig atria. However, as shown in our previous paper (2), neuraminidase treatment is needed for obtaining reproducible data and higher yields of \( \beta \)-adrenoceptor binding sites.

With \( \beta_1 \)-selective \( \beta \)-blockers, especially with practolol and atenolol, there was a tendency for \( pA_2 \) obtained by the biological assay method to be greater than \( K_i \) obtained with the radioligand binding assay method. This may probably be due to the use of \( ^3H \)-DHA as a radioligand. As is well known, \( ^3H \)-DHA can bind to both \( \beta_1 \) and \( \beta_2 \) adrenoceptors; and as Minneman et al. (11) already reported, rat cardiac muscle contains both \( \beta_1 \) and \( \beta_2 \) adrenoceptor sites. None of the three new \( \beta \)-blockers used in the present study were selective \( \beta \)-blockers, and as expected, no gross dissociation between \( pA_2 \) and \( K_i \) was observed with these three compounds.

As the basic structure, \( \beta \)-blockers possess either an aryloxypropanolamine moiety and have substituents at the para or ortho position of aryloxypropanolamine (12). It is known that the chemicals (propranolol, pindolol, oxprenolol or alpenolol) with substituents at the ortho position show higher affinity to \( \beta \)-adrenergic receptor binding sites and lower selectivity than the compounds with substituents at the para position (atenolol, practolol, acebutolol, metoprolol or sotalol). In this respect, S-596 and N-696 are unique in that the aryl nucleus was replaced by the heterocyclic nucleus. It was found that the potency of N-696 was intermediate, equal to that of acebutolol, while S-596 with a heterocyclic nucleus and a N-substituted thiopropanolamine moiety attached to a heterocyclic nucleus (13) had the lowest values of \( K_i \) or highest values of \( pA_2 \). K-351 with a pyran nucleus at the para position of aryloxypropanolamine showed potencies slightly higher than those of non-selective \( \beta \)-blockers. Thus, judging from the results of the present experiments, we may add the following structures as important structures for \( \beta \)-blocking actions in addition to the aryloxypropanolamine or aryloxyethanolamine of well known \( \beta \)-blocking antagonists, i.e., an thiazolethicpropanolamine or a heterocyclic nucleus instead of an aryl nucleus.

Acknowledgments: The authors wish to thank Miss R. Nakagawa for help in preparing the manuscript.

References

8. Nakazawa, M., Katano, Y., Nakagawa, Y., and Imai, S.: \( \beta_1 \)-selectivity of acebutolol hydrochloride, a new \( \beta \)-blocking agent. Pharmacol-


