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Letters

Novel Lead Generation through Hypothetical Pharmacophore Three-Dimensional Database Searching: Discovery of Isoflavonoids as Nonsteroidal Inhibitors of Rat 5α-Reductase

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Abstract: A hypothetical pharmacophore of 5α-reductase inhibitors was generated and served as a template in virtual screening. When the pharmacophore was used, eight isoflavone derivatives were characterized as novel potential nonsteroidal inhibitors of rat 5α-reductase. This investigation has demonstrated a practical approach toward the development of lead compounds through a hypothetic pharmacophore via three-dimensional database searching.

Introduction. Dihydrotestosterone (DHT) plays an essential role in the pathogenesis of benign prostate hyperplasia (BPH), prostatic cancer, androgenic alopecia, acne, and hirsutism.[1] The two enzymes, types I and II NADPH-dependent isozymes, of steroid 5α-reductase catalyze the reduction of testosterone (T) to DHT in several human tissues.[2] Although there are many inhibitors of 5α-reductase reported,[3−5] finasteride (**1**, Proscar) is the only clinically useful 5α-reductase inhibitor to treat BPH and androgenic alopecia. Finasteride is a potent type II inhibitor with 200-fold less potency against the type I isozyme.[6] However, the steroidal structure of finasteride may incur hormonal adverse effects, and it is imperative to search for novel nonsteroidal inhibitors of 5α-reductase.

To our knowledge, few systematic studies appeared even though several classes of steroidal and nonsteroidal inhibitors have been reported. It is of considerable interest to know how structurally different inhibitors are able to induce the same enzyme inhibitory activity. In general, it is agreed that these compounds may possess the key structural elements that are essential for interacting with the enzyme active sites. Undoubtedly, a direct Docking program onto the three-dimensional (3D) structure of enzyme active sites will facilitate the new lead discovery. Because 5α-reductase is a membrane-associated enzyme, its X-ray crystal structure is not available. Consequently, the direct structure-based drug design approach is impossible in this case. We assumed that if we can generate hypothetical enzymatic active sites, it will provide a ligand template for a 3D database search and may facilitate the lead compound discovery. This way may provide a new drug candidate via a series of interactive action between lead selection and lead optimization as shown in Scheme 1.

Molecular modeling has become a useful tool for finding lead compounds in recent years.[7] There are few molecular modeling studies reported on inhibitors of 5α-reductase; nevertheless, they were all focused only on the conformations of the substituents[3,8] or skeleton[9] of steroid. The catalyst program has been used to establish a hypothetical enzyme active site[10] by analyzing a variety of known inhibitors. Thereupon, we investigated a series of 5α-reducatase inhibitors via computer-aided analysis to generate general hypothetical receptor sites, which expectantly are serving as a template, and to perform a 3D Chemical database search for the discovery of novel inhibitors of 5α-reductase.

Rat 5α-Reductase Inhibition Assay. The inhibition assay was performed according to the method reported by Rusell et al.[11] with some minor modifications. To 200 μL of 20 mM sodium phosphate buffer (pH 6.5) containing 5 μM of [4-^{14}C]-testosterone (2.10 GBa/mmol), 200 μM of NADPH, and 0.05% of Tween-80 were added various amounts of tested compound. The resulting

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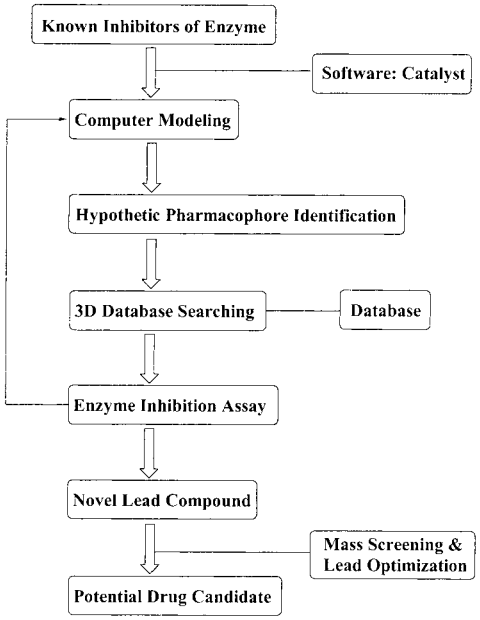
Scheme 1



solution was incubated at 37 °C for 30 min. The reaction was initiated with the addition of 20 μg of rat liver microsome. After incubation, the reaction was stopped with the addition of 1 mL of acetone. The supernatant was removed and dried. The residue was spotted on a TLC plate (silica gel) and developed with dichloromethane/ether 1:1. Radioactivities were recorded with a Bioscan γ-ray detector. The amount of product formed was calculated correspondingly.

Hypothetic Pharmacophore Generation. This study was performed using the software package Catalyst installed on a Silicon Graphics Origin2000 server. Sixteen structurally different compounds[5] were selected as the training set for the pharmacophore generation of human type II 5α-reductase inhibitors, and their structures with IC_{50} values are shown in Supporting Information. A wide range of IC_{50} (0.18 nM to 6.3 μM) is chosen in order to create a general phormacophore that can differentiate compounds as the active or inactive inhibitors for 5α-reductase. To find nonsteroidal inhibitors, we chose minor amounts of steroidal compounds and a majority of nonsteroidal compounds in the training set. Standard parameters were utilized, and the conformational model of each molecule was generated by the Best command in Catalyst, utilizing a 20 kcal mol^{-1} limit for conformational energy. The training set was analyzed to generate 10 hypotheses comprising all or any of the following features: hydrogen bond acceptors and hydrophobic groups. Overlays of all compounds with pharmacophores were completed by using the Best Fit command in Catalyst.

After hypothetic pharmacophore generation, it was employed as a template for searching through the available NCI database by the Catalyst program. We found that three hits possessed the isoflavone core structure in the first 300 hits. Therefore, we searched through the NCI database to find all isoflavone compounds that would map to the corresponding hypothesis. The Best Flexible Search Databases command was applied.

Results and Discussion. 1. Analysis of Hypotheses. The 16 structurally different compounds in the training set were minimized, and then the conformational models were computed. Depending on the flexibility of the compound, different numbers of conformer were generated, and results are listed in Supporting Information. As one would expect, compounds with a steroidal skeleton had fewer conformers while the others possessed the maximal number of 251 conformers.

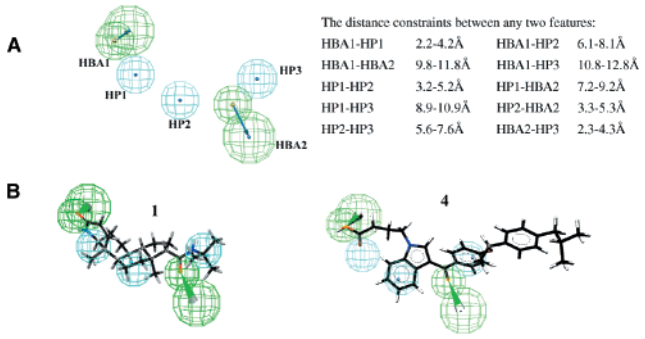


Figure 1. (A) Pharmacophore of type II 5α-reductase inhibitors containing two hydrogen bond acceptors (HBA) and three hydrophobic groups (HP). (B) Mapping pictures of the generated pharmacophore to compounds **1** and **4**.

The Catalyst program was then requested to generate 10 hypotheses that served as the hypothetical receptor active sites of type II 5α-reductase based on the 16 compounds **1**−**16**. The correlations, total costs, and features of the 10 hypotheses are compiled in Supporting Information. For hypothesis generation, Catalyst calculates the ideal and null hypotheses. The ideal hypothesis comprises the error cost being minimal and the slope of the activity correlation line being 1. On the other hand, the null hypothesis contains high error cost and the slope of correlation is zero. The greater the cost difference between a hypothesis and the null hypothesis, the more useful the hypothesis. The first two highest-scoring hypotheses have the smallest cost differences (Δ_{ideal} = 12.04 and 12.12, respectively) with the ideal hypothesis and have the largest cost differences (Δ_{null} = 21.72 and 21.65, respectively) with the null hypothesis. They possess almost exactly the same correlation coefficients. These indicate that the two hypotheses lie closer to the ideal hypothesis. Furthermore, these two hypotheses possess a coefficient (the ratio of predicted activity vs actual activity) closest to 1. The other eight hypotheses have larger Δ_{ideal} and smaller Δ_{null}, and the correlation coefficients are also decreased. Therefore, we chose to further analyze the two highest-scoring hypotheses. By analyzing the generated hypotheses, we found that the two highest scoring hypotheses both contain two hydrogen bond acceptors and three hydrophobic groups. In fact, the two highest scoring hypotheses are very similar to each other,[12] and accordingly, we chose the highest-scoring hypothesis as being the pharmacophore of human type II 5α-reductase inhibitors. The 3D pharmacophore consists of two hydrogen bond acceptors (HBA1 and HBA2) and three hydrophobic groups (HP1-3). The pharmacophore and the distance constraints are shown in Figure 1A.

2. Validation of Generated Pharmacophore. The estimated inhibitory activities of the training set by our hypothesis model illustrated in Table 1 of the Supporting Information were similar to the measured activities in general. Because our goal was to find a general

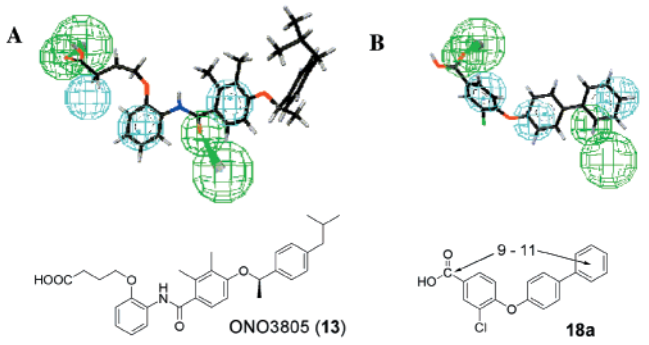


Figure 2. Generated pharmacophore mapped to (A) ONO3805 (**13**) and (B) benzoic acid **18a**.

pharmacophore that could bind to the enzyme, we chose active compounds **1**, **2**, **4**, and **5** to validate our model in view of the fact that they possessed different chemical structures. The mapping pictures are shown in Figure 1B and Supporting Information. Our model was fitted to the steroidal compounds **1** and **2** superbly with all features being correspondingly mapped. For nonsteroidal compounds **4** and **5**, the pharmacophore was fitted to the compounds quite well except for one hydrophobic feature not mapped (HP1 in **4** and HP2 in **5**). It should be noted that one large hydrophobic group of the longer compounds, such as **4** and **6**−**9**, was not included in our current model.

Holt et al. suggested that ONO3805 (**13**) acted as a ligand to interact with the active site of the 5α-reductase[13] and was designed as a bisubstrate analogue such that the large lipophilic group mimics the steroidal skeleton and the carboxylic acid interacts with NADPH. Kumazawa et al.[14] also reported a series of potent nonsteroidal inhibitors such as KF18678 (**17**) on the basis of this concept. Because the Catalyst program is designed to generate the hypothetical pharmacophore that is essential for interacting with the active sites of receptor, this category of inhibitors should correspond to our pharmacophore features. Inhibitor ONO3805 (**13**) is in our original training set. One can see from Figure 2A that HBA1 and HBA2 are fitted to the carboxylic acid and carbonyl group, respectively. HP2 and HP3 are also fitted to the benzene rings very well. Our hypothetical active sites mimic the carboxylic acid side and the space bridge with hydrogen bond acceptor and hydrophobic groups. However, our pharmacophore cannot illustrate the large hydrophobic group that was designed to mimic the steroid skeleton. We also modeled KF18678 into the pharmacophore, and it is shown in Supporting Information. All the mapping sites corresponding to those of ONO3805. Similarly, the large hydrophobic group is still located outside our pharmacophore. Nevertheless, these results demonstrate that at this stage our model can mimic the active sites of the bridge and the phosphate site of the enzyme. To fully describe the active sites of 5α-reductase, the pharmacophore needs to be further modified to include the large hydrophobic group that can mimic steroid molecule.

Recently, Igarashi et al.[15] reported a series of benzoic acid derivatives and indole derivatives as potent human 5α-reductase inhibitors. On the basis of the structure−activity relationships and modeling of the two series, they concluded that the distances between the centroid of benzene ring A and the carboxyl group of the derivatives of both benzoic acid and indole series needed to be in the range of 9−11 Å. Because the constraint distance of HBA1-HP3 in our pharmacophore model is 10.8−12.8 Å, we expected that the carboxyl group and benzene ring would correspond to HBA1 and HP3, respectively, in our model. Hence, Igarashi's two potent compounds 4-(biphenyl-4-yloxy)chlorobenzoic acid (**18a**) and 4-[(1-benzyl-1*H*-indol-5-yl)oxy]-3-chlorobenzoic acid (**18b**) were selected and superimposed onto our hypothetical pharmacophore. The superimpositions of compounds **18a** and **18b** onto the pharmacophore reveal a good correlation and are shown in Figure 2B and Supporting Information. Likewise, the estimated IC_{50} values were 170 and 8.6 nM for **18a** and **18b**, respectively, which were comparable with the measured IC_{50} of 0.87 and 0.44 nM, respectively. Clearly, these results lend some supports to our hypothetic pharmacophore.

3. Isoflavonoids. During the study, the generated hypothesis was searched through the NCI database, and we found three hits with the isoflavone skeleton. Because some isoflavone compounds were used as phytotherapeutic agents, it prompted us to explore the potential of isoflavone derivatives as 5α-reductase inhibitors. Many isoflavonoids act as phytoalexins, which play an important role in the defense against fungal infection. Isoflavonoid derivatives also possess a diverse range of biological activities including antimicrobial, oestrogenic, antioxidant, stimulating nerve growth, and insecticidal activities. A perusal of the literature indicated that phytotherapeutic agents may be useful in treating BPH, and among them, the most potent extract genistein has an IC_{50} of 35 μM against type II 5α-reductase in noncompetitive mechanism.[16]

Consequently, we created an in-house database that consisted of 151 isoflavone core compounds in the NCI database. The pharmacophore was mapped to the in-house database, and there were 37 hits found. The initial results indicated that these isoflavones might be a potential novel class of nonsteroidal 5α-reductase inhibitors. Eight isoflavone derivatives **19a**−**h** (Figure 3) were generously supplied by the original authors.[17] Therefore, they were chosen to demonstrate the fitting with the pharmacophore (Supporting Information) and were subjected to enzymatic inhibition assay.

On the whole, these isoflavones are divided into two categories in accordance with the estimated human type II 5α-reductase IC_{50} and fitting model. The first category containing **19a**−**d** (IC_{50} of 10−27 nM) is predicted to be much more potent than the second category containing **19e**−**h** (IC_{50} of 0.4−23 μM). More interestingly, the hydrogen bond acceptor HB2 fits the carbonyl group of **19a**−**d** in the first category whereas the carbonyl group of **19e**−**h** is located on the other side in the second category (Supporting Information).

The experimental IC_{50} values of **19a**−**h** against rat 5α-reductase are in the range 6.9−49 μM (Figure 3). These results intimate the potential of isoflavone derivatives as a new class of nonsteroidal 5α-reductase inhibitors that are compliant with the phytotherapeutic agents.[16] Nonetheless, these isoflavone derivatives all have IC_{50} values against rat 5α-reductase on a micromolar scale, and they are not as potent as previously reported inhibitors. Previous investigations[15] have reported compounds with very low or even no inhibitory

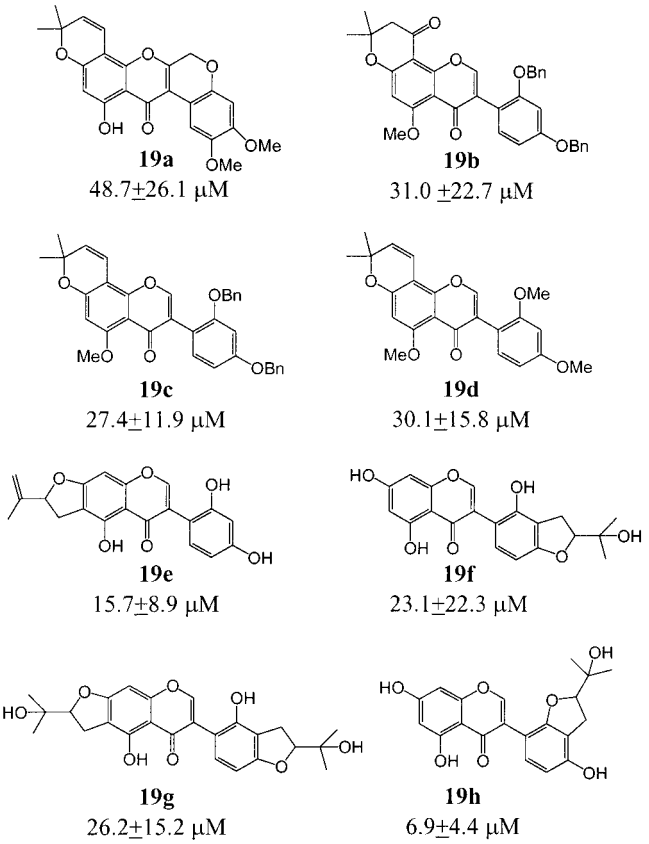


Figure 3. Structures of isoflavone derivatives **19a**−**h** and IC_{50} values against rat 5α-reductase.

activity on rat 5α-reductase but with high inhibitory activity on human 5α-reductase. It should be noted that, according to our generated pharmacophore, compounds **19a**−**d** were illustrated as highly potential inhibitors for human type II 5α-reductase. The lower inhibitory activity of **19a**−**h** against rat 5α-reductase might be due to the species difference. For that reason, the enzyme inhibitory assays of **19a**−**h** against human 5α-reductase are currently ongoing.

Conclusion. We have generated a pharmacophore using the Catalyst program for the active sites of 5α-reductase. Employing the generated pharmacophore, we have characterized isoflavone derivatives as a novel class of nonsteroidal 5α-reductase inhibitors. Because these isoflavonoids did not superimpose the hypothetic pharmacophore very well, we are currently actively conducting research in the design and synthesis of a series of isoflavone derivatives based on the features of the active sites generated by molecular modeling and the concept of a bisubstrate analogue between the NADP coenzyme and steroids. In such ways, it would be a new tool for finding other more potent and selective inhibitors of 5α-reductase.

In summary, this investigation has demonstrated a practical approach toward the development of lead compounds through a hypothetic pharmacophore via 3D database searching. Continuing optimization on these lead compounds may lead to a potential drug candidate against 5α-reductase for the treatment of BPH. The greatest advantage of this approach is the use of fewer compounds of the training set instead of thousands of known compounds to generate new lead. We did not attempt to define the pharmacophore of 5α-reductase, but we did a successful virtual screening for lead generation via a small group of known compounds.

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Supporting Information Available: Tables 1 and 2 of computational data and figures of mapping pictures. This material is available free of charge via the Internet at http://pubs.acs.org.

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