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Conjugates of Catecholamines

II. *In Vitro* and *in Vivo* Pharmacological Activity of *N*-Alkyl-Functionalized Carboxylic Acid Congeners and Amides Related to Isoproterenol

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SUMMARY

In this study, 10 congeners of isoproterenol were systematically synthesized and pharmacologically tested in both *in vitro* and *in vivo* systems. The aim was to produce compounds that were more potent and had effects different from those of the parent compound and that could ultimately be attached covalently to inert peptide carriers offering versatility in size, pK, and other physicochemical properties. The congeners synthesized were tested in the S49 mouse lymphoma assay for their ability to stimulate cyclic AMP accumulation and were shown to have potencies relative to isoproterenol ranging from 4 orders of magnitude less potent to 4 orders of magnitude more potent than isoproterenol in eliciting this response. Some of the congeners were also tested in a guinea pig isolated atrial preparation, a rat blood pressure assay, a guinea pig bronchodilation assay, and an anesthetized dog preparation. In these assays, the congeners were shown to have the same types of activities as in the S49 cell assay. The results of these studies indicate that structural modifications distant from the catecholamine moiety dramatically alter the pharmacological profile of the congeners versus the parent compound, resulting in a series of ligands with a wide range of activities.

INTRODUCTION

Our previous studies (1) have shown that biologically active catecholamines (ligands) can be attached covalently to complex but pharmacologically inert polymers, peptides, or protein carriers (2) without the loss of some of the biological activity of the ligand. The pharmacological properties of the resultant conjugates were characteristic of, but less potent *in vitro* than, the parent compound.

Because theoretically tissue target-directed catecholamines could have therapeutic advantages over the nonspecific, widely distributed free ligand, we initiated a systematic series of experiments aimed ultimately at the construction of conjugates that might be tissue-targeted. The first aim of our studies was to synthesize potent congeners of isoproterenol. We reasoned that the more potent the ligand before conjugation, the more likely the potency of the conjugate would be greater than isoproterenol. We would then proceed to synthesize pharmacologically inactive carriers that offered versatility in size, pK and other physicochemical properties to which congeners would be covalently linked. This report summarizes the pharmacological testing of a series of congeners and model derivatives of isoproterenol that could serve as more potent ligands than isoproterenol.

METHODS

Synthesis of Congeners

The synthesis of a series of carboxyl-functionalized congeners related to isoproterenol has been described fully by Jacobson *et al.* (1). In this novel series of drug derivatives, the *N*-isopropyl group of the catecholamine, isoproterenol, was extended by a linear methylene chain terminated by a carboxylic acid group. The compounds were synthesized via the reduction amination of norepinephrine with methyl ketoacids and were designed specifically for covalent attachment to carriers such as oligopeptides. Amide model compounds were synthesized either by direct condensation of the congeners with amines, or, more often, via reductive amination of norepinephrine with the appropriate, preformed methyl ketoamide (1) in order to model the attachment of the congeners to peptide carriers and also for the purpose of optimizing the length of the spacer group (1).

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All of the resulting compounds were screened for *beta*-adrenergic properties and compared with the parent compound, isoproterenol, in generating accumulation of cyclic AMP intracellularly in the S49 mouse lymphoma cell. In addition, we compared the relative potencies of several of these compounds to displace a radiolabeled antagonist, [^{125}I]CYP,[5] from *beta*-adrenergic receptors in membranes derived from the S49 cells. To characterize further the interactions of these selected compounds with *beta*-receptors, we also determined their relative potencies at *beta*-receptors in rat lung and heart using [^{125}I]CYP. Rat lung contains predominantly $beta_2$-receptors (~85%), whereas rat heart contains mainly $beta_1$-receptors (>90%) with few measurable $beta_2$-receptors (3, 4). Compounds were selected for testing in these assays so that examples of drugs more and less potent than isoproterenol in the biological assays would be represented.

Other *in vitro* tests included measurement of the effects of test compounds on the heart rate and force of contraction in the isolated guinea pig atria. Some of the compounds as well as isoproterenol were also tested *in vivo* for their effect on rat and dog blood pressure, heart rate and force of contraction, and guinea pig bronchodilation.

The sequence of the testing process started with the routine use of the S49 assay for general screening of all compounds. The most potent compounds as well as some of the least active (used as controls) were then screened in the rat blood pressure assay. Compound 2 was chosen for the binding studies because its effects on S49 cells were greater than those of isoproterenol and because its structure was the basis for the synthesis of other, more active compounds. Compound 3 was tested along with Compound 2 in blocking experiments in order to determine whether their relative agonistic potencies as compared to isoproterenol were exclusively related to the *beta*-receptor and would therefore be blocked by precalculated concentrations of propranolol. Compound 3 was chosen for more extensive study because it was the most potent compound that we had used in the assays.

In Vitro Studies

S49 mouse lymphoma cell assay. S49 mouse lymphoma cells were centrifuged and resuspended at a density of 2–2.5 × 10^6/ml in Dulbecco's modified Eagle's medium (13.3 μg/liter) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) (DMEH) plus 0.1% bovine serum albumin (5). They were next incubated at 37° for 10 min without drug and were then added to tubes with or without test compounds for an additional 6 min. The reaction was stopped by putting the tubes on ice. After centrifugation to form a pellet of cells, the pellets were resuspended and boiled. Aliquots were then used for the competitive binding assay described by Gilman (5). Increased levels of cyclic AMP were plotted as a function of the log of the concentration of the catecholamines. Thus, K_a (the association constant in molarity units which represents one-half the concentration that produced maximal effect) and E_{max} (maximal response in picomoles of cyclic AMP per 10^7 cells) values were obtained for each test compound using the mean of three to five experiments with each ligand. The ratio of the K_a values for the compound and for isoproterenol tested at the same time is considered an indicator of relative *in vitro* potency.

S49 mouse lymphoma beta-blocking assay. This assay was carried out in the same fashion as the agonist assay with only one difference: the test compound was administered at a concentration equivalent to the effective dose that produced 90% E_{max} response (ED_{90}) in production of cyclic AMP, and the antagonist propranolol was administered at the same time in concentrations ranging from 10^{-14} to 10^{-4} M. The concentration of propranonol required to block 50% of the ED_{90} of the test compound was then calculated.

[^{125}I]CYP binding assays. Binding assays were conducted in membrane fractions from rat heart, rat lung, and S49 cells prepared as previously described (4, 6). Membranes were resuspended in buffer containing 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 7.5). Assays were performed in a total volume of 0.15 ml at 37° for 60 min (S49 and heart) and 30 min (lung), by which time equilibrium had been achieved. Incubations were terminated by washing membrane preparations with 20 ml of buffer over glass-fiber filters. Radioactivity on the filters was determined in a *gamma* counter at an efficiency of 70%. In the competition curve experiments, the concentration of [^{125}I]CYP was at its $K\Delta$ value for each of the tissues. CYP was iodinated as described by Engel *et al.* (7). The EC_{50} of each competitor was determined by analyzing competition curves using the four-parameter logistic equation as described in ref. 8.

In Vivo Studies

Rat blood pressure assay. Male Sprague-Dawley rats weighing 280–350 g were anesthetized with pentobarbital sodium (60 mg/kg i.p.). The left femoral vein and artery were cannulated. A Statham pressure transducer (P233Db) was connected to the arterial cannula for measurement of blood pressure. The femoral vein was utilized for administration of test compound. Dose-response curves for the effects on blood pressure were obtained by administering doses of the test compound ranging from 10^{-12} to 10^{-8} moles/kg i.v. (minimum of 10 animals per dose). Doses were always administered in progressively increasing amounts after the blood pressure had reached baseline. The maximal decrease in blood pressure was measured and expressed as a percentage change from baseline. The ED_{50} for decrease in blood pressure, the relative potency of the test compounds as compared with isoproterenol, and the time interval between the administration of the drug and the point at which the blood pressure reached its maximal decrease were then calculated.

Isolated guinea pig atria assay (9). Male guinea pigs were killed by a blow to the head. The heart was removed, and the left and right atria were dissected free of ventricular tissue. Each atrium was individually attached to a tissue holder and suspended in separate 25-ml organ baths containing Krebs-Henseleit solution having the following composition (millimolar): NaCl (118), KCl (4.7), $CaCl_2$ (2.7), $MgSO_4 \cdot 7H_2O$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), glucose (5.6). This solution was gassed with 5% CO_2 in oxygen and maintained at 37°. The final pH of the solution was 7.4. The free ends of each atrium were attached to isometric force transducers, and an initial diastolic tension of 0.5–1.0 g was applied to each tissue. Inotropic activity was measured from the left atrium electrically driven at a constant rate of 2.5 Hz with square wave pulses (5-msec duration and threshold voltage plus 50%). Chronotropic activity was measured from the spontaneously beating right atrium by means of a cardiotachometer triggered by the tension signal. A 30-min equilibration period preceded each experiment.

Cumulative dose-response curves for the agonists were then constructed. In order to determine inotropic responses in the left atria, increasing doses were administered until a maximal response was obtained. The inotropic responses were allowed to plateau between each successive dose. To establish chronotropic responses in the right atria, the tissue was exposed to increasing doses of the agonist for a period of 2 min. A 30-min washout period followed each cumulative dose-response test. Three consecutive cumulative dose-response curves were obtained for isoproterenol in all tissues. This was followed by a cumulative dose-response curve for the test compound (Compound 3). The first two dose-response curves served to condition the tissue and were not used for calculating data.

In a separate group of control tissues, a fourth isoproterenol dose-response curve was obtained instead of a curve for the test compound. The control experiments were performed in order to correct for the small changes in tissue sensitivity which occurred over time between the third and fourth dose-response curves. The sensitivity of the fourth isoproterenol curve was slightly less than the third isoproterenol curve. By determining the percentage change between the third and fourth isoproterenol dose-response curves at each of five concentrations and then averaging these values, the relative degree of change between these two curves could be assessed. A 7% difference occurred in terms of the inotropic dose-response curves and less than a 1% difference in terms of chronotropic responses. This factor was then applied to the third isoproterenol curve obtained in the treatment groups in order to predict the response to isoproterenol if it had been given at the same point in time as the test agonist. The isoproterenol dose-response curve thus calculated was used as the standard reference. Responses were calculated by measuring the agonist-induced increases above predose

[5] The abbreviation used is: [^{125}I]CYP, radiolabeled cyanopindolol.

levels and expressing these as percentages of the maximal isoproterenol response.

Guinea pig bronchodilation assay. Male guinea pigs (Hartley strain Charles River) weighing 300–500 g were anesthetized with urethane (2 g/kg i.p.), and a polyethylene cannula was inserted into the jugular vein for drug administration. Tracheal pressure was recorded from a cannula inserted into the trachea and connected to a Statham transducer. After surgical preparation of the animals, a period of time was allowed for pulmonary functions to stabilize. Spontaneously breathing animals were exposed for a 5-min period to aerosol solutions of test drug (varying concentrations, % w/v) or to distilled water. All compounds were administered by inhalation using a Monaghan (Model 750) ultrasonic nebulizer. Fresh aqueous solutions of the test compounds were prepared and introduced into the chamber of the nebulizer. The output of the nebulizer was made available to the animal by directing a bias flow of aerosol through a Y-tube connected to the tracheal cannula. At the end of the exposure period, the animals were paralyzed with succinylcholine (1.2 mg/kg, i.v.) and mechanically respirated (Harvard rodent respirator at 40 breaths/min and a 2.5-ml tidal volume). Animals were then challenged with a maximally constricting dose of histamine (200 μg/kg) delivered i.v. 30 sec after administration of the succinylcholine.

Anesthetized dog assay. Three male beagle hounds were used in the study. The animals were anesthetized with a combination of barbital (300 mg/kg) and pentobarbital (15 mg/kg) given i.v. Arterial blood pressure was monitored from the left carotid artery with a Statham transducer. To monitor contractile force, the chest was opened through the right fifth intercostal space, and a strain gauge arch was sutured to the surface of the right ventricle. Artificial respiration was maintained by a Bird Mark 8 respirator. The chest was closed by suturing, and the animal was allowed to breathe spontaneously. A polyethylene catheter was inserted into the left saphenous vein to permit injection of drug. Heart rate was measured with a Hewlett-Packard cardiotachometer. The electrical signal form monitoring heart rate was taken from the R wave of Lead II. After the preparation was completed, a stabilization period of 30 min was allowed to elapse before control readings were taken. All monitored parameters were recorded on a Hewlett-Packard 8-channel, Series 7758A, direct-writing recorder.

The drugs used in the study were prepared daily in 0.9% NaCl solution, protected from light and kept at 0°. Cumulative dose-response curves for isoproterenol (0.01, 0.05, 0.10, 0.30 μg/kg i.v.) and for Compound 3 (0.005, 0.015, 0.05 μg/kg i.v.) were generated in the same animal. The increase in contractile force was calculated in terms of the percentage change from predose control. A dose which increased contractile force by at least 80% in these initial studies was selected and administered in one animal to assess the duration of the effect.

RESULTS

The results of the *in vitro* S49 mouse lymphoma cell assay are shown in Table 1. The carboxylic acid congener of isoproterenol (Compound 1) was about 4 orders of magnitude less potent than the parent compound in eliciting cyclic AMP accumulation in S49 cells (Table 1). The *p*-toluide congener derivative (Compound 2) proved to be approximately 1 order of magnitude more potent than isoproterenol, and the *p*-trifluoromethylanilide derivative (Compound 3) was 4 orders of magnitude more potent than isoproterenol in this assay (Table 1).

The results of the binding studies using [^{125}I]CYP are shown in Table 2. The relative potencies of three compounds were compared with isoproterenol in membranes from S49 cells, rat heart, and rat lung by measuring the EC_{50} for each compound as described under Methods. In the potency series, Compound 3 > 2 > isoproterenol > 5 was observed in each tissue and was identical to the potency as determined by cyclic AMP accumulation. Similar potencies for these compounds in heart ($beta_1$) and lung ($beta_2$) suggested that none of the drugs has substantial *beta*-receptor subtype selectivity.

The series of *p*-toluide derivatives (Compounds 2, 8, 9, and 10) was synthesized in order to study the effect of changing the length of the methylene chain (the spacer group) on accumulation of cyclic AMP in S49 cells (Table 1). Compound 2, with four methylenes adjacent to the carbonyl group, was clearly more active than the other members of the series, i.e., approximately 1 order of magnitude more active than isoproterenol and 2 orders greater than the other members of this series.

A variety of structural analogues of Compound 2 (Table 1) was also synthesized and tested. In Compound 6, the *p*-methyl group was extended by three methylene groups to provide a *para-n*-butyl derivative whose *in vitro* activity was increased to 3 orders of magnitude greater than isoproterenol. Compound 7 (Table 1) contained an electron-donating methoxyl group in the *para* position and was found to be approximately equipotent to isoproterenol. When an electron-withdrawing group, trifluoromethyl, was placed in the *para* position (Compound 3), the activity increased to 4 orders of magnitude greater than isoproterenol.

Table 1 shows the effects of positioning the trifluoromethyl group of Compound 3 at the *para* position or the *meta* (Compound 4) or *ortho* (Compound 5) positions. When the trifluoromethyl group was *meta* or *ortho*, the *in vitro* activity decreased dramatically. The *meta* derivative, Compound 4, was approximately as active as isoproterenol, but the *ortho* derivative, Compound 5, was roughly 4 orders of magnitude less active (Table 1). The enhanced *in vitro* biological activity of Compound 3 is therefore not a simple function of the electron-withdrawing substituent on the aromatic amide but is likely to be related to other effects, such as conformational effects in which, for example, the two aromatic rings may be stacked.

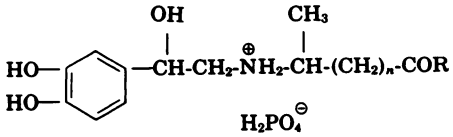
Compounds 2 and 3 were tested to determine whether their *beta*-agonist action could be blocked by propranolol. These studies showed that a 10^{-8} M concentration of propranolol was required to block 50% of a 10^{-8} M concentration (ED_{90}% E_{max}) of Compound 2, whereas the same 10^{-8} M concentration of propranolol was required to block 50% of a 10^{-10} M concentration (ED_{90}% E_{max}) of Compound 3 and a 10^{-7} M concentration of isoproterenol.

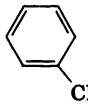
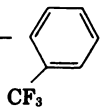
Dose-response studies comparing the inotropic and chronotropic effects of the *p*-trifluoromethylanilide derivative (Compound 3) with isoproterenol effects in the isolated guinea pig atria were performed (Table 3). Compound 3 was more potent than isoproterenol in this assay. The relative potencies of these compounds were determined by calculating the dose required to produce 50% of the maximal inotropic and chronotropic responses obtained with isoproterenol (ED_{50}). As shown in Table 3, the inotropic activity of the test compound was 4.7 times greater than isoproterenol, whereas the chronotropic response of the analogue was 2.4 times greater than the parent compound. Because of our experimental design, we do not know whether there was a differential effect on rate versus force of contraction in these experiments. For determination of rate, the tissues were exposed to the agonist for 2 min, and equilibrium of the rate changes did not always occur. The changes in rate were determined after a 2-min exposure rather than at plateau

TABLE 1

In vitro biological activity of congeners and model derivatives of isoproterenol

Biological activity was measured by cyclic AMP accumulation in S49 cells. Relative activity is expressed as the ratio of the K_a for isoproterenol to K_a for the test compound.



Compound	R	n[a]	Activity relative to isoproterenol
1. Carboxylic acid	—OH	4	1.6×10^{-4}
2. *p*-Toluide	—NH—  —CH_3	4	5.4×10^{1}
3. *p*-Trifluoromethylanilide	—NH—  —CF_3	4	1.1×10^{4}
4. *m*-Trifluoromethylanilide	—NH— 	4	9.8×10^{-1}
5. *o*-Trifluoromethylanilide	—NH— 	4	2.5×10^{-4}
6. *p*-Butylanilide	—NH—  —$(CH_2)_3CH_3$	4	3.2×10^{3}
7. *p*-Methoxylanilide	—NH—  —OCH_3	4	3.1
8. *p*-Toluide	—NH—  —CH_3	2	7.1×10^{-1}
9. *p*-Toluide	—NH—  —CH_3	3	3.6×10^{-1}
10. *p*-Toluide	—NH—  —CH_3	5	1.9×10^{-1}

[a] n = Number of methylene groups in the spacer group.

because the peak response developed too slowly and the time to the peak response developed too inconsistently to judge when the next highest dose should be given. Thus, a timed exposure seemed more appropriate for a cumulative dose-response curve. However, inotropic responses were allowed to plateau between successive doses. Thus, the values for inotropic activity more accurately reflect relative potency. The maximal efficacy of each compound was equivalent for both inotropic and chronotropic actions.

We also compared the time required to reach a maximal inotropic response, and the duration of effect after washout for both inotropic and chronotropic responses. The *p*-trifluoromethylanilide derivative (Compound 3) required a significantly ($p < 0.05$) greater time than isoproterenol to reach maximal inotropic response (Table 3); its response lasted significantly longer after washout than that with isoproterenol. Similarly, chronotropic responses following washout of isoproterenol returned to predrug levels, and the chronotropic response following the test compound remained higher. Note that the inotropic response of Compound 3 was protracted beyond that of its chronotropic effect (Table 3).

A carboxylic acid congener (Compound 1), representative of this series of compounds, and two model derivatives (Compounds 2 and 3), which were shown to be more potent than isoproterenol *in vitro*, were also studied *in vivo* by assessing the change in blood pressure produced after i.v. administration to anesthetized rats. The results, shown in Fig. 1, indicate that all of these compounds produced dose-dependent reductions in blood pressure with variations in potency and efficacy. The carboxylic acid congener (Compound 1) was the least potent and efficacious, with an ED_{50} and 95% confidence limits of 1.61×10^{-8} (range 0.55×10^{-8} to 1.77×10^{-8}) moles/kg i.v. and a potency of 0.16 as compared with

TABLE 2

EC_{50} of agonists for beta-receptors labeled with [^{125}I]CYP

The EC_{50} for each compound in competition for [^{125}I]CYP binding sites was determined as described under Methods. The EC_{50} values are presented as arithmetic mean ± standard error of the mean for four separate experiments.

Compound	EC_{50}		
	S49 cells	Heart	Lung
		nM	
Isoproterenol	2200 ± 1200	200 ± 80	270 ± 100
p-Trifluoromethylanilide (Compound 3, Table 1)	84 ± 13	21 ± 4	40 ± 8
p-Toluide (Compound 2, Table 1)	250 ± 100	33 ± 14	76 ± 14
o-Trifluoromethylanilide (Compound 5, Table 1)	3650 ± 550	490 ± 100	460 ± 230

isoproterenol. The *p*-toluide congener derivative (Compound 2) was slightly more potent than isoproterenol, with an ED_{50} of 1.12×10^{-9} (range 0.84×10^{-9} to 1.4×10^{-9}) moles/kg i.v. and a potency relative to isoproterenol of 1.62. On the other hand, the *p*-trifluoromethylanilide derivative (Compound 3) proved to be significantly ($p < 0.05$) more potent than isoproterenol, with an ED_{50} of 5.4×10^{-10} (range 4.97×10^{-10} to 6.33×10^{-10}) moles/kg i.v. and a potency relative to the parent compound of 3.35. The potency, as would have been predicted from the testing in S49 cells, of the *p*-toluide and *p*-trifluoromethylanilide derivatives was greater than that of the carboxylic acid congener.

In addition to the differences noted above, the time interval from the administration of the ED_{50}-equivalent dose to the maximal decrease in blood pressure was significantly ($p < 0.05$) longer for the carboxylic acid congener (Compound 1) and the *p*-trifluoromethylanilide derivative (Compound 3) than for isoproterenol (isoproterenol = 0.55 min; Compound 1 = 1.43 min; Compound 2 = 0.72 min; Compound 3 = 0.85 min).

The *p*-trifluoromethylanilide derivative (Compound 3) and isoproterenol were studied in the anesthetized dog for changes in contractile force, heart rate, and blood pressure. Compound 3 increased contractile force by at least 80% after a dose of 0.05 μg/kg i.v. (6.87×10^{-11} moles/kg). A similar change in force occurred after 0.03 μg/kg i.v. of isoproterenol (1.21×10^{-10} moles/kg). Compound 3 is also increased heart rate (Fig. 2).

The inotropic and chronotropic response produced by Compound 3 (0.05 μg/kg i.v.) lasted considerably longer than did twice the dose of isoproterenol (0.1 μg/kg i.v.). The response to isoproterenol disappeared in a matter of 2 or 3 min, whereas the effects of the congener persisted for 60–80 min (Fig. 2).

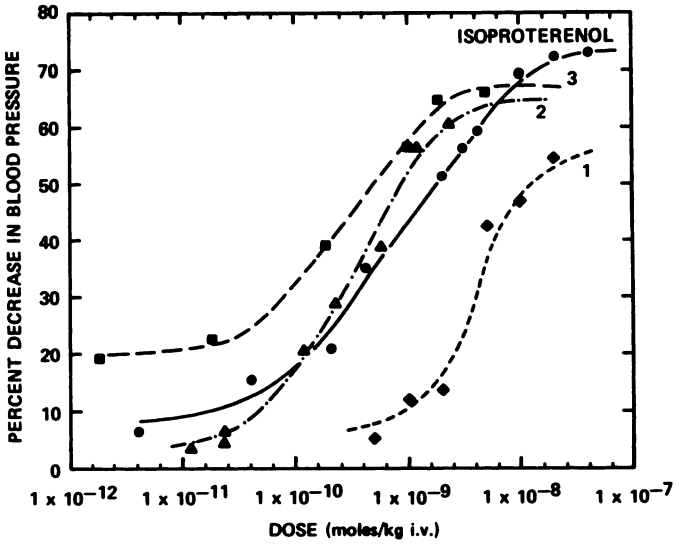


FIG. 1. *Effects of congener derivatives of isoproterenol on blood pressure in the rat*

Blood pressure was determined as described under Methods. Results were calculated as mean percentage decrease in blood pressure: isoproterenol, $N = 12$; carboxylic acid (Compound 1), $N = 7$; *p*-toluide (Compound 2), $N = 10$; *p*-trifluoromethylanilide (Compound 3), $N = 10$. See Table 1 for details.

TABLE 3

Chronotropic and inotropic activity[a] of isoproterenol and p-trifluoromethylanilide congener derivative in the isolated guinea pig atrial preparation

Inotropic and chronotropic activity were determined as described under Methods. The units in C represent percentage of predrug values. They were calculated by measuring the respective inotropic or chronotropic values 30 min following washout of the highest concentration of drug. These numbers were then expressed as a percentage of the values obtained just before starting the cumulative dosing procedure (predrug values).

A. Relative potency	Inotropic response		Chronotropic response	
	ED_{50}	Potency ratio	ED_{50}	Potency ratio
Isoproterenol	1.14×10^{-8} M	1	6.1×10^{-9} M	1
p-Trifluoromethylanilide	3.0×10^{-9} M	4.7	2.5×10^{-9} M	2.4
B. Time to maximal response	**Inotropic response**			
Isoproterenol	579 18 (sec)			
p-Trifluoromethylanilide	1344 369[a] (sec)			
C. Washout pattern[b]	**Inotropic response**		**Chronotropic response**	
Isoproterenol	66.4 5.2		98.7 4.8	
p-Trifluoromethylanilide	600 120[a]		143 16	

[a] $p < 0.05$ (paired *t*-test: test compound versus calculated isoproterenol response).

[b] Mean ± standard error of the mean calculated as percentage change from predrug values to values recorded 30 min after the last dose.

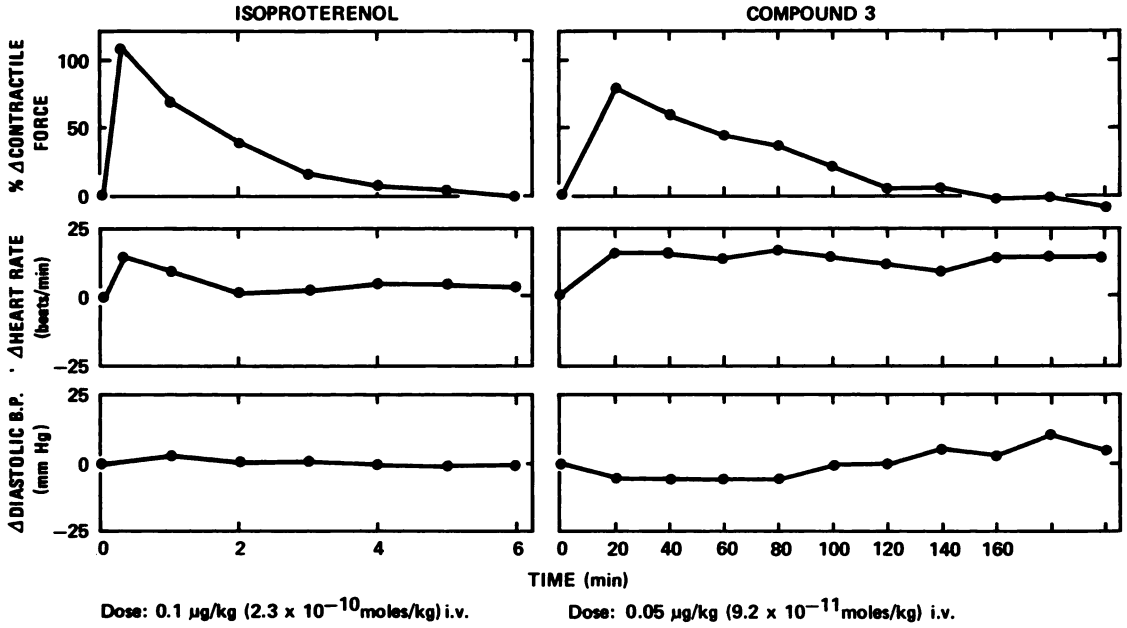


FIG. 2. *Duration of action of isoproterenol and p-trifluoromethylanilide congener derivative of isoproterenol in the dog*
Contractile force, heart rate, and diastolic blood pressure were measured as described under Methods.

The same congener derivative (Compound 3) and isoproterenol were administered as an aerosol to anesthetized guinea pigs. The animals were challenged with a maximal constrictor dose of histamine, and the ability to inhibit histamine-induced bronchospasm was measured. Figure 3 illustrates the bronchodilation produced by Compound 3 and isoproterenol. Isoproterenol as an aerosol (IC_{50} = 0.00017%) was 20 times more potent than Compound 3 (IC_{50} = 0.0034%).

DISCUSSION

These studies were designed to answer several questions about the pharmacological activity of a series of structurally related congener derivatives of isoproterenol. The *in vitro* S49 mouse lymphoma cell assay was used as a first screen for *beta*-adrenergic activity of the compounds as well as to assess the relative potencies of the congeners and derivatives. This cell line is responsive to both $beta_1$- and $beta_2$-adrenergic stimulation with greater sensitivity of $beta_2$ effects (3). In the assay the drug is administered directly to the media that contain the cells, and therefore transport of the drug plays no significant role in the cellular response to drug stimulation. The same is also true for the binding studies. The potency of three of the compounds relative to isoproterenol was predictable by the concentrations of [^{125}I]CYP displaced from the *beta*-receptors in the binding studies (Table 2). When these are combined with tests of the relative potency of blockade of accumulation of cyclic AMP by propranolol, they lead us to the conclusion that all of the *in vitro* effects of each compound can be explained by their action on *beta*-adrenergic receptors. On the other hand, the effects observed in the *in vivo* rat and dog blood pressure assays, the isolated guinea pig atrial preparation, and the guinea pig bronchodilation assays are a composite of drug-receptor interaction plus absorption, biodisposition, and metabolism of the drug. The full effects of the drugs were competitively and completely inhibited by propranolol in each test system examined. Propranolol was not tested in either the guinea pig assay or the bronchopulmonary study. Thus, despite differences and changes in potency shown by the different assay systems, all of the effects of the drugs tested appeared to be exclusively related to their *beta*-mimetic actions.

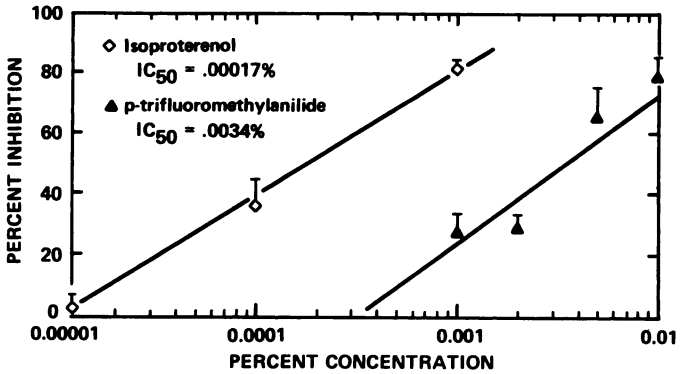


FIG. 3. *Concentration-response curve for aerosolized isoproterenol and p-trifluoromethylanilide congener derivatives against histamine-induced bronchoconstriction*
Compounds were administered by aerosol for an exposure time of 5 min. After an interval of 30 sec following initiation of dosage, histamine (200 μg/kg) was administered as challenge. Each point represents the mean (± standard error of the mean) of determinations made on four animals.

The structure-activity relationship studies with the S49 cells (Table 1) on the series of *p*-toluide derivatives (Compounds 2, 8, 9, and 10) indicate that changes in the length of the methylene chain (the spacer) can affect biological activity. Structural modifications of the *p*-toluide group in Compound 2 showed that the presence of an electron-withdrawing group in the form of a *p*-trifluoromethyl group (Compound 3), in lieu of the *p*-methyl group, markedly increased potency (Table 1). Finally,

alterations in the position of the trifluoromethyl group (Table 1) on the ring indicate that the position of this group on the ring is an important determinant of potency. Thus the potency decreased dramatically if the *p*-trifluoromethyl group was anywhere but in the *para* position. The *in vitro* enhancement of potency was therefore not simply a function of having an electron-withdrawing substituent on the ring. It appears that steric factors may also be involved.

Direct comparison of the *in vitro* and *in vivo* results suggests that a general qualitative agreement in the bioactivity exists across these models. The striking differences are in the magnitude and duration of the effects when the drugs are given *in vivo* or in the isolated guinea pig atrium *in vitro* preparations. Changes of up to 4 orders of magnitude can be observed in the S49 mouse lymphoma cells assay (Table 1), whereas in the rat blood pressure model the greatest difference is seen for Compound 3 (Fig. 3), whose potency is 3.35 times and thus significantly ($p < 0.05$) greater than isoproterenol's. The *in vitro* results are more a function of the net effect of the drug-receptor interactions unbuffered by compensatory actions of other organs, whereas the *in vivo* findings are determined by a combination of direct drug-receptor interaction, physiological responses of the animal to those effects, and drug pharmacokinetics and metabolism. They are likely to be accounted for on the basis of different pharmacokinetics, pharmacodynamics, and/or metabolism than with isoproterenol. The relative contribution of each factor that will account for our inability to quantify potency, duration of effect, and extent of efficacy in the S49 cells versus other test systems is yet to be determined. To the extent that discrepancies can be explained on one of these bases and related to chemical structures, subsequent drug derivatives might be constructed to take therapeutic advantage of the unique structural sensitivity of these molecules.

The effects of the *p*-trifluoromethylanilide congener derivative (Compound 3) on the isolated guinea pig atrium preparation and the anesthetized dog for inotropic and chronotropic effects showed that congener derivatives of isoproterenol can be constructed that have greater potency as well as duration of action than the parent compound in both *in vitro* and *in vivo* models of cardiovascular function.

When Compound 3 was compared with isoproterenol in the *in vivo* guinea pig bronchodilation assay, the congener was found to be 20 times less potent than isoproterenol, indicating an apparent selectivity for $beta_1$-receptors rather than $beta_2$-receptors of the compound when given by this route. The mechanism of this predisposition for only one receptor-related effect is entirely unknown. We do not know the drug's penetrability or absorbability across the bronchial mucosa and have not tested for systemic effects in the absence of bronchodilatatory activity. Discovering the mechanisms of such unanticipated actions and capitalizing on them chemically and pharmacologically certainly seems sensible.

The dramatic dependence of the pharmacological profile of the congener derivatives versus the parent compound on modifications distant from the catecholamine moiety suggests that modulating activity by linking drugs to a variety of carriers may have potential application in the development of other new drugs besides derivatives of catecholamines. These experiments as well as other evidence (10) from our laboratories also suggest that carriers such as small peptides or even large proteins can be attached covalently to these congeners, resulting in a new class of drug. Whether this class has tissue specificity, is as potent as some congener derivatives, and has as long a duration of action as some others remains the subject for future study.

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REFERENCES

1. Jacobson, K. A., D. Marr-Leisy, R. P. Rosenkranz, M. S. Verlander, K. L. Melmon, and M. Goodman. Conjugates of catecholamines. I. N-alkyl functionalized carboxylic acid congeners and amides. *J. Med. Chem.* **26**:492–499 (1983).
2. Melmon, K. L., M. S. Verlander, L. Krasny, M. Goodman, N. Castagnoli, and P. Insel. The chemistry and pharmacology of catecholamines and other low molecular weight ligands conjugated to carriers, in *Proceedings of the 4th International Catecholamine Symposium (1978)* (E. Usdin and J. Barchas, eds.), Vol. 1. Pergamon Press, New York, 483–485 (1979).
3. Hancock, A. A., A. L. De Lean, and R. J. Lefkowitz. Quantitative resolution of *beta* adrenergic receptor subtypes by selective ligand binding: application of a computerized model fitting technique. *Mol. Pharmacol.* **16**:1–9 (1979).
4. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. Simultaneous determination of $beta_1$ and $beta_2$ adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmacol.* **16**:34–46 (1979).
5. Gilman, A. G. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**:305–312 (1970).
6. Insel, P. A., M. E. Maguire, A. G. Gilman, H. R. Bourne, P. Coffino, and K. L. Melmon. *Beta* adrenergic receptors and adenylate cyclates: products of separate genes? *Mol. Pharmacol.* **12**:1062–1069 (1976).
7. Engel, G., D. Hoyer, R. Berthold, and H. Wagner. (±)-^{125}Iodo cyanopindolol, a new ligand for beta adrenoceptors: identification and quantitation of subclasses of beta adrenoceptors in guinea pig. *Naunyn Schmiedebergs Arch. Pharmacol.* **317**:277–285 (1981).
8. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
9. Broadley, K. J. An analysis of the coronary vascular responses to catecholamines using a modified Langendorff heart preparation. *Br. J. Pharmacol.* **40**:617–629 (1970).
10. Verlander, M. S., K. A. Jacobson, R. P. Rosenkranz, K. L. Melmon, and M. Goodman. Some novel approaches to the design and synthesis of peptide-catecholamine conjugates. *Biopolymers* **22**:531–545 (1983).

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