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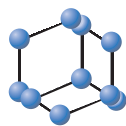


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Identification of Small Molecule as a High Affinity β_2 Agonist Promiscuously Targeting Wild and Mutated (Thr164Ile) β_2 Adrenergic Receptor in the Treatment of Bronchial Asthma



Srinivas Bandaru^{1,2+}, Mallika Alvala²⁺, Jyothy Akka¹, Someshwar Rao Sagurthi⁴, Anuraj Nayarisseri^{3,5}, Sanjeev Kumar Singh*⁵ and Hema Prasad Mundluru¹

¹Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad - 500 016, India; ²National Institute of Pharmaceutical Education and Research, Hyderabad - 500 037, India; ³In Silico Research Laboratory, Eminent Biosciences, Indore - 452 010, India; ⁴Department of Genetics, Osmania University, Hyderabad- 500 007, India; ⁵Computer Aided Drug Designing and Molecular Modeling Lab, Department of Bioinformatics, Alagappa University, Karaikudi-630 003, Tamil Nadu, India

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Abstract: Background: A subset of asthmatics shows refractoriness to Salbutamol owing to ADRB2 gene C→T polymorphism (rs 1800888) that substitutes Thr to Ile at the position 164 in the β_2 adrenergic receptor leading to sub-optimal binding of Salbutamol. The present study aims to associate the Salbutamol (200 mcg) refractoriness with the polymorphism and select the best existing agonist with optimal binding affinity against wild and mutated receptor and further identify high affinity compound, irrespectively targeting wild and mutated receptor through virtual screening methods. **Methods:** Responders to Salbutamol were categorized, if percentage reversibility was greater than or equal to 12% in them, while those showing reversibility less than 12% were non-responders. The genotyping for polymorphism was performed by ARMS PCR method. Established agonists with consistent binding affinity against wild and mutated receptors formed query compound to identify high affinity molecule from Phase database through 7 point pharmacophore based screening. **Results:** Polymorphism was significantly associated with non-responders ($p < 0.05$) demonstrating it as a major factor of Salbutamol refractoriness. Results from Glide Docking showed that Fenoterol had highest affinity for mutated receptor and stood as second best (after Salbutamol) high affinity agonist for wild receptor among the established β_2 agonists. Therefore Fenoterol formed a query molecule (7 point pharmacophore) in identification of high affinity compound for virtual screening process. **Conclusion:** Compound CACPD2011a-0001278239 identified through virtual screening against 4 million compounds in phase database was shown to irrespectively target both wild and mutated β_2 adrenergic receptor with high and consistent affinity which was par greater than established β_2 agonists.



Sanjeev Kumar Singh

Keywords: ADRB2 polymorphism, β_2 AR receptor mutation, salbutamol, fenoterol, 7 point pharmacophore modeling, virtual screening.

1. INTRODUCTION

Asthma is a complex inflammatory disorder that currently affects about 300 million people worldwide and approximately 250,000 people die of asthma annually [1]. According to the cross sectional National Family Health Survey (NFHS)-3, (IIPS and Macro International, 2007), 25-30 million people have been positively diagnosed for asthma in India, and around 2 out of every 100 individuals have asthma in South Indian states [2].

There has been substantial increase in the treatment options in the management of asthma, of which β_2 agonists hold prime importance. Inhaled β_2 agonists have been established as first line treatment for the management of acute asthma because of their excellent bronchodilator effects, low level of side effects and wide therapeutic range [3, 4]. β_2 -agonists form an effective and well tolerated bronchodilator in patients with asthma [5-7]. International guidelines on asthma management (National Heart, Lung, and

Blood Institute National Asthma Education and Prevention Program, 2007) [8, 9] currently recommends rapid-onset inhaled β_2 -AR agonists such as salbutamol, terbutaline, or formoterol alone for symptom relief and for the pretreatment of exercise-induced bronchoconstriction in all patients with asthma [10-13].

The use of beta 2 agonists dates back for more than 2000 years wherein alkaloids from 'ma huang' plant (*Ephedra equisetina*) were administered for symptomatic treatment of asthma. The alkaloid, later identified as ephedrine, was regularly used for the short-term treatment of respiratory symptoms. Although a powerful bronchodilator, in the later years, ephedrine was not considered ideal bronchodilator as it had slow onset of action and had considerably lower potency, in addition to regular use showed tolerance in patients. At the beginning of 19th century, epinephrine was introduced into clinical practice for the treatment of acute asthma [14]. Although highly efficacious, epinephrine was far from the perfect bronchodilator, because in addition to the desired therapeutic effect of bronchodilation, it caused hypertension and tachycardia, and was nonselective for α -AR's and β -AR's that had its effects on cardiovascular system. Moreover, because of high metabolic instability, it had a short duration of action and had to be given parenterally [15]. In the early 1940s, isoproterenol - a nonselective β -AR agonist was

*Address correspondence to this author at the Computer Aided Drug Designing and Molecular Modeling Lab, Department of Bioinformatics, Alagappa University, Karaikudi-630 003, Tamil Nadu, India;

E-mail: skysanjeev@gmail.com

[†]Authors contributed equally.

introduced for the airway diseases and became the standard-of-care bronchodilator, however its use was complicated and regular doses showed adverse effects which actually were due to activation of the β_1 -AR in the heart [15], which elicits tachycardia and predisposes patients to cardiac dysrhythmias. Considering the adverse effects caused by non-selective bronchodilators, the modern era of selective β_2 -AR agonists came up with the discovery of Salbutamol (called albuterol in United States) by Sir David Jack and colleagues at Allen and Hanburys (now part of GlaxoSmithKline) [16]. Salbutamol has negligible α -AR activity at recommended clinical doses and demonstrates a substantial greater selectivity between β_2 - and β_1 -ARs than any other product previously available [17]. The discovery of Salbutamol was such a breakthrough that it revolutionized asthma treatment and management within months of appearing in the market. Since its discovery past 52 years, Salbutamol still remains the first line of treatment for bronchoconstriction treatment globally [18].

Bronchodilators like Salbutamol show its action by binding to β_2 AR which is a GPCR (G Protein Coupled receptor) protein. Persistent activation of a β_2 AR is achieved through the binding of both an agonist and a G protein at opposite ends of the receptors, relative to the lipid bilayer [19]. β_2 AR are coupled to G_s , where stimulation by a β_2 -AR agonist activates adenylyl cyclase and increases cAMP levels. cAMP increases protein kinase A (PKA) activity, which phosphorylates downstream protein modulators [20]. The overall activation of this signal transduction pathway can lead to the inhibition of phosphoinositol hydrolysis, a fall in intracellular Ca^{2+} levels, and the activation of large conductance K^+ channels. The hyperpolarization of airway smoothens the muscle and as a result of opening the K^+ channels, it can lead to the relaxation of airway smooth muscle [21, 22].

Asthma is one of the most common respiratory diseases with multi-factorial origin, wherein both environmental risk factors and genetics play confounding factors in the disease etiology and severity [23-25]. While several environmental risk factors have been identified, several genes have also been shown to be associated with pathological modulation in asthma. Of particular relevance, ADRB2 surfaced as an important gene in asthma pathophysiology. ADRB2 gene codes for Beta 2 adrenergic receptor (β_2 AR), which is expressed by airway smooth muscle cells, is known to interact with hormones and small molecule compounds which brings about bronchodilation [26].

Unfortunately, a subset of patients shows sub-optimal response to β_2 agonists owing to polymorphisms within the ADRB2 gene located on chromosome 5q31. In studies utilizing site-directed mutagenesis and recombinant expression, the amino acid substitutions in the receptor have been found to have a significant impact on the functioning of the receptor leading to pathological outcomes [27]. Of particular importance the C>T base exchange at the 491 position in ADRB2 gene results in the replacement of amino acid Threonine with Isoleucine at 164th position in the β_2 adrenergic receptor (β_2 AR). Structural studies revealed that the mutation occurs near to the agonist binding site at the fourth transmembrane spanning domain of the receptor (Fig. 1) [28]. Since the mutation occurs close to active site, the impact of mutation has its functional consequence like decreased ligand-receptor interactions and depressed coupling of β_2 AR to adenylyl cyclase. The mutation resulting in an impact on drug binding, has its effects on the dose-response curve, where the curve shifts to the right indicating the low affinity of the drug to the receptor thereby diminishing the desired effect [28]. In addition, Ile-164 receptor showed an approximately three-fold decreased affinity for β_2 agonists like Isoproterenol, Epinephrine, and Norepinephrine [28]. Previous studies suggests that the conformation of the receptor is altered such that the spontaneous toggling to the activated state and agonist stabilized activation is significantly decreased in the Ile-164 receptor. One interpretation is that the Ile substitution at residue 164 has perturbed the role of Ser

165 resulting in both its inability to interact with the β hydroxyl group of β_2 agonists and maintaining the proper receptor conformation [29].

Considering the crucial role of Thr164Ile polymorphism in altering drug-receptor interactions, we speculate that this particular polymorphism may affect the response to β_2 agonists in asthmatics. Therefore, we sought to identify the possible association of the polymorphism with response to Salbutamol - a high efficacy β_2 agonist in asthmatics and to select the best existing short acting β_2 agonist with optimal binding affinity against wild and mutated (Thr164Ile) receptor and further identify a small molecule which irrespectively targets both wild and mutated (Thr14Ile) β_2 adrenergic receptor with high affinity, through virtual screening approaches.

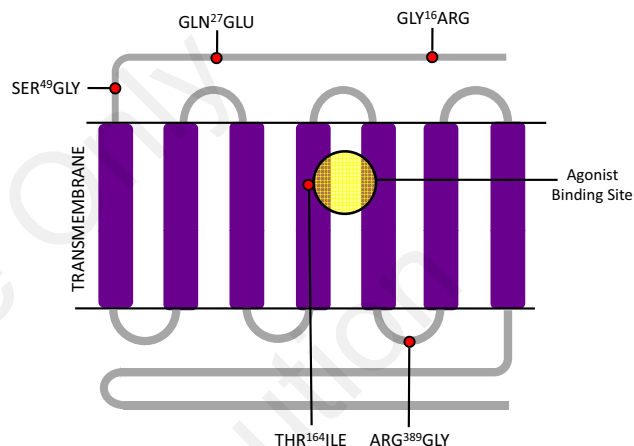


Fig. (1). Localization of non-synonymous polymorphisms in human β_2 adrenergic receptor. Thr164Ile polymorphism which lies close to the agonist binding has its functional consequence in decreased ligand-receptor interactions and depressed coupling of β_2 AR to adenylyl cyclase. (Figure adapted with permission from Bandaru et al., *Gene*. 2016; 592(1): 15-22).

2. MATERIALS AND METHODS

2.1. Study Subjects

398 patients diagnosed by physician, were recruited from Government General and Chest Hospital (Gandhi Medical College and Osmania Medical College, Hyderabad, India). No patient had evidence of chronic obstructive pulmonary disease, bronchiectasis or any other lung disorder. Only adults, 20 years and above, were recruited in the study. Since children do not cooperate well with pulmonary function test, which may lead to false positive results in lung function variables, they were excluded from the present study. Epidemiological details such as age, gender, age of onset, addictions, inhabitation and allergic information were taken through a personal interview with the help of a standard questionnaire. The study protocol was approved by the ethical committee at our institution. An informed and voluntary consent was obtained from all the subjects selected for the study.

2.2. Assessment of Salbutamol Response

To assess the bronchodilator response, inhalation of the 200 μ g of Salbutamol (Asthalin, Cipla[®]) was administered using a metered dose inhaler and a spacer. Spirometry (Viasys Healthcare, Micro-medical Limited, Kent, England) was performed before and (15 min) after Salbutamol dose, according to the American Thoracic Society Standards [30]. Pulmonary function test results were expressed as a percentage of the predicted normal value using Hankinson's age-adjusted and race adjusted prediction equation [31]. The percentage reversibility change in the Forced Expiratory Volume (FEV_1) was calculated by Knudson equation [32] as given below:

%Reversibility =

$$\frac{\text{Max. FEV}_1 \text{ Post bronchodilator} - \text{Max. FEV}_1 \text{ Pre bronchodilator} \times 100}{\text{Max. FEV}_1 \text{ Pre bronchodilator}}$$

Responders to Salbutamol were those patients, who demonstrated percentage reversibility greater than or equal to 12%, while, patients demonstrating reversibility less than 12% were categorized as non-responders [33].

2.3 Genotyping of ADRB2 Thr164Ile C 491 T (rs 1800888) Polymorphism

Genomic DNA was isolated from whole blood samples from all the patients by the salting out procedure [34]. The genotype for ADRB2 Thr164Ile (C 491 T substitution rs 1800888) was determined by means of amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method. Briefly, two complementary reactions were established for each allele consisting of target DNA. A 662 bp region in the ADRB2 gene was targeted for amplification. The primers used were as follows: Antisense (Common Forward) 5' CACAGCAGTTTATTTCTTT 3' Sense (Reverse - C allele specific) 5' TGGATTGTGTCAGGCCTTAC 3' Sense (Reverse - T allele specific) 5'TGGATTGTGTCAGGCCTTAT 3'. The optimized reaction mixture of 20 μ l contained 100 ng of genomic DNA, 1 $\times$ reaction buffer, 1.5 μ M MgCl₂, 30 μ M of each dNTP, 0.16 μ M of each primer, and 0.3 U of Taq DNA polymerase. The PCR amplification reaction consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30s, 61°C for 20s, and 72°C for 30s. The final extension step was at 72°C for 6 min. The PCR products were electrophoresed on 2 % agarose gel stained with ethidium bromide and were visualized under ultraviolet light with a 100-bp ladder (Fig. 2).

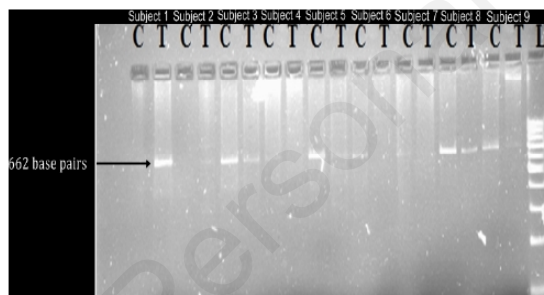


Fig. (2). A 662 -bp region in exon 1 of the ADRB 2 gene amplified by Allele Specific PCR. Two adjacent lanes are specified for genotyping for each subject. Amplified bands in the lane marked with only “C” among the two lanes specified for subject represents CC (Thr/Thr) genotype of subject. Likewise, amplified bands in the lane marked with only “T” among the two lanes specified for subject represents TT (Ile/Ile) genotype of subject. While Bands in the both C and T lanes represent subject is heterozygous with CT (Thr/Ile) genotype. “L” is 100 base pair ladder. (Figure adapted with permission from Bandaru *et al.*, *Gene*. 2016; 592(1): 15-22).

2.4. Selection of Short Acting $\beta 2$ Agonists (SABAs)

Ten established short acting $\beta 2$ agonists (SABAs) viz. Fenoterol, Ritodrine, Procaterol, Terbutaline, Salbutamol, Isoetharine, Pirbuterol, Metaproterenol, Isoprenaline and Bitolterol were selected for present study in attempt to find the best short acting $\beta 2$ agonist against wild and mutated receptor (Thr164Ile) $\beta 2$ AR.

2.5. Structure Retrieval, Optimization and Protein Preparation

The three dimensional structure of all the ten short acting $\beta 2$ agonists were retrieved from NCBI's PubChem database. All structures were optimized and cleaned in three dimensional format using MARVIN VIEW [35]. The three-dimensional structure of

$\beta 2$ AR was retrieved from Protein Data Bank [PDB: 3NY8] [36]. Prior docking of compounds, the protein was optimized and prepared by removing all bound crystal water molecules and adding hydrogen bonds. Explicit hydrogen were created, bond orders, hybridizations and charges were assigned wherever missing. The resulting structure was energy minimized using OPLS-2005 force field by protein preparation wizards of Schrödinger suite -2013.

2.6. Analysis of Mutation in $\beta 2$ Adrenergic Receptor Structure

The structure of wild $\beta 2$ adrenergic receptor was mutated by substituting amino acid isoleucine in place of threonine at 164th position by Molegro Virtual Docker software 2010.4.0.0. The structure obtained from mutation was further energy minimized and optimized. The change in the protein structure accounting to amino acid mutation was analyzed by employing an online server - Site Directed Mutator (SDM) [37]. SDM - a statistical potential energy function that helps in analyzing single nucleotide polymorphisms (SNPs) that affects the structure or function of proteins and predicts the probability of malfunctioning of receptors in terms of protein stability [38, 39].

2.7. Molecular Docking of Established Short Acting $\beta 2$ Agonists (SABAs)

Glide, version 6.1, (Schrödinger, LLC, New York, NY, 2013.Inc. 2012) which includes highly efficient OPLS force field and scoring functions provided a flexible platform for docking all the structures of short acting $\beta 2$ agonists. Schrödinger's proprietary GlideScore multi-ligand scoring function combined with composite Emodel score was used to rank the poses of each ligand [40-42]. On the basis of Glide Score, best short acting $\beta 2$ agonist with optimal binding affinity was selected against wild and mutated receptors.

2.8. Pharmacophore Based Virtual Screening

2.8.1. Development of Pharmacophore Model

We developed 7 point pharmacophore fingerprints (AADDPRR-geometric locations of hydrogen bond acceptors (A), hydrogen bond donors (D), hydrophobic groups (H), positive ionizable centers (P) and aromatic rings (R)) for the established $\beta 2$ agonist having balanced affinity against wild and mutated receptor which served as query compound for virtual screening. The Pharmacophore was developed as implemented into Phase module of Schrödinger suite [43]. A compound was defined as an analogue if the Tanimoto coefficient (Tc) [44] to query compound was >0.60. Analogues were searched against phase database which includes 4 million compounds composed of commercial libraries from the actual edition of the top vendor databases viz., Maybridge, ChemDiv, ChemBridge, Enamine, Asinex, , Specs, IfLab, Interbioscreen, AMRI, UkrOrg [45-54] and open databases like PubChem, zinc databases [55] and various commercial (annotated) databases [56, 57] which includes compounds with biological activity data from preclinical to clinical candidates and preferably with efficacies below 1 μ M. Second step involved clustering of the compounds into major chemotypes which were reviewed to remove the highly redundant structures through shape similarity screening (compounds with shape similarity >0.6 were considered redundant).

2.8.2. Calculation of the Physico-chemical Parameters and Property-based Filtering

Physicochemical descriptors which are useful in property-based drug discovery were used to refine the pharmacophore based similarity search [58, 59]. The compound library was screened to retrieve compounds which followed Lipinski [60] as well as Veber rules [61]. The physico-chemical parameters (Mwt, LogP, H-bond donors/acceptors, rotatable bonds and topological polar surface area) were calculated by the online calculation suit PClient of Virtual Computational Chemistry Laboratory [62].

2.8.3. Diversity Based Selection

In order to further retrieve non-redundant compounds with non-overlapping chemical features, the library compounds obtained from the previous step was loaded in the Canvas Suite of Schrödinger for diversity based selection (similarity metric - Soergel distance; compounds selection algorithm - sphere exclusion; sphere size - 0.5; initialization - random with random seed, cut off ≥ 0.6).

2.8.4. Structure Based Virtual Screening

The library of screened molecules from the previous step was docked at agonist binding site of wild and mutated (Thr164Ile) β_2 adrenergic receptor. The docking was performed using three modes: High throughput virtual screening (HTVS), Standard Precision (SP) and Extra precision (XP). HTVS and SP docking use the same scoring function; however, HTVS reduces the number of intermediate conformations throughout the docking funnel, and also reduces the thoroughness of the final torsional refinement and sampling. XP does more extensive sampling than SP: XP employs a more sophisticated scoring function that gives more refined results than the SP GlideScore, with greater requirements for ligand-receptor shape complementarity, and weeds out false positives that SP lets through [63]. In the first step, docking of all the screened library of molecules was performed using HTVS mode. 10% of all the molecules with best docking score obtained in HTVS were again docked using SP mode. Similarly, 10% of the molecules obtained from SP were docked into the protein with XP mode. Using rational workflow for virtual screening from HTVS to SP to XP helped us in enriching the data with fewer compounds but with higher accuracy of docking scores.

2.9. Statistical Analyses

Hardy-Weinberg equilibrium was tested for ADRB2 Thr/Ile variant. Any deviation in the allele and genotype frequencies between response phenotypes were tested for statistical significance using Yates corrected χ^2 test and Odds Ratio at 95% C.I. Lung function end points were compared using ANOVA and student test (with Hartley's f test for equality of variance). All the p values were two sided, and the level of significance was considered at $p < 0.05$. Statistical analyses were performed using Open EPI online statistical software [64] (Open EPI v 2.3.1, from department of Epidemiology, Rollins School of public health, Emory University, Atlanta, GA 30322, USA) and SNP stat web server (http://bioinfo.iconcologia.net/en/SNPStats_web) [65].

3. RESULTS AND DISCUSSION

There has been a steep increase in the world wide prevalence of asthma that shoots up by 50% every decade [66]. In India itself 25-30 million people suffer from asthma and 57,000 deaths were attributed to asthma, and have been seen as leading cause of morbidity and mortality in rural India [67]. One important attribute for the rising morbidity of asthma, is the variation in drug response, which in addition to other factors is an account of genetic variability. The genes encoding drug metabolizing proteins and receptors vary in individual patients implying the same drug may not show optimal effect in all. One of the well documented pharmacogenetic differences in individuals is the improper or refractory response to β_2 agonists. β_2 -Adrenergic receptors (β_2 ARs) expressed on airway smooth muscle are the targets for β_2 agonists that are used in the treatment of bronchospasm. The substitution of T allele in place of C allele at 491 position in the ADRB2 leads to mutation (Thr164Ile) in the receptor, which displays decreased agonist binding and depressed coupling of β_2 AR to adenyl cyclase.

A total of 398 physician diagnosed asthmatics were recruited for the study. The demographic characteristics of patients are presented in Table 1. In the study, we found no significant difference in number of males and females in patient, however, the number of females were marginally higher. The mean duration from which the

patients were suffering was found to be 10.5 years. 165 (41.45%) patients had a family history of asthma whereas 233 (58.55 %) showed absence of family history.

Table 1. Demographic variables of asthmatics.

Patients N = 398	
Gender	n (%)
Males	185 (46.48)
Females	213 (53.52)
Age (in years) [†]	
Males	46.44 $\pm$ 11.24
Females	42.24 $\pm$ 09.85
Duration of asthma (in years) [†]	10.5 $\pm$ 3.00
Family History of bronchial asthma	n (%)
Present	165 (41.45)
Absent	233 (58.55)
[†] Figures expressed as mean	
Table adapted with permission from Bandaru <i>et al.</i> , <i>Curr Top Med Chem.</i> 2015; 15(1): 50-6).	

Out of 398 asthmatics 261 (65.58%) patients were responders to Salbutamol while 137 (34.42%) patients were non-responders (Table 2). The lung function variables (FEV₁, PEF and FVC) with respect to severity classes and response phenotype are represented in Table 2. We found significant difference in the values of lung function endpoints, especially in FEV₁, within the response phenotypes (t-Test for FEV₁ within response phenotype; F Stat = 2.54, p value <0.001). In addition to this, the mean FEV₁ percentage reversibility was significantly declined in non-responding patients confirming that these patients demonstrate refractoriness to Salbutamol (t-Test for mean percentage FEV₁ reversibility within response phenotype; F Stat = 2.91, p value <0.001). By performing pulmonary function test, we observed that Salbutamol was ineffective to bring about significant bronchodilation (less than minimal threshold of 12% FEV₁ reversibility) and such patients were stratified as non-responders and since β_2 AR happens to be an important pharmacological drug target, we further advanced to evaluate whether Thr164Ile polymorphism influences declined response patterns to Salbutamol. As anticipated, we found the significant association of the polymorphism with Salbutamol (200 μ g) refractoriness in asthmatics. In the study, the frequency of the recessive genotype and allele was respectively 39.41% and 59.93% in non-responders as opposed to 30.27 and 47.51% in responders. From the risk estimate calculations, we found that the recessive allele offers 1.139 folds higher risk to non-responders than to its responding counterparts. Therefore, from the present analysis we inferred that the polymorphism marks as one of the main determinant of Salbutamol refractoriness and hence for the patients bearing the polymorphism, Salbutamol may not be a prescribed choice for bronchodilation in severe asthma groups.

Further, we tested the implication of the polymorphism for Salbutamol refractoriness in asthmatics (Table 3). The frequency of recessive genotype (Ile/Ile) in non- responders was noticeably higher (39.41%) opposed to 30.27% in responders. Further recess-

Table 2. Lung function variables in asthma response phenotypes.

Response Phenotype	n (%)	FEV ₁ (Mean $\pm$ SD)	PEF (Mean $\pm$ SD)	FVC (Mean $\pm$ SD)
Responders	261 (65.58)	64.82 $\pm$ 5.42	48.84 $\pm$ 4.85	51.26 $\pm$ 2.40
Non-responders	137 (34.42)	52.66 $\pm$ 3.4	40.21 $\pm$ 6.21	51.55 $\pm$ 4.21
Mean percentage FEV₁ reversibility (Mean% $\pm$ SD)				
Responders	25.55 $\pm$ 4.1			
Non-responders	10.15 $\pm$ 2.4			
t-Test for FEV ₁ within response phenotype: p value < 0.001				
t-Test for mean percentage FEV ₁ reversibility within response phenotype : p value < 0.001				
(Table adapted with permission from Bandaru <i>et al.</i> , <i>Inflammation</i> . 2015; 38(6): 2146-55).				

sive allele “Ile” frequency was 56.93 % in non-responders compared to 47.51 % in responders. From co-dominant model of inheritance it can be noticed that in comparison to responders, non-responders harbor the recessive variant considerably at a higher frequency (Responders *vs.* non-responders, Thr/Thr *vs.* Ile/Ile OR = 1.79 CI = (1.07 - 3.02), $\chi^2 = 4.35$ p = 0.0370). Further, the intense impact of polymorphism on Salbutamol refractoriness can be observed from recessive model as well (Responders *vs.* non-responders, Ile/Ile *vs.* Thr/Thr + Thr/Ile, OR = 0.67 CI = (0.43 - 1.03), $\chi^2 = 2.99$, p = 0.0843). Therefore, apparent from logistic regression analysis considering different inherent models, it becomes quite evident that the polymorphism is significantly associated with non-responding phenotypes and noteworthy stating that the polymorphism offers significant refractoriness to Salbutamol in asthmatics. In concordance to our investigation, pioneering studies have proved that the profound effect of Thr164Ile polymorphism on refractoriness can be attributed to ineffective binding of B₂ agonists in the mutated receptor [68]. Studies using recombinant cells have also found that the Thr164Ile allele has four times less ligand affinity leading to depressed formation of the agonist-receptor-Gs complex and consequently 50% reduction in agonist-stimulated adenylyl cyclase activity [69].

Mutational analysis of the mutated (Thr164Ile) β_2 adrenergic receptor by statistical potential energy function in Site Directed Mutator (SDM) predicted that mutation enhances the stability of the protein and also leads to malfunctioning of the receptor (pseudo $\Delta\Delta G = 2.79$). In addition, the solvent accessible surface area reduced to 41.9% in mutated receptor compared to 58.3% in wild receptor. The RMSD calculated between wild and mutated receptor was 5.6299. The superimposed structures are shown in figure 3. The keen perusal of the agonist binding area revealed that the wild receptor had cavity volume of 404.48 Å³. Thr to Ile substitution at 164th position leads to global perturbation of the receptor increasing the cavity volume to 520.704 Å³. The superimposed cavities are shown in Fig. (4).

Through extensive docking of all the established agonists, we observed that the binding affinity (Glide Score) of Salbutamol was best (-16.697) against wild β_2 receptor, while for mutated receptor it was Fenoterol which showed the best (-15.419) binding affinity (Table 4).

It is worthy to note that Salbutamol, although being a powerful inducer of the adrenergic receptor, however, fails to induce the receptor at the mutated states and the rationale behind the declined induction can be attributed to reduced affinity against mutated receptor (ratio of binding affinity of Salbutamol Wild receptor to Salbutamol mutated receptor = 2.127). The declined binding of Salbutamol to mutated receptor well explains the reason behind the

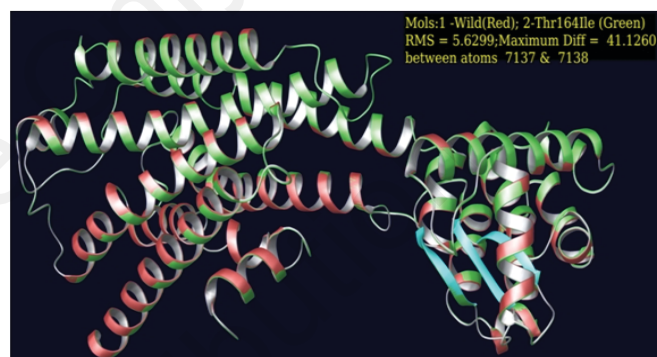


Fig. (3). The super imposed secondary structures of Wild (red) and Thr164Ile (green) mutated receptor. The substitution leads to global perturbation in the structure (RMS= 5.6229). (Figure adapted with permission from Bandaru *et al.*, *Curr Top Med Chem*. 2015; 15(1): 50-6).

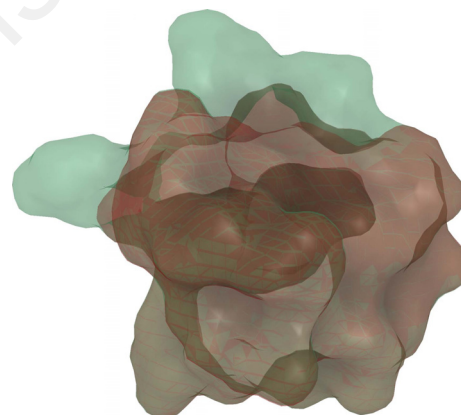


Fig. (4). The overlapping cartoon depicts binding cavities of wild (red) and Thr164Ile (green) receptor. The cavity of mutated surface has more volume than the wild receptor. (Figure adapted with permission from Bandaru *et al.*, *Curr Top Med Chem*. 2015; 15(1): 50-6).

Salbutamol refractoriness in asthmatics bearing Thr164Ile mutation. From extensive binding analysis we observed that Fenoterol had balanced and consistent binding affinity against wild and mutated receptor (ratio of binding affinity of Fenoterol Wild receptor to Fenoterol mutated receptor = 1.01). However, Fenoterol although being consistent in two receptor states and in addition being high affinity agonist for mutated receptor than Salbutamol (ratio of binding affinity of Fenoterol mutated receptor to Salbutamol mutated receptor = 1.97) ; it is not as much efficient binding

Table 3. Genotype and allelic distribution in responders and non-responders.

Inheritance Model	Genotype	Responders (N= 261) n (%)	Non-Responders (N= 137) n (%)	Statistics
Codominant	Thr/Thr	92 (35.25)	35 (25.55)	Ref
	Thr/Ile	90 (34.48)	48 (35.04)	OR = 1.40, CI = (0.83 - 2.38), $\chi^2 = 1.29$, p = 0.258
	Ile/Ile	79 (30.27)	54 (39.41)	OR = 1.79, CI = (1.07 - 3.02), $\chi^2 = 4.351$, p = 0.037*
Dominant	Thr/Thr	92 (35.25)	35 (25.55)	Ref
	Ile/Ile+Thr/Ile	169 (64.75)	102 (74.45)	OR = 1.58, CI = (1.00 - 2.51), $\chi^2 = 3.460$, p = 0.031*
Recessive	Ile/Ile	79 (30.27)	54 (39.41)	Ref
	Thr/Ile+Thr/Thr	182 (69.73)	83 (60.59)	OR = 0.67, CI = (0.43 - 1.03), $\chi^2 = 2.991$, p = 0.084
Allele Frequencies	Thr	274 (52.49)	118 (43.07)	Ref
	Ile	248 (47.51)	156 (56.93)	OR = 1.46, CI = (1.09 - 1.96), $\chi^2 = 6.01$, p = 0.014*

*p<0.05; OR= Odds Ratio

Table 4. Docking scores (Glide Score) of short acting β 2 agonists (SABA) against β 2AR wild and mutated (Thr164 Ile) receptor. The scores are arranged in descending order of affinity.

Affinity order of β 2 agonists in wild receptor		Glide XP G score	Affinity order of β 2 agonists in mutated receptor		Glide XP G score
1	Salbutamol	-16.697	1	Fenoterol	-15.419
2	Fenoterol	-15.597	2	Ritodrine	-13.413
3	Ritodrine	-12.784	3	Procaterol	-11.373
4	Procaterol	-9.395	4	Pirbuterol	-10.359
5	Pirbuterol	-9.303	5	Isoetharine	-10.094
6	Isoetharine	-9.208	6	Isoprenaline	-8.863
7	Terbutaline	-8.267	7	Terbutaline	-8.734
8	Metaproterenol	-7.798	8	Salbutamol	-7.803
9	Isoprenaline	-7.124	9	Bitolterol	-6.638
10	Bitolterol	-5.676	10	Metaproterenol	-6.572

Glide XP G Score: Glide Extra precision Glide Score

candidate as Salbutamol in wild state of the receptor. It would therefore mean that, to bring about better management of asthma, Salbutamol needs to be administered to patients bearing wild receptor while Fenoterol to patients bearing mutation. At the clinical grounds the procedure may be thought so, however it should be a cumbersome process as each patient needs to be screened for mutation and then be prescribed a suitable β 2 agonist. To overcome such “cumbersome” process, we thought to identify a molecule which can irrespectively target both wild and mutated receptor with similar, enhanced and balanced affinity. Since Fenoterol had almost balanced affinity for both wild and mutated receptor, we assumed that analogues of Fenoterol can be derived based on its pharmacophoric features so that we identify a molecule which will be bestowed with high affinity as of Salbutamol in wild receptor and as of Fenoterol in mutated receptor. To attain this, we developed pharmacophore based virtual screening considering Fenoterol as a query molecule. 7 point pharmacophore

(AADDPRR) was model was developed for Fenoterol (Fig. 5) in order to obtain molecules akin to Fenoterol from a Phase database of Schrödinger's suite which consists of 4 million compounds indexed from various commercial chemical database.

In the virtual screening process, as mentioned afore, Fenoterol formed the query structure to screen for the compound against phase database based on 7 point Pharmacophore. 982262 out of 4 million compounds were obtained similar to pharmacophore of Fenoterol. In order to obtain non-redundant compounds, the compound obtained in the previous step were clustered into major chemotypes, and compounds with similarity of above 0.6 were weeded off ;therefore the number of compound obtained in this step were 912540. The physico-chemical properties are important for drug metabolism therefore the compounds were filtered adhering to Lipinski and Veber rule, so the screened compound now were 86248. Next step involved diversity based selection of compounds

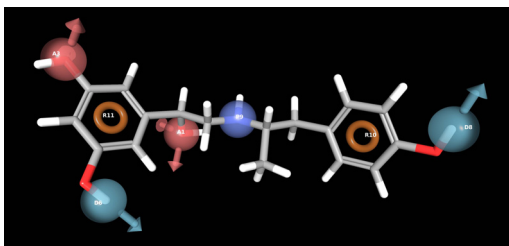


Fig. (5). 7 point Pharmacophore model (AADDPRR) of Fenoterol. (AADDPRR-geometric locations of hydrogen bond acceptors (A), hydrogen bond donors (D), hydrophobic groups (H), positive ionizable centers (P) and aromatic rings (R)).

which weeds of compounds with overlapping chemical features, therefore applying this selection, the compounds were further reduced to 7402. All these 74012 now formed a library created from ligand based virtual screening. This compound library was now subjected to structure based virtual screening which includes

highly efficient docking algorithms like HTVS (High Throughput Virtual Screening), SP (Standard Precision) and XP (Extra Precision). The entire 74012 compound obtained from ligand based virtual screening were docked against wild and mutated (Thr164Ile) β_2 adrenergic receptor with HTVS algorithm. 10% *i.e.* 7400 of the docked molecules obtained from HTVS were again docked against the respective receptors with improved docking precision algorithm-SP. In the final step 10% *i.e.* 740 compounds obtained from SP were now docked with XP mode to get final set of high affinity compound devoid of false positives and enhanced binding affinity scores. 74 molecules were obtained in the final step (Fig. 6 and 7). It is interesting and quite worthy to note that, out of 74 compounds, a compound "CACPD2011a-0001278239" (5-{2-[4-(4-hydroxyphenyl)-1, 2, 3, 6-tetrahydropyridin-1-yl] acetamido} benzene-1, 3-dicarboxamide) (Fig. 8) was retrieved, which had highest and similar affinity amongst the 74 molecules against both in wild and mutated receptors.

Table 5 and Fig. (9) shows the comparative analysis of docking scores of Salbutamol, Fenoterol and CACPD2011a0001278239 in

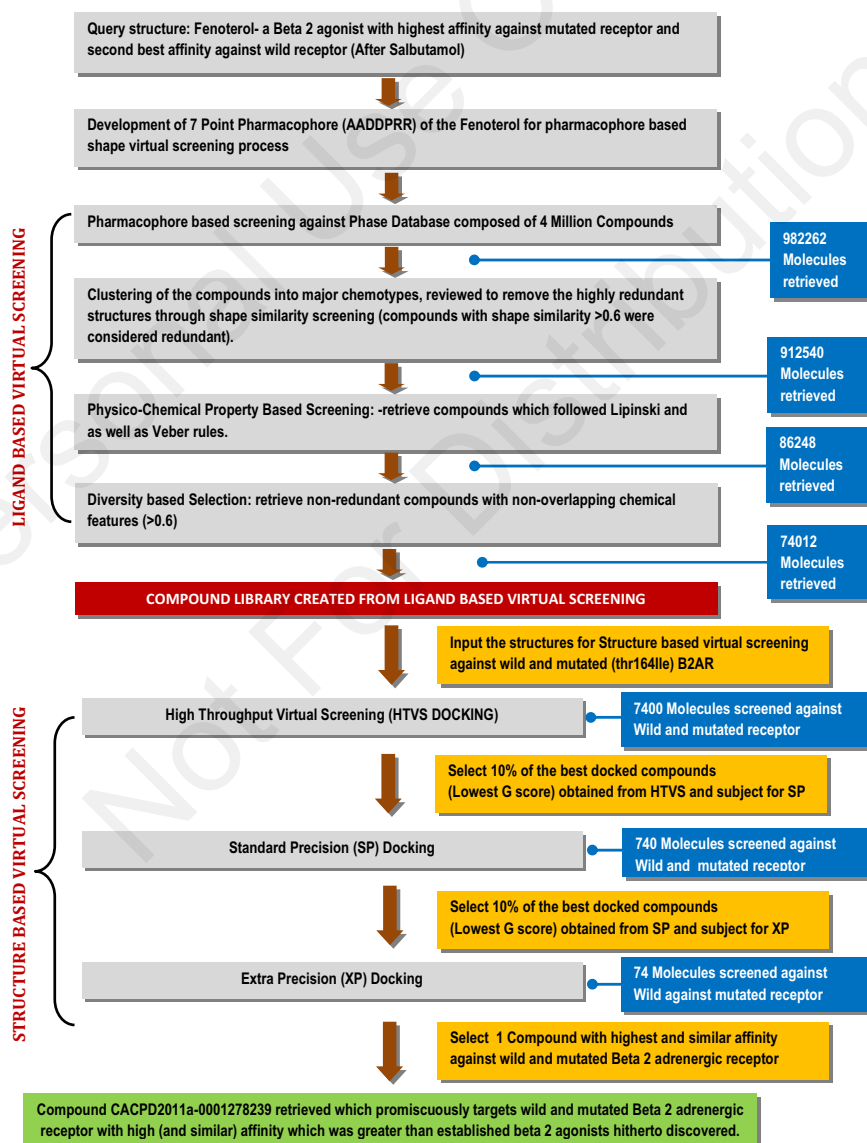


Fig. (6). Steps in ligand and structure based virtual screening methods.

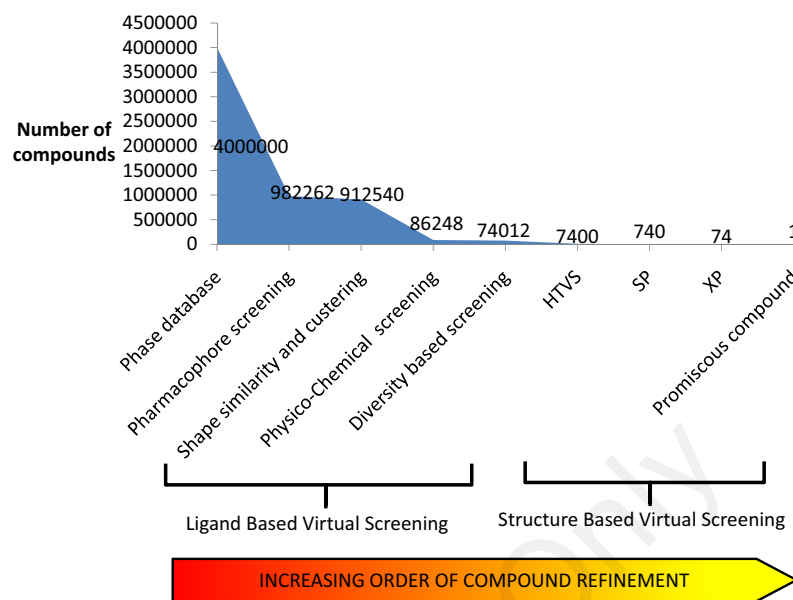


Fig. (7). Refinement of compounds through ligand and structure base virtual screening method.

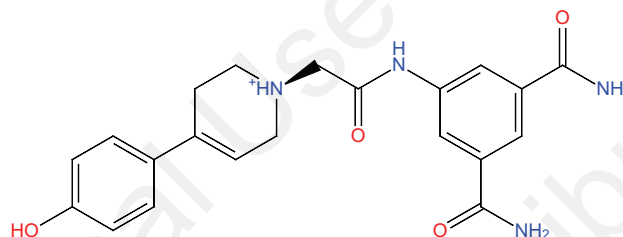


Fig. (8). Molecular structure of high affinity screened compound CACPD2011a-0001278239. (5-[2-[4-(4-hydroxyphenyl)-1,2,3,6-tetrahydropyridin-1-yl] acetamido] benzene-1,3-dicarboxamide). Presence of dicarboxamide groups and amide linkage tethering tetrahydropyridin and benzene-1,3-dicarboxamide group in the structure makes it a good hydrogen bond donating compound.

wild and mutated (Thr164Ile) β_2 adrenergic receptor. Evident from the docking scores, CACPD2011a0001278239 shows highest affinity against wild and mutated receptor than established agonists Salbutamol and Fenoterol. In addition, CACPD2011a0001278239 is consistent in its affinity in both the wild and mutated states testifying its potential to irrespectively bind with both wild and mutated states without any structure based preference (ratio of binding affinity of CACPD2011a0001278239 Wild receptor to CACPD2011a0001278239 mutated receptor = 1.031). It can be thought that CACPD2011a0001278239 can compensate the affinity drawbacks of Salbutamol in mutated receptor (ratio of binding affinity of CACPD2011a0001278239 mutated receptor to Salbutamol mutated receptor = 2.17) and can also act similar to it (Salbutamol) in wild receptor (ratio of binding affinity of CACPD2011a0001278239 wild receptor to Salbutamol wild receptor = 1.056). Further affinity of CACPD2011a0001278239 is 1.102 folds higher than Fenoterol in mutated receptor which (Ratio of binding affinity of CACPD2011a0001278239 mutated receptor to Fenoterol mutated receptor = 1.102). From the comprehensive analysis, it can be noted that CACPD2011a0001278239 can form high affinity β_2 agonist promiscuously and irrespectively targeting both wild and mutated states of β_2 adrenergic receptor.

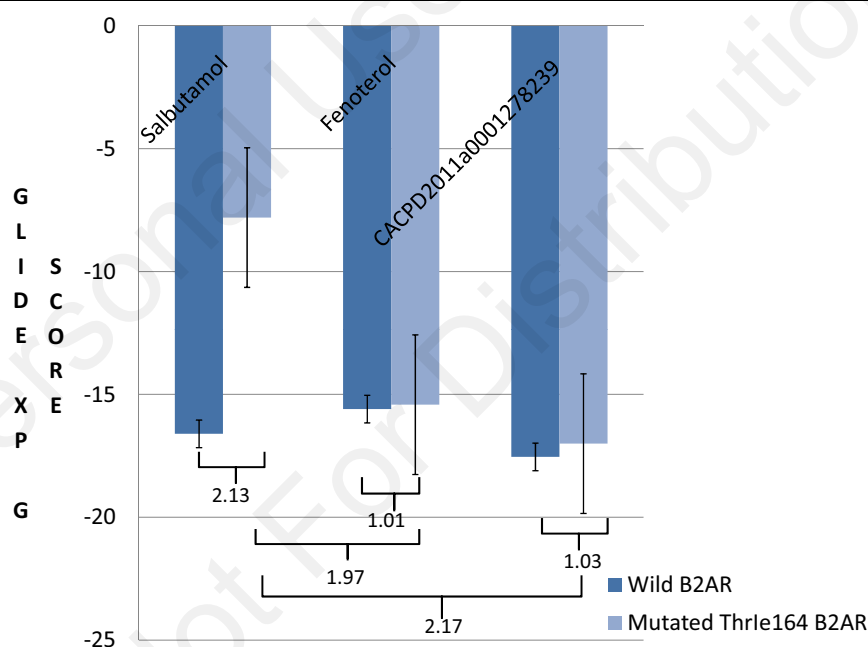
The affinity score of established agonists Salbutamol, Fenoterol and virtually screened CACPD2011a0001278239 - three major compounds in the study can be well attributed to their interaction profiles as shown in Fig. (10) and Table 6. A close perusal at the

interaction profiles in wild and mutated receptors expounds the declined affinity of Salbutamol in mutated states. In the mutated receptor, Salbutamol forms 2 hydrogen bonds as opposed to 4 hydrogen bonds in wild state of the receptor. In addition, Salbutamol fails to form π - π interactions and salt bridges in mutated receptor as it forms in wild receptor, furthermore, van der Waal interactions are declined when Salbutamol interacts in the mutated receptor. The superior interactions of Fenoterol in mutated receptor than Salbutamol can again be attributed to higher number of hydrogen bonds and presence π - π interactions and salt bridges which Salbutamol fails to demonstrate in the mutated receptor. Although interaction of Fenoterol is almost consistent in both wild and mutated states, it shows better interaction in mutated states. In wild states of the receptor, it forms 2 hydrogen bonds while in mutated receptor it forms 3 hydrogen bonds. This observation also explain rationale for affinity score of Fenoterol being next to Salbutamol in wild state of the receptor which forms 4 hydrogen bonds. CACPD2011a0001278239 compound identified in the study demonstrates par excellent interaction profile than Salbutamol and Fenoterol. The similar affinity score in both wild and mutated receptor is quite proportional to its interaction profile. Except for salt bridge formation (which is missing in mutated receptor that hardly contributes to affinity), the number of hydrogen bonds, π - π interactions electrostatic contacts and van der Waal contacts are equal in both the states. In addition, CACPD2011a0001278239 shows much better interaction profile than Salbutamol in wild receptor and Fenoterol in the mutated receptor. In-depth perusal of the molecular structure of CACPD2011a0001278239 (Fig. 8) further expounds the basis of its high affinity against both the receptors. Incorpora-

Table 5. Comparative analysis of docking scores of Salbutamol, Fenoterol and CACPD2011a0001278239 in wild and mutated (Thr164Ile) β_2 adrenergic receptor.

XPG Glide Score	Wild	Mutated	Docking ratios of compounds in wild and mutated states
Salbutamol	-16.602	-7.803	Salbutamol _{wild} /Salbutamol _{mutated} = 2.127
Fenoterol	-15.597	-15.419	Fenoterol _{wild} /Fenoterol _{mutated} = 1.01
CACPD2011a0001278239	-17.541	-17.002	Fenoterol _{mutated} /Salbutamol _{mutated} = 1.97
			Salbutamol _{wild} /Fenoterol _{wild} = 1.06
			CACPD2011a0001278239 _{wild} /CACPD2011a0001278239 _{mutated} = 1.031
			CACPD2011a0001278239 _{mutated} /Salbutamol _{mutated} = 2.17
			CACPD2011a0001278239 _{wild} /Salbutamol _{wild} = 1.056
			CACPD2011a0001278239 _{mutated} /Fenoterol _{mutated} = 1.102
			CACPD2011a0001278239 _{wild} /Fenoterol _{wild} = 1.124

The compound with subscripts represents the docking score of the compound in wild/mutated receptor.

**Fig. (9).** Docking scores of Salbutamol, Fenoterol and CACPD2011a0001278239 in wild and mutated (Thr164Ile) β_2 adrenergic receptor. Figures represent the docking ratios of the compound in wild and mutated states of the receptor.

tion of dicarboxamide groups in the structure makes CACPD2011a0001278239 a good hydrogen bond donor. In addition, the amide linkage tethering tetrahydropyridin and benzene-1, 3-dicarboxamide group further acts as strong hydrogen bond donor to the surrounding residues in both the receptors. In can be well noticed that hydrogen bond donating dicarboxamide groups are completely absent in Salbutamol as well as in Fenoterol- the rational which underscores the basis of declined affinity of these agonists in receptors.

From the clinical perspective, it may be well assumed that Salbutamol can be prescribed to asthmatics harboring wild β_2 adrenergic receptor while Fenoterol would be the preferred choice in patients with mutated receptor, however, as mentioned earlier, disease management would be cumbersome as each patient needs to be

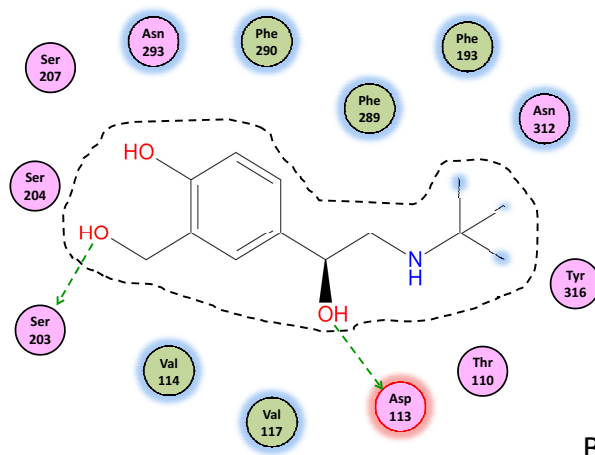
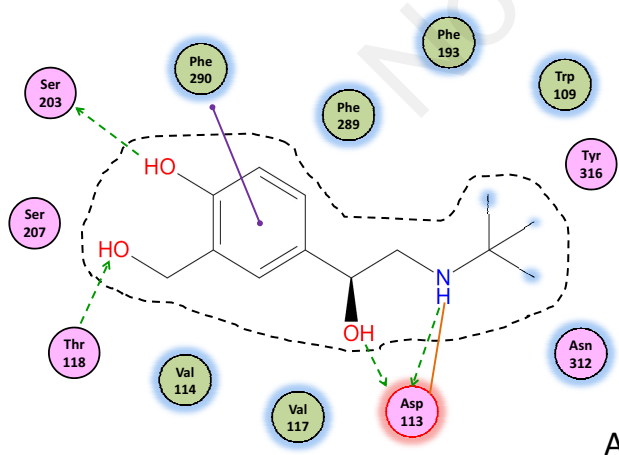
screened for the presence of polymorphism. The present study is a future step in better management of asthma wherein a patient may not be screened for mutation and a single β_2 agonist can be administered to any patient irrespective of Thr164Ile polymorphism, and “CACPD2011a0001278239” therefore forms such potential single β_2 agonist. The present study can be put forth in pharmacodynamic and pharmacokinetic studies for further understanding and correlating our findings in *in vitro* and *in vivo* systems.

CONCLUSION

In the present study we report ADRB2 Thr164Ile polymorphism as an important variant to bring about Salbutamol refractoriness in severe asthmatics. Through extensive virtual screening approaches, we identified CACPD2011a0001278239 as a potential

Table 6. Interaction profiles of Salbutamol, Fenoterol and CACPD2011 a0001278239 in wild and mutated states of $\beta 2$ adrenergic receptor

	Wild $\beta 2$ AR					Mutated (Thr164Ile) $\beta 2$ AR				
	H Bonds (n)	π - π Stacking (n)	Salt Bridges (n)	Electrostatic Contacts (n)	van der Waal Contacts (n)	H Bonds (n)	π - π Stacking (n)	Salt Bridges(n)	Electrostatic Contacts (n)	van der Waal Contacts(n)
Salbutamol	4 Asp 113, Asp113, Thr 118, Ser 203	1 Phe 290	1 Asp 113	6 Tyr 316, Asn 312, Asp 113, Ser 203, Ser 207, Thr 118	6 Trp 109, Phe 193, Phe 289, Phe 290, Val 117, Val 114	2 Asp 113, Ser 203	0	0	7 Asn 312, Thr 110, Asp 113, Ser 203, Ser 204, Ser207, Asn 293	5 Val 117, Val 114, Phe 290, Phe 289, Phe 193
Feneterol	2 Asn 312, Ser 203	1 Phe 290	1 Asp 113	8 Cys 191, Asn 312, Tyr 316, Tyr 308, Ser 203, Ser 204, Asp 113, Thr 110	5 Trp 109, Phe 193, Val 114, Phe 290, Phe 289	3 Asn 312, Tyr 316, Ser 203	1 Phe 290	1 Asp 113	6 Asn 312, Tyr 316, Asp 113, Ser 207, Ser 204, Ser 203	6 Phe 289, Val 117, Val 114, Phe 290, Phe 193, Trp 109
CACPD2011 a0001278239	4 Asp 192, Trp 313, Gly 90, Asn312	1 Phe 290	1 Asp 113	8 Ser 204, Ser 203, Asp 192, His 93, Gly 90, Try 316, Asn 312, Asp 113	6 Phe 290, Val 114, Phe 289, Phe 193, Ile 94, Trp 313	4 Asp 192, Trp 313, Gly 90, Asn312	1 Phe 289	0	9 Ser 203, Ser 204, Ser 207, Try 316, Asp 192, His 93, Gy 90, Asn 312, Asp 113	6 Phe 289, Phe 193, Val 114, Ile 94, Trp 313, Phe 290
n= number of bonds										



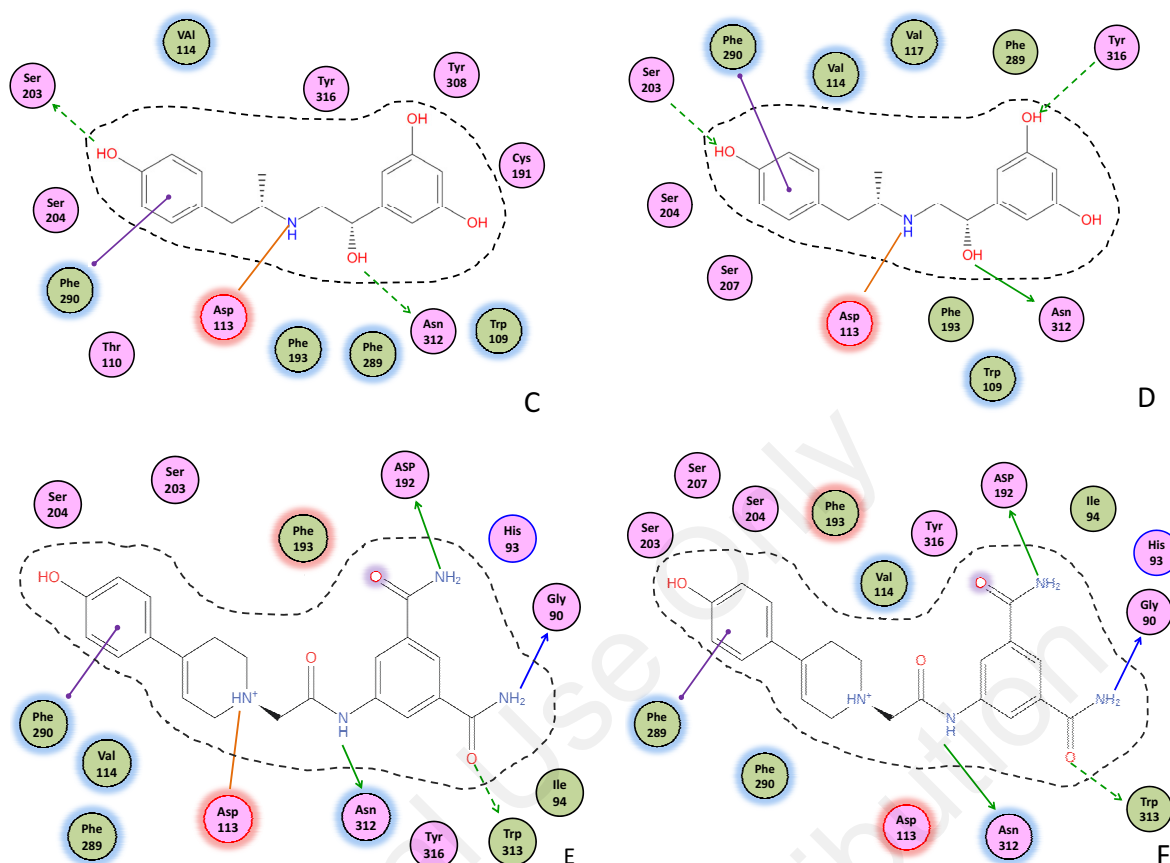


Fig. (10). Molecular interaction diagrams of Salbutamol in (A) Wild (B) mutated receptor Fenoterol in (C) Wild (D) mutated receptor and CACPD2011 A0001278239 in (E) Wild (F) mutated states of β_2 adrenergic receptor.

molecule promiscuously targeting both wild and mutated (Thr164Ile) β_2 adrenergic receptors with similar and consistent affinity, than any established β_2 agonist prescribed for clinical treatment of asthma.

LIST OF ABBREVIATIONS

ADRB2	=	Adrenoceptor B 2 gene [Homo sapiens]
ANOVA	=	Analysis of Variance
C.I.	=	Confidence Interval
Emodel	=	Energy Model
FEV ₁	=	Forced Expiratory Volume in 1 second
G	=	Gibbs Energy
Ile	=	Isoleucine
mcg	=	Microgram
Open EPI	=	Open Source Epidemiologic Statistics for Public Health
OPLS	=	Optimized Potentials for Liquid Simulations
OR	=	Odds Ratio
Thr	=	Threonine
XPG Score	=	Extra Precision Glide Score
β_2 AR	=	B 2 Adrenergic Receptor
χ^2	=	Chi squared test

SOFTWARE, SUITES AND WEB SERVERS USED

Quantity[®] 1D analysis software, BioRad laboratories was used for Gel documentation. Open Source Epidemiologic Statistics for

Public Health (OpenEPI) Version 3.01 server was used calculating for genotype and allele related statistical analysis. Structures of β_2 agonists downloaded from PUBCHEM (NCBI). All the structures were optimized in MarvinView 5.6.0.2, (1998-2011, Copyright[®] ChemAxon Ltd). Mutation of the protein was carried out in MOLEGTRO VIRTUAL DOCKER 2010.4.0.0. Accelrys Discovery Studio[®] Visualizer 3.5.0.12158 (Copyright[®] 2005-12, Accelrys Software Inc.) was used as a visualization platform. Docking calculations were carried using GLIDE v 6.0 program of Schrödinger suite 2013 (Copyright[®] 2005-2014 Schrödinger, LLC). Phase Module of Schrödinger suite 2013 was used for virtual screening process.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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