Abstract —The commercially available long acting anti-asthmatic drug, formoterol exists as a racemate of four enantiomers ((R,R)-, (R,S)-, (S,R)- and (S,S)-. The study describes several comparisons between two completely different enantiomers, (R,R)- and (S,S)- based on their 
1) cell surface binding, # 2) cAMP elevation ability, # 3) G-protein activation and # 4) inhibition of DNA synthesis in PC3 cells. The presence of high affinity \( \beta_2 \)-adrenergic receptor (\( K_d \sim 30 \) pmol/L) was confirmed by competition binding of \(^{125}\)I-cyanopindolol with increasing concentration of (R,R)-formoterol using both intact PC3 cells and isolated plasma membrane. Replacing (R,R)- by (S,S)- yielded no significant binding interaction proving its ineffectiveness toward the \( \beta_2 \)-adreno-receptor. While both were capable of eliciting prolonged cAMP generating activity in intact PC3 cells, the EC\(_{50}\) values (R,R- = 10.5 pM, R,S- = 11.0 pM and S,S = 1000 pM) varied nearly 100 fold in favor of (R,R)- and (R,S)-. Propanolol effectively inhibited cAMP elevation in intact cells in both the cases as also agonist stimulated incorporation of \([\gamma^{32}\text{P}]-\text{GTP-AA} \) (Guanosine triphosphate azidoanilide) by (R,R)- in isolated PC3 membrane, but failed in both events in the presence of (S,S)- enantiomer although incorporation of \([\gamma^{32}\text{P}]-\text{GTP-AA} \) is specific for both the enantiomers. The unique discriminatory behavior is further observed in presence of muscarinic agonist, carbachol, which potentiated cAMP generation by (R,R)- nearly 2–3 fold, but was unable to do so in the presence of (S,S)-. These facts confirmatively indicate that cAMP elevation by (S,S)- in PC3 cells occurs entirely via a different pathway than its (R,R)-counterpart. Interestingly, both enantiomers can effectively lower the DNA synthesis, showing superior efficacy of (R,R)-.

Keywords : Binding, \( \beta_2 \)-Adrenergic Agonist, Androgen, Formoterol, Prostate Cancer, PC3, LnCaP, Du145, G-Protein, \( \beta \)-Adrenergic Receptor, Enantiomer.
ABBREVIATION

Cyclic Adenosine Mono-phosphate (cAMP); Cyclic Guanosine Mono-phosphate (cGMP); Inositol-triphosphate / Polyphosphoinositide (IP3), Phospholipase C β (PLCβ); Protein Kinase C (PKC); Guanosine triphosphate (GTP); Isobutyl Methyl Xanthine (IBMX); GTP binding protein (G-protein); G-protein coupled receptor (GPCR); Intracellular Calcium ([Ca^{+2}]_I); AdenosineTriphosphate (ATP); Deoxy Ribonucleic Acid (DNA); Guanosine-5’-[γ-Thiol]Triphosphate (GTPγS); Trichloro acetic acid (TCA); Tosyl-lysyl chloro ketone (TLCK); Tosyl-phenyl chloro ketone (TPCK); Phenyl methyl sulfonyl fluoride (PMSF); Dimethylsulfoxide (DMSO); 3-(4,5- dimethyl-Thiazole-2Yl)-2,5-diphenyl-Tetrazole (MTT), Guanosine-5’[γ-^{32}P] – Triphosphate– Azido anilide (γ-^{32}P-GTP-AA); Sodium Dodecyl Sulfate (SDS). Phosphate Buffered Saline (PBS).

INTRODUCTION

Formoterol, a widely known β₂-sympathomimetic, is commonly marketed as racemic mixture for the treatment of status asthmaticus. It is proven to be a potent and long acting β₂-adrenergic receptor agonist that dilates airway passages by relaxing the tracheal smooth muscle [1,2,3,4,5,6]. The molecule bears two chiral centers thereby producing four enantiomers thereby which only (R,R)- and (S,S)- are shown (Fig. 1). C₁ and C₂ are the two chiral centers. The first chiral center (C₁) is crucial for β₂-adrenergic action (boxed region) and bears similarity to the natural agonist, R-epinephrine. In the recent advent of chiral separation technology, all four individuals have been isolated with high level of purity and one of them is commercially marketed as BROVANA (R, R-Formoterol) for treating asthma with greater success and efficiency [7].

Like other β₂-agonists, R,R- Formoterol binding to β₂-adrenergic receptors increases intracellular cAMP level. These receptors are categorized as hetero-trimeric G-protein coupled receptor (GPCR) family. Binding to the β-agonist activates receptor linked G-protein(s) Gα (subcategory and defined as stimulatory) by uncoupling the α from its βγ subunits. The dissociated Gα associating with GTP activates membrane bound adenyllylcyclase to generate cAMP from intracellular ATP. In the airways, cAMP generation results in the relaxation of the tracheal smooth muscle and subsequent inhibition of inflammatory mediator release from mast cells and eosinophils, thereby, alleviating asthmatic symptoms. Interestingly, additional information showed that in PC3, intracellular cAMP elevation either by adding dibutyryl cAMP or β₂-adrenergic receptor stimulation caused growth retardation by
lowering the DNA synthesis, which may be considered beneficial for treating androgen independent prostate cancer in future [8,9].

Utilizing measurements of intrinsic activity and radioligand ($^{125}$I-Cyanopindolol) binding assays, it was demonstrated that (R,R)-Formoterol exhibited extreme selectivity over (S,S)- toward $\beta_2$-adrenergic receptor [10,11,12]. Moreover, the (R,R)-enantiomer (Fig. 1, the first chiral center C1 having R-configuration) shares an identical spatial and chiral moiety with endogenously occurring natural R-epinephrine and thus subsequently behaving as a $\beta_2$-adrenergic agonist. Conversely, the (S)-configuration (Fig. 1), at C1, bears reverse stereo-chemical arrangement and thus considered to be a neutral entity toward the $\beta_2$-adrenergic receptor. Until recently, like others, the biological activity of the enantomer, (S,S)-formoterol, has escaped evaluation because

![R,R-Formoterol](image1.png)

![S,S-Formoterol](image2.png)

![R-Epinephrine](image3.png)

Fig. 1. The stereochemical configurations of (R,R)- and (S,S)-formoterols along with that of the natural agonist, R-Epinephrine.
it was presumed to be inert. But several current studies establish few different biological activities between (R,R)- and (S,S)- formoterol [12,13,14,]. Therefore, further information are certainly needed to unravel the exact physiological impact of (S,S)- enantiomer. Undeniably, its presence in asthma relieving medication is least desirable. But if properly identified, (S,S)- may be introduced for other medicinal purposes. Our current preliminary study showed that (S,S)-enantiomer also lowers the DNA synthesis in PC3 cells like its (R,R)- counterpart but to a lesser extent. Regarding others, (R,S)- behaves identical to (R,R)- but as recorded in medicinal trial it lacks the long acting role in human system [7]. Presumably, the R- configuration in C2 allosterically helps proper anchoring of the molecule to cell membrane. Presumably, the role of (S,R)- will not be much different from (S,S)- which therefore creates no inertest in the current study.

The dissimilar effect of an enantiomer was also previously reported in case of S – albuterol, that as opposed to R-, S – enantiomer elevates intracellular calcium ([Ca+2]i) by poly-phosphoinositide (IP3) hydrolysis, which allows further calcium influx by nimodipine sensitive L-type channel [15]. Thus the fatal bronchial hyper-responsiveness observed during asthma attack due to frequent use of albuterol is associated with intracellular calcium elevation resulting in subsequent contraction of the tracheal muscle. It was well proven that the actions of both albuterol enantiomers are entirely different [15].

Based on that assumption, the current study was undertaken to characterize few properties of two distinctly opposite enantiomers of formoterol, (R,R)- and (S,S)-, their binding behavior, the G-protein activation and intracellular cAMP elevation in the androgen independent human prostate cancer cell line, PC3, which are replete with β2-adrenergic receptors. Additional studies were also conducted on steroid dependent human prostate cancer cell lines DU 145 and LnCAP which also showed specific (R,R)- Formoterol binding not viewed in case of the (S,S)- analogue.

**EXPERIMENTAL**

**Materials :** (R,R)-formoterol (Lot # 121697A; IN-0360) and (S,S)-formoterol (Lot # QF 780-365 D, IN–02308) were supplied by Sepracor Inc. (Marlboro, MA). The purity of the respective compounds was 99.98 % and 99.99%, respectively. Both compounds were solubilized in DMSO and 1 mM fresh stocks were prepared for use in most experiments. 125I-Cyanopindolol and cAMP radio-immune assay kits were purchased from Amersham Corp. (Chicago, IL). GTPγS (Sigma Chemical Co., St Louis, MO) and [γ 32 P]-GTP-azido anilide (Affinity Labeling Technology Inc. Louisville, KY) were freshly prepared prior to each experiment and used immediately.
Carbachol, propanolol and atropine were obtained from Research Biochemical International (San Diego, CA) and dissolved in buffer prior to use. $^3$H-methyl thymidine was purchased from New England Nuclear (Boston, MA), diluted with 70% ethanol, aliquoted and stored at −70 °C freezer for use in future.

**Methods:** *Cell Culture* — PC3 cells were originally supplied by the American Type Culture Collection (ATCC) and were cultured according to the method reported previously with slight modification [16,17]. Cells were grown in F12K medium supplemented with 1 mM Glutamine, 100U/ml Penicillin-G and 10% fetal calf serum. In all experiments, cells were cultured for approximately two days to reach 70 to 80% confluence before conducting any experiment. The viability of the cells was measured by trypsinization and MTT assay and counted to determine cell number [17]. All experiments were conducted within seven passages of cell culture.

*Plasma Membrane Preparation* — PC3 membranes were prepared from freshly cultured cells according to the previously described method [16]. Cells were washed with 300 mM ice cold sucrose solution buffered with 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM bacitracin, 1 mM o-phenanthroline, 1 mM TLCK, 1 mM TPCK, 1 mM PMSF, 1 mM benzamidine, 0.1 µM leupeptin. Washed cells were scrapped from the dish using 300 mM ice cold sucrose solution and pelleted by centrifugation at 1000 rpm using Danon refrigerated centrifuge (ICN Corp., La Jolla, CA). The pellet consisting of approximately $2 \times 10^8$ cells was homogenized in 50 ml ice-cold buffer by 20 strokes in a Dounce homogenizer (0.1 mm passage) at 400 rpm. The homogenate was centrifuged at 1000 $\times$ g in a Beckman centrifuge armed with a J-21 rotor for 10 minutes at 4°C. The decanted supernatant was further centrifuged at 30,000 $\times$ g for 20 minutes. The pellet was subsequently washed to remove sucrose before use in the experiment. The protein content of the prepared membrane was quantified with the reagent from Biorad (Boston, MA).

*Binding Studies on to Cells* — The binding of $^{125}$I-Cyanopindolol with PC3 cells was performed in a 24 well-cultured plate at 37°C inside a cell culture incubator under aseptic condition. Briefly, the cells were washed twice with 1ml serum free F12K media and were incubated in presence of $10^5$ cpm of radio-ligand with increasing concentrations of non-radio labeled competitor in 1ml volume for about an hour inside a cell culture incubator. In all cases, the binding media was selected as F12K without serum. Approximately, $250 \times 10^3$ cells were used in each well, which was previously detected as the most confluent stage [16]. At the end of incubation, the supernatant was aspirated and the plated cells were washed three times by 1 ml of ice-cold medium followed by dissolution in 1 ml 1M NaOH at room temperature with gentle shaking on an orbital shaker for an hour. Afterwards exact 0.5 ml fluid from each well was counted in a γ counter. Each data point was conducted in quadruplicate and the non-specific binding was assessed as cpm (counts per minute) bound in
presence of 100 µM unlabeled ligand, which was (R,R)- or (R,S)-Formoterol. The specific binding was considered as total minus the non-specific binding. The number of sites per cell was calculated by taking into account approximately 1000 cpm or ~ 2.0 fmol (2.0 × 10^{-15} \text{ mol}) by considering the specific activity of ^{125}\text{I} - Cyanopindolol as tentatively assigned by the supplier (Amersham Corp.).

**Binding To PC3 Plasma Membrane** — Binding to cell membrane was conducted using fresh preparation from cultured cells. Approximately 5 – 7 µg of protein were used in each tube incubated with ~ 10^5 cpm ^{125}\text{I}- cyanopindolol in presence of varying contents of unlabeled ligands in 1.0 ml of 0.05 M Tris-HCl + 5 mM MgCl2, pH – 7.5 buffer with constant orbital shaker at 37ºC. The non-specifics were counted as cpm bound in presence of 100 – 200 µM unlabeled respective ligands and thus subsequently deducted from the bound phase to determine the specific binding. The results were expressed as % specific binding. The reactions were terminated by washing with 10 vol of same ice cold buffer filtering three times over GF-B glass filter supplied by Whatman (VWR, Boston) using 32 channel Brandell cell harvester (Brandell, Gaithersburg, MD) as previously described [16]. The filters were auto punched and later counted in a 16-channel γ counter (Beckman, CA). Each point is in triplicate and each plot is a combination of three experiments (N = 3). All data were processed extensively by using softwares.

**Binding Data Analysis** — The competition binding data were critically analyzed by following the equation (1), the Cheng and Prusoff’s equation [18].

\[
K_i = \frac{IC_{50}}{1 + \frac{[I]}{K_D}}
\]  

(1)

\(K_i\) – inhibitory constant, \([I]\) – inhibitor concentration, \(K_D\) – affinity or dissociation constant of the inhibitory ligand and IC_{50} - inhibitor concentration at 50 % suppression during radio-ligand binding. The number of binding sites (B max) was calculated according to Scatchard’s analysis using the following hyperbolic function (implanted within software) [19,20].

\[
[L_R] = \frac{B_{max}}{K_D + [L]} 
\]  

(2)

where \([LR]\) is the receptor bound ligand concentration and \([L]\) is the concentration of the free ligand.

It is then converted to linear form or more generalized Scatchard’s equation

\[
\frac{[LR]}{[L]} = \frac{B_{max}}{K_D} - \frac{[LR]}{K_D}
\]  

(3)

Thus plotting \([LR]/[L]\) against \([LR]\) will provide \(K_D\) from the slope and \(B_{max}\) from the X- axis intercept. I also used multiple binding sites analysis, mostly two sites, which is a modification of the above equation.

\[
[LR]_{total} = \frac{B_{max1}}{K_{D1} + [L]} + \frac{B_{max2}}{K_{D2} + [L]}
\]  

(4)
All analyses was conducted by using the same software Prizm – 5 version from GRAPHPAD (Sandiego, CA).

**cAMP Measurement** — The exact quantity of PC3 cells were plated in 24 well plates to approximately 250,000 cells / well. After attaining ~ 70 to 80 % confluence, the cultured cells were washed once with 37 ºC pre-warmed serum free F12K medium at 1 ml/ well, incubated further with the same F12K medium and maintained at 37ºC for 30 minutes. Test compounds were prepared in F12K (serum free) and added at 100 x concentration. In general, reactions were allowed to proceed for 20 minutes at 37ºC and were terminated by the addition of 1 ml ice cold 10% TCA. During kinetic studies, reactions were conducted for a desired period of times ranging from 5 to 90 minutes. cAMP generation was assayed by radio-immune assay using the kit from Amersham [9,21]. Unless mentioned, in most reactions, the use of IBMX, a phospho-diesterase inhibitor, was intentionally avoided. All measurements were performed in quadruplicate and each experiment was conducted at least three times. The final results were expressed as average ± SEM with simultaneous application of ANOVA.

**32P-GTP-AA Binding To PC3 Plasma Membrane** — 32P-GTP-AA incorporation was conducted by slight modification of the previous protocol [22]. PC3 cell membranes prepared as above were washed twice with Tris-saline buffer, pH 7.5 (100 mM NaCl + 20 mM KCl + 20 mM Tris-HCl) supplemented with 10 mM MgCl2. Membranes (5 μg) were suspended in 1ml of Tris-saline buffer and incubated at 37ºC for 30 minutes. The incubation was continued for another 30 minutes following addition of the formoterol enantiomers. 32P-GTP- AA reagent was then added to a final concentration of 0.2 nM and kept in the dark at room temperature for 10 minutes with constant shaking. The membrane was then washed twice with ice-cold buffer and microfuged at 4ºC for 5 minutes. Finally, the pellet was dissolved in 1ml of 1M NaOH + 2% SDS followed by mixing with 10 ml scintillation fluid and counted in a scintillation counter to determine the incorporation of 32P-GTP- AA. Specific incorporation was determined by incubating a separate group of membranes with freshly prepared 100 μM GTPγS in the presence of 32P-GTP- AA.

**3H- Thymidine Incorporation in DNA** — The adopted experimental protocol was a modification of previously published report [16]. Approximately 5,000 cells were plated in each well of a 24 well plate in F12K medium supplemented with 10 % fetal calf serum (FCS) for 48 hours. The medium was then aspirated and cells were washed twice with serum free F12K and allowed to starve overnight in the same FCS free medium condition. The drugs were added in different doses along with fixed amount of 1μCi 3H- thymidine (15 – 20 Ci / mol, H3- methyl thymidine, New England Nuclear, Boston, MA) and further incubated overnight in exact 1.0 ml volume using the same medium at 37ºC in a cell culture incubator. In the end, cells
were washed with ice-cold isotonic PBS, pH – 7.5 by aspiration and then washed with ice-cold 10 % TCA followed by ice-cold PBS again. After removing PBS, the cells were left exposed to 10 % TCA for 1.0 hour at 4ºC and then washed thrice again with ice-cold PBS and later dissolved in 0.3 M NaOH +1.0 % SDS in exact 1.0 ml by keeping over slow orbital shaker at room temperature for at least 3 hours. 250 µl of aliquots from each well were then counted in a Beckman scintillation counter after mixing well with 10 ml of scintillation fluid in screw capped glass scintillation vials. In all cases each concentration was studied in quadruplicate and the average counts per minute (cpm) ± SEM were converted to % incorporation ± SEM and then plotted against increasing dose of drug concentrations.

Data Analysis : The software, Prizm-5 or its INPLOT version from Graphpad Inc (Sandiego, CA) was used for conducting all the statistical analyses (± SEM), ANOVA and other binding parameters (K_d) including the EC_{50} values [22].

RESULTS AND DISCUSSION

Separation of enantiomers enabled the confirmation that the (R,R)-enantiomer is a specific β_2-adrenergic receptor agonist while the enantiomer, (S,S)-Formoterol is inactive due to stereo-chemical differences [3]. However, the fatal hyper-responsiveness observed among the frequent anti-asthmatic drug users raises a serious concern regarding their effect in the racemic mixture [23]. Recently, we established that S-albuterol acts differently in comparison to the R-analogue which is widely known as a potent β_2-adrenergic receptor agonist [15]. In isolated bovine tracheal cells, R-albuterol showed high affinity binding and elevated intracellular cAMP, which produces relaxation of the tracheal smooth muscle causing asthma relief. On the contrary, S-albuterol elevated intracellular [Ca^{+2}]) by activating PLC-β thereby generating IP_3 and caused further influx of Ca^{+2} into the cell via nimodipine sensitive L-type channel. The huge rise of intracellular Ca^{+2} produced contraction and shortening of the tracheal cells [15]. Therefore, the hyper-responsiveness observed during the use of previously marketed (racemic) albuterol could be attributed due to the presence of S-enantiomer. It is thus inferred that the physiologic effect of an enantiomer could be very different other than being inert.

In the present study, I indicate several pharmacological differences between (R,R)- and (S,S)-formoterol in the action of these on PC3 cells. Comparable to tracheal smooth muscle, PC3 expresses both β_2-adrenergic and muscarinic (most subclasses) receptors in large quantities [24,25]. Using the non-specific β-adreno-receptor ligand 125I-Cyanopindolol the high affinity binding of (R,R)- and (R,S)-Formoterol (K_d ~ 30 pmol/L) was recorded, as opposed to its (S,S)- counterpart (K_d >10 µmol/L). The binding of (R,R)- and (R,S)- is sensitive to GTPγS treatment
Formoterol Binding and cAMP Elevation in PC3 Cells

Binding Data of Intact Cells and Plasma Membrane — Analysis of the competition binding data of $^{125}$I-cyanopindolol in presence of increasing concentration of (R,R)-Formoterol (Table 1, Fig. 2) showed significantly high affinity binding ($K_d = 24.1 \pm 0.7$ pmol/L, $N = 4$ observations, $p = 0.002$, $\pm$ SEM) with intact PC3 cells and plasma membrane ($K_d = 50.0 \pm 5.2$ pmol/L, $N = 3$, $\pm$ SEM), in close agreement with others using iso-proterenol [23]. The binding of (R,S)-formoterol showed comparable affinity ($K_d = 32.4 \pm 1.1$ pmol/L, $N = 4$ observations, $p = 0.001$, $\pm$ SEM). On the contrary, no significant binding was noticed at any reasonable doses in the case of the (S,S)-enantiomer except at extremely high concentrations ($10^{-100}$ μM) when only 5 – 10% suppression in binding was observed, which certainly was not accounted as receptor specific.

As per further interest, other human prostate cancer cell lines, androgen dependent LnCAP and gluco-corticoid dependent DU145 also showed specific as well as significant binding affinity (~ 2.25 and ~2.40 nmol/L, respectively) with considerable capacity (~ 9–12 × 10$^4$ sites / cell) toward (R,R)-Formoterol. Undoubtedly, among them PC3 expressed the highest level (~ 24 × 10$^4$ sites / cell). Analysis by computer assisted dual sites model indicated that 40 % of them are in high affinity ($K_{d1} \sim 30$ pmol/L, whereas the rests (60 %) belong to the low affinity

![Fig. 2. The competition binding result of Formoterol enantiomers with $^{125}$I-cyanopindolol at 37°C. [Data analyzed and plotted within 95% confidence level using the software, Prizm – 5].](image-url)
state, $K_{d2} \sim 200$ nmol/L (Table 1).

Table 2 shows the sensitivity of (R,R)- and (R,S)- Formoterol binding to GTP$_{i/S}$ treatment. The significant alteration in affinity and capacity was noticed with prior treatment of GTP$_{i/S}$ specially, to the high affinity sites. It is known that the binding of non-hydrolyzable GTP$_{i/S}$ to hetero-trimeric G-protein associated to cytosolic domain of the receptor allows dissociating away G$_{\alpha}$ subunit, which subsequently

**TABLE 1.**

The binding parameters of $^{125}$I-cyanopindolol and (R,R)-Formoterol in human prostate cancer cell lines at 37 °C, PC3, LnCAP and DU145 calculated and analyzed within 95% confidence [N = 3 experiments ± SEM; p ~ 0.001, ANOVA] following two sites model by using Prizm 5.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Site -1 ($K_{d1}$)</th>
<th>Site - 2 ($K_{d2}$)</th>
<th>% of Site -1*</th>
<th>% of Site - 2*</th>
<th>Total Number of Sites/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>0.025 ± 0.007</td>
<td>200.0 ± 8.0</td>
<td>40.0</td>
<td>60.0</td>
<td>24.0 ± 5.2 × 10$^4$</td>
</tr>
<tr>
<td>LnCAP</td>
<td>2.250 ± 0.800</td>
<td>1800 ± 102</td>
<td>30.0</td>
<td>70.0</td>
<td>12.0 ± 1.3 × 10$^4$</td>
</tr>
<tr>
<td>DU145</td>
<td>2.400 ± 0.900</td>
<td>2200 ± 175</td>
<td>29.0</td>
<td>71.0</td>
<td>9.0 ± 1.7 × 10$^4$</td>
</tr>
</tbody>
</table>

*The data in column 4 and 5 are of 95% confidence

**TABLE 2.**

Competition binding between $^{125}$I-cyanopindolol and (R,R)- or (R,S)- Formoterol using PC3 plasma membrane in presence and absence of 100 µM GTP$_{i/S}$ at 37°C. The (S,S)-enantiomer showed no significant specific binding. Over 100 µM concentration ~ 10% displacement was observed which was considered as non-specific.

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>Condition</th>
<th>$K_d$ (pmol/L)</th>
<th>$B_{Max}$ (fmol/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R,R-) Formoterol</td>
<td>No GTP$_{i/S}$</td>
<td>$K_{d1} = 50.0 \pm 5.2$</td>
<td>$B_{Max 1} = 1.5 \pm 0.12$</td>
</tr>
<tr>
<td></td>
<td>+100 µM GTP$_{i/S}$</td>
<td>$K_{d1} = 162 \pm 15$</td>
<td>$B_{Max 1} = 0.81 \pm 0.10$</td>
</tr>
<tr>
<td>(R,S-) Formoterol</td>
<td>No GTP$_{i/S}$</td>
<td>$K_{d1} = 59.0 \pm 2.9$</td>
<td>$B_{Max 1} = 1.6 \pm 0.06$</td>
</tr>
<tr>
<td></td>
<td>+100 µM GTP$_{i/S}$</td>
<td>$K_{d1} = 287 \pm 77$</td>
<td>$B_{Max 2} = 2.2 \pm 0.24$</td>
</tr>
</tbody>
</table>

*The data in column 4 and 5 are of 95% confidence
lowers the ligand binding affinity, the event not being expected in intact viable cells. So expectedly, the results without GTPγS treatment were nearly as similar to that seen in case of intact PC3 cells indicating receptor’s viability and attachment to the G-protein(s).

The data confirms the specific high affinity β₂-adrenergic receptors on the prostate cancer cell lines and the order of affinity (Kₐ) and binding capacity follow the order, PC3 > LnCAP > DU145. Sincerely, the R- configuration at C1 (Fig. 1) is proven to be necessary as seen previously in case of R-albuterol interaction with bovine trachea [15].

Dose-dependent cAMP Elevation, Kinetics and Effect of Propanolol — Fig. 3 shows both enantiomers of formoterol elevated cAMP level in intact PC3 cells in a dose-dependent manner when studied within 20 minutes period of time. Maximal cAMP generation was attained at ~ 10⁻⁸ M by both (R,R)- and (R,S)- Formoterols, while for (S,S)- the maximum response occurred at ~ 10⁻⁶ M, at less magnitude. Interestingly, the EC₅₀ value for cAMP generating ability is in close proximity to high affinity binding of the receptor (Kₐ ~ 30 pmol/L). The calculated EC₅₀ values, (R,R)- = 10.5 ± 0.9 pM, (R,S)- = 11.0 ± 1.4 pM and (S,S)- = 1000 ± 15 pM, (p < 0.003, N = 4, ± SEM) indicate that the (R,R)- is ~100-fold more efficacious
than the (S,S)- distomer. Additionally, the maximum magnitude of cAMP generation at plateau level (~ 250 pmol / well for (R,R)- and for (S,S)- ~ 100 pmol / well) is also significantly different (~ 2.5 fold), proving further effectiveness of (R,R)-

The phospho-diesterase inhibitor (IBMX) was avoided due to possible interference (~ 5 – 7% difference in cAMP level observed in presence and absence of either 10 μM IBMX or theophylline when incubated for 10 minutes at 37°C prior to the agonist addition).

Fig. 4 demonstrates that cAMP generation by 1.0 μM (R,R)- and (S,S)-formoterols reached a constant level within ~ 30 minutes and remained almost constant for a long period of time (90 minutes). The level (~250 pmol / well) was ~ 2.5 times higher for (R,R)- than the (S,S)- (~100 pmol / well) counterpart but nearly the same (~300 pmol / well) in the case of (R,S)-. No significant deterioration (less than 10%) was observed despite the absence of a phosphodiesterase inhibitor in the incubating medium. The initial phase of cAMP generation rate (25.0 ± 1.2 pmol / min ± SEM) of (R,R)- is also ~ 3 fold higher than (S,S)-formoterol (8.0 ± 0.3 pmol / min ± SEM ) but identical (24.9 ± 0.4 pmol / min ± SEM ) to the (R,S)- analogue (Fig. 4).

In addition to the above differences, several other disparities on qualitative aspects were also witnessed. Supplementary experiments (Fig. 5) showed that prior addition of non-selective β-adrenergic receptor antagonist, propanolol (1.0 μM) blocked the cAMP generation (90 %; N = 3, p < 0.005) by (R,R)- (1.0 μM) but

![Fig. 4. The time dependent cAMP generation by (1 μM) (R,R)-, (R,S)- and (S,S)- Formoterols in PC3 cells at 37°C in absence of IBMX [N = 3 experiments].](image-url)
Formoterol Binding and cAMP Elevation in PC3 Cells

failed (N = 3, p > 0.05) in the case of (S,S)-(1.0 μM), which certainly indicates that (S,S)- does not belong to the category of β-adreno-receptor agonist and its action may propagate in a different way.

Based on these facts one can infer that the β-adrenergic action is primarily located at the R- configuration of the first chiral center (C1) of the molecule since both R,R – and R,S- enantiomers exhibits almost identical pharmacological behavior (Fig. 1). The role of the other chiral center, C2 is not well understood. Few unpublished data suggest that R – conformation at C2 may help anchoring the molecule on proper orientation enabling for long lasting effect.

**Agonist Stimulation of [γ^{32}P] GTP-AA Binding to PC3 Cell Membrane —** Assessment of a G-protein’s involvement during agonist stimulation was determined by evaluating the binding of [γ^{32}P]-GTP-AA (non-hydrolyzable analogue of GTP) to PC3 cell membranes. As seen in Fig. 6, the presence of (R,R)- or (S,S)-formoterol (1 μM) stimulated specific incorporation of [γ^{32}P]GTP-AA into PC3 membranes that was inhibited (> 90%) by pre-treatment with 100 μM GTPγS. A discernible dissimilarity was further observed in the presence of β-adrenergic receptor blocker, propranolol (1 μM). Propranolol enabled to prevent the incorporation by (R,R)-formoterol (> 60%) but failed completely in preventing (S,S)-formoterol stimulation of [γ^{32}P]-GTP-AA incorporation.

The data in Fig. 6 represents an average of N = 4 experiments. 100 μM
GTPγS blocked (80 %, p < 0.001, N= 4) incorporation by both the enantiomers. The incorporation by (R,R)- is inhibited (70 %) by 1.0 μM propanolol (pro) (p < 0.001, N = 4). No inhibition is observed in the case of (S,S)-Formoterol. The incorporation by propanolol alone as a control was insignificant (p > 0.05) compared to the basal incorporation level. ~ 20% increment by propanolol is noticed in the presence of the (S,S)- enantiomer (Analyzed by ANOVA).

The specific incorporation of [γ^32P]-GTP-AA in PC3 cell membrane indicated that both (R,R)- and (S,S)- formoterol can activate membrane linked G protein(s) (Fig. 6). The incorporation is agonist specific because in both the cases, 100 μM GTPγS completely blocked the incorporation. But the disability of propanolol to block the incorporation by (SS)- is keeping in line with the conjecture for its non-inclusion to the β-adreno-receptor agonist family. Currently, it is beyond the scope to identify the exact link between G-protein activation and cAMP elevation by the distomer. But for certainty it is inferred that action of (S,S)- follow a different pathway, which may be a focus of future investigation.

Carbachol Potentiation of cAMP Generation — Previous demonstration in PC3 cells showed that co-stimulation of β2-adrenergic receptor by isoproterenol along with Gq linked receptor agonist results in the synergistic production of cAMP [9,21]. Since PC3 expresses muscarinic receptors that are linked to Gq carbachol stimulation creates synergism in the cAMP production by (R,R)- Formoterol, as shown in Fig. 7 [24].
The results indicate that the presence of carbachol (1 μM), potentiates cAMP production ~ 2 – 3 fold along with (R,R)- (0.1 and 1.0 μM) as compared to (R,R)- alone. In each case the potentiation by carbachol is almost 2-3 fold at all doses of (R,R)- or (R,S)-Formoterol (N = 7, p < 0.001 – 0.005, by INPLOT, ANOVA). The incident was also observed placing (R,S)- instead of (R,R)-.

The occurrence of synergistic cAMP production was recorded earlier in the case co-stimulation of cells expressing both Gs and Gq / Gi linked receptors [9,21]. So far two major pathways were identified which include activation of the protein, kinase C (PKC) or the involvement of βγ subunits of G-protein [9]. In some cells, PKC activation by phorbol ester produces synergism in cAMP production in response to β-adrenergic stimulation [25]. Since, PC3 Cell also expresses muscarinic receptors therefore carbachol is able to activate membrane associated Gq or Gi [24]. The Gq activation can generate di-acyl-glycerol and subsequent calcium elevation [Ca^{2+}], which in turn can activate PKC [26]. Similar potentiation is also possible by the Gi activation and the simultaneous catalytic effect of Gβγ subunits [27]. The nature of adenylyl cyclase(s) present in PC3 Cells is also one of the decisive factors behind the synergism [21,28]. Our unpublished data showed that besides muscarinic receptor activation by carbachol, activation of neuropeptide (neuropeptide) receptor that are linked to either Gi or Gq produced similar dose-dependent potentiation in PC3 Cells.

Fig. 7. Carbachol (1 μM) potentiation of cAMP production by 10 nM (R,R)- unobserved for (S,S)-Formoterol in PC3 cells at 37°C.
when added in the presence of (R,R)-formoterol [29]. Previously, we reported that co-stimulation by Neurotensin, a hypothalamus derived neuropeptide and Isoproteronol causes synergistic cAMP production in PC3 Cells [9]. The effect was unobserved when the (S,S)- enantiomer was added in place of the (R,R)- along with the neurotensin. The potentiation phenomenon provides further distinctive support for the (S,S)- Formoterol not being a β₂ adreno-receptor agonist.

**Effect on H³-Thymidine Incorporation** — The concentration dependent lowering of H³-Thymidine incorporation in DNA was observed for all the three enantiomers (Fig. 8). The efficacy was similar for (R,R)- and (R,S)-Formoterols (EC₅₀ ~ 1.0 nM) whereas for (S,S)-, the value is ~ 100 nM. But in all the cases a long plateau is observed starting at 0.01 – 10 nM, which is unexplainable at this moment. Within that range the value remains constant. In case of (S,S)- only ~ 20 % suppression was noticeable whereas for others the value is double, ~ 40 %. This is the most unique feature displayed by the (S,S)- enantiomer unlike its counterpart (R,R)- or (R,S)- regarding its ability to lower DNA synthesis in PC3 cells regardless of their other differences (Fig. 8). This lowering could be attributed due to intracellular cAMP elevation in PC3 cells [9].

![Fig. 8. Dose-dependent inhibition of H³-Thymidine incorporation in PC3 DNA by (R,R)-, (R,S)- and (S,S)- Formoterols at 37°C. Expressed as % inhibition ± SEM [N = 4 experiments].](image-url)
CONCLUSION

In brief, the similarities observed between (R,R)- and (S,S)- were #1) elevation of intracellular cAMP, #2) specific \([\gamma^{32}P]\)-GTP-AA incorporation and #3) lowering of DNA synthesis whereas the observable differences were #1) Lack of binding to \(\beta\) adrenergic receptor, #2) severe disparity (~ 100 fold) between the EC_{50} values and the level of cAMP production, #3) discriminatory role of propanolol in preventing (R,R)- but not (S,S)-Formoterol during cAMP generation, #4) synergistic production of cAMP by carbachol in the presence of (R,R)- but not in the presence of (S,S)-. The events lead to hypothesize that despite brief similarities, the interacting pathways of two enantiomers are totally different.

ACKNOWLEDGEMENT

The author is deeply indebted to the Department of Surgery / Division of Urology, University of Massachusetts Medical Center for providing the facility for the work and the office space during my tenure. Special thanks are offered to Dr Robert Blute Jr., the division chief of Urology for supporting the prostate cancer research program. He also extends his gratitude to Dr. Yael Schwartz of Sepracor Inc. (Marlboro, MA) for providing the Formoterols.

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