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Identification of Previously Unrecognized Antiestrogenic Chemicals Using a Novel Virtual Screening Approach

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Abstract

The physiological roles of estrogen in sexual differentiation and development, female and male reproductive processes, and bone health are complex and diverse. Numerous natural and synthetic chemical compounds, commonly known as endocrine disrupting chemicals (EDCs), have been shown to alter the physiological effects of estrogen in humans and wildlife. As such, these EDCs may cause unanticipated and even undesirable effects. Large-scale *in vitro* and *in vivo* screening of chemicals to assess their estrogenic activity would demand a prodigious investment of time, labor, and money and would require animal testing on an unprecedented scale. Approaches *in silico* are increasingly recognized as playing a vital role in screening and prioritizing chemicals to extend limited resources available for experimental testing. Here, we evaluated a multistep procedure that is suitable for *in silico* (virtual) screening of large chemical databases to identify compounds exhibiting estrogenic activity. This procedure incorporates *Shape Signatures*, a novel computational tool that rapidly compares molecules on the basis of similarity in shape, polarity, and other bio-relevant properties. Using 4-hydroxy tamoxifen (4-OH TAM) and diethylstilbestrol (DES) as input queries, we employed this scheme to search a sample database of ~200 000 commercially available organic chemicals for matches (hits). Of the eight compounds identified computationally as potentially (anti)estrogenic, biological evaluation confirmed two as heretofore unknown estrogen antagonists. Subsequent radioligand binding assays confirmed that two of these three compounds exhibit antiestrogenic activities comparable to 4-OH TAM. Molecular modeling studies of these ligands docked inside the binding pocket of estrogen receptor α (ERα) elucidated key ligand–receptor interactions that corroborate these experimental findings. The present study demonstrates the utility of our computational scheme for this and related applications in drug discovery, predictive toxicology, and virtual screening.

Introduction

Estrogen is an essential hormone in biological processes such as sexual differentiation and development, reproduction, and bone health. Numerous natural and synthetic chemicals have

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been shown capable of activating or antagonizing estrogenic responses in various species (1–6). These chemicals are called endocrine disrupting chemicals (EDCs) in view of their potential to disturb the normal endocrine processes of estrogen and other endogenous hormones (7) and, thereby, give rise to deleterious health effects (8,9). The *in vitro* and *in vivo* (anti)estrogenic actions of chemical compounds, such as DDT, PCBs, and various insecticides and herbicides, have been well documented. Moreover, these compounds have been implicated in exerting a detrimental influence on the reproductive capability of wildlife as well as affecting normal development and gender-specific differences (10–14). Given the large and growing number of chemicals that pose a risk in this regard, there is a great need for fast, reliable tools to identify potential EDCs. Along with the development of various *in vitro* and *in vivo* assays, *in silico* (virtual) screening and related molecular modeling approaches present an efficient option to meet this global challenge (15–17).

Existing computational approaches (e.g., QSAR) for the rapid screening of large numbers of potential EDCs can require extensive computer resources. Furthermore, they usually require the availability of a significant amount of experimental data so that appropriate molecular descriptors and end points can be defined. An alternative method, *Shape Signatures*, has recently been introduced for the identification of lead compounds in drug discovery (16–19). The *Shape Signatures* method is computationally fast and easy to use in that it avoids specification of complex structural queries or molecular alignment schemes and can accommodate an almost limitless number and variety of molecular entities (organic/organometallics, neutral/charged species, etc.). The method, thus, lends itself to rapid comparison of large databases of chemical compounds with each other or with a known bioactive molecule of interest (the query compound).

In this study, we incorporated *Shape Signature* within a multistep computational scheme to screen large libraries of compounds in search of chemicals that exhibit heretofore unrecognized estrogenic or antiestrogenic activity. Experiments confirm that this novel computational scheme identified two compounds exhibiting significant antiestrogenic activity among a subset of eight chemicals retrieved from commercially available databases. Subsequent molecular modeling of ligand–receptor interactions helped to rationalize the molecular basis for the observed antiestrogenic effects of these two chemicals. The present results encourage more general use of this virtual screening protocol for the identification of chemicals that pose a potential hazard to humans and wildlife.

Experimental Procedures

Computational Methods

Two prototypical synthetic estrogen receptor active compounds, viz., the agonist diethylstilbestrol (DES) and the antagonist 4-hydroxy tamoxifen (4-OH TAM), were selected as queries for the present study. The bioactive conformations of 4-OH TAM and DES were extracted directly from the X-ray crystal structure of their respective complexes with the human ERα ligand binding domain (pdb accession: 3ERT and 3ERD). Both compounds were saved as mol2 files in Sybyl v7.0 (Tripos, Inc., St. Louis, MO) and then uploaded to our in-house server.

Shape Signatures are probability distributions, expressed as histograms, derived from a ray-trace of the volume enclosed by the solvent accessible surface of a small-molecule compound (16). They are rotationally invariant descriptors that can be used to rapidly compare molecules for shape similarity. The method can easily accommodate additional biorelevant properties defined on the molecular surface such as electrostatic charge. Large Shape Signature databases of chemicals can be screened easily and rapidly using simple metrics to identify small

molecules for diverse applications in virtual screening, drug discovery, and predictive toxicology.

The compounds examined for their potential (anti)estrogenic activity comprised a database of >200 000 commercially available organic compounds marketed by Aldrich, ASINEX, BIONET, Leadquest, Maybridge, and Sigma. The low-energy molecular structure of each compound was generated from the corresponding SMILES string using CORINA (http://www.molecular-networks.com/). The computational method and procedures for the conversion of these compounds to their Shape Signature representation has been reported previously (16); the histograms of the query and database compounds were compared using the chi square (χ^2) metric. The deviation between the histograms provided a dissimilarity score between the two molecules being compared.

The 100 top-scoring hits for each of the two queries (DES and 4-OH TAM) based on their 1D Shape Signature scores were combined to yield an initial hit list of 200 compounds. Our present focus was to identify novel estrogen receptor active compounds; hence, we excluded all known (anti)estrogenic compounds from further consideration. The only exception was 4-OH estradiol (4-OH E2), which we retained as an example of a known estrogen that was identified by the *Shape Signature* method. Jarvis–Patrick clustering of the 200 hits represented by 2D MACCS structural keys (MACCS-II, MDL, Ltd., San Leandro, CA) as implemented in the Molecular Operating Environment (MOE, Chemical Computing Group, Inc., Montreal, Quebec, Canada) program resulted in partitioning into 15 categories by (sub)structure similarity.

Using the ligand–receptor docking program GOLD (20), representative compounds from each of the 15 clusters were docked inside the ligand binding site associated with the agonist-bound (3ERD) and antagonist-bound (3ERT) ERα ligand binding domain (LBD) crystal structure. The orientations adopted by 4-OH TAM (3ERT) and DES (3ERD) within the ERα crystal structure were employed as guides to position the docked test compounds. The top-ranked conformation of each ligand was selected from 25 independent dockings, and the corresponding GOLD score was then used to pick chemical compounds for biological evaluation. The Ligand Explorer software (http://users.sdsc.edu/~qzhang/ligand/help/) was used to inspect ligand–receptor binding interactions. The entire screening process is summarized in Figure 1.

Estrogenic Activity Assays

The eight top-scoring hits obtained from GOLD docking, together with two positive controls (DES and 17β-estradiol) and one negative control (the antiestrogen 4-OH tamoxifen), were acquired from the following vendors: 17β-estradiol, 4-OH TAM, and 4-OH estradiol, Sigma-Aldrich, St. Louis, MO; 5386105 and ASN_3780064, ASINEX Corp., Winston-Salem, NC; 3T_0238, 8R_0263, and MS_1105, BIONET, Key Organics Ltd., Camelford, Cornwall, UK); SP-00944, Maybridge, via Ryan Scientific, Inc., Isle of Palms, SC; and 3257–4404, ChemDiv, San Diego, CA. A stock solution of each compound was prepared in DMSO; the DMSO concentration in the final assay was less than 2.5%.

(Anti)estrogenic activity was determined using the NR peptide ERα ELISA kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions. The nuclear extract from the MCF-7 breast cancer cell line was provided as the source of the ERα. The concentration of each test (or control) ligand was 25 μM. The amount of activated ERα was determined using a GENios multifunction microplate reader (TECAN U.S. Inc., Research Triangle Park, NC) to measure the light emitted after the addition of the provided chemiluminescence reagent. The light intensity of the ERα sample with no added ligand was defined as 100%; thus, readings would show >100% for agonists (e.g., DES) and <100% for antagonists (e.g., 4-OH TAM). Each

ligand was tested three times, with duplicate readings for each test. Error bars represent the standard deviations of the three determinations.

ERα Competitive Binding Assays

Binding of the putative ERα agonist/antagonist was determined by a radioligand displacement assay. Purified full length ERα (10 ng) was mixed with [6, 7-^{3}H]-estradiol (specific activity 44 Ci/mmol, Amersham Biosciences) and the test compound (not radiolabeled) in 50 mM Tris-HCl at pH7.5, 1 mM EDTA, 20% Glycerol, and 1 mM DTT buffer. Final concentrations were 25 nM of the ^{3}H-estradiol and 10 nM of the test compound in a total solution volume 1 mL. After 1 h of incubation on ice, the assay was terminated by filtering through Whatman GF/B filter paper. Filters were then soaked in an Ecoscint liquid scintillation mixture (National Diagnostics, Somerville, NJ) to remove the ERα/ligand complex. Radioactivity was measured using a Beckman LS 1071 counter. Results were normalized to the negative control (vehicle only, defined as 100%), and all test compounds were reported as the percentage of bound radiolabeled estradiol retained. Duplicate readings were taken of three independent experiments for each compound; error bars represent the standard deviations of the three experiments.

Results

The Shape Signatures of the >200 000 commercially available chemicals were compared with the Shape Signatures of DES (agonist) and 4-OH TAM (antagonist). Scores ranged from ~0.05 (very similar) to 2.0, which is the upper limit for molecules that have totally dissimilar Shape Signatures. As expected, our search identified many known estrogenic compounds. Because the purpose of the present study was to identify novel (anti)estrogenic compounds, we excluded known (anti)-estrogens. We did, however, retain 4OH-estradiol (4-OH E2) as an instance of a known estrogen that was identified by the *Shape Signature* method. Among the remaining compounds, the top-scoring 100 matches for each query compound (cutoff score <0.1) were selected to yield a total of 200 compounds for further evaluation. Jarvis–Patrick clustering analysis was performed in the MOE program to divide these 200 hits into 15 categories on the basis of chemical structure.

Using GOLD, representative compounds from each of these classes were docked inside the ligand binding pocket of both the ERα-agonist (3ERD) and the ERα-antagonist (3ERT) X-ray crystal structures. We noted that most of the compounds had better docking scores to the antagonist form of ERα, thus predicting them to be antagonists. On the basis of their GOLD docking scores and vendor availability, we selected eight of the compounds for experimental testing.

The results of the (anti)estrogenic activity assays are shown in Figure 2A. The chemiluminescence measured for ERα alone, that is, no ligand, is normalized to 100% activity; all other assays were then reported as a percentage of this activity. The control compounds gave the expected results: 4-OH TAM behaved as a strong antagonist (36% activity), whereas DES (179%) and 17β-estradiol (149%) behaved as strong agonists. The test compounds MS_1105 (53%), and 3257_4404 (69%) exhibited appreciable ERα antagonist activity. Compounds 5386105 and 8R-0263 exhibited weak antagonist activity (82% and 78%, respectively). The 4-OH E2 showed exceptionally high agonist activity (196%). The remaining three test compounds displayed marginal antiestrogenic activity.

To evaluate our computational approach more quantitatively, we compared the GOLD scores of all eleven test and control compounds with their measured (anti)estrogenic activities. A plot (Figure 2B) of the GOLD score versus the absolute difference of the %ERα activation between the compound and the control (Abs (100 – $\%ER\alpha_{compound}$)) revealed a statistically significant

correlation (R^2 = 0.65), thereby adding confidence to our computational approach. Moreover, the plot reveals a clear trend between stronger anti(estrogenic) activity and a higher GOLD score among this structurally diverse set of 11 agonists and antagonists. Two apparent outliers from the linear regression line are 4-OH TAM and 4-OH E2. These deviations are attributed to the positive bias of the GOLD scoring function toward hydrophobic interactions; consequently, the larger 4-OH TAM molecule (MW = 378.5) would score above and the smaller 4-OH E2 molecule (MW = 288.4) below the linear fit.

To understand the relationship between the ligand–ERα binding affinity and (anti)estrogenic activity of our test compounds, we measured the competitive binding of selected compounds. We used purified ERα for these binding assays, unlike the nuclear extract used for the estrogenic assay. By measuring the residual radioactivity after allowing the test compound to displace bound [6,7-^3H] estradiol from the purified ERα, we were able to determine the binding affinity of the test compound. A lower level of residual bound radioligand corresponds to stronger binding of the test compound. These experiments revealed that the test compounds identified as ERα antagonists indeed displaced the radioligand (Figure 3). For MS_1105, only 66% of the [6, 7-^{3}H] estradiol remained. This is comparable to the reduced levels of radioactivity produced by the positive controls, 17β-estradiol (62%) and 4-OH TAM (48%), which are known to have extremely high affinity to ERα. The 3257–4404 yielded somewhat lower displacement (90% retained).

We next employed molecular modeling techniques to explore the specific interactions responsible for the binding affinities of the various ligands. After docking MS_1105 and 3257–4404 inside the ERα binding pocket, we compared their binding poses with those of 17β-estradiol and 4-OH TAM as determined by their crystal structures in complex with ERα. The MS_1105 and 3257–4404 adopted an extended conformation spanning ~12Å, similar to that of the high affinity control molecules. Their precise orientation, however, resembled that of 4-OH TAM more closely than that of 17β-estradiol, in agreement with our experimental findings that these two test compounds function as ERα antagonists rather than agonists.

Close inspection of the four ERα-bound ligands revealed significant differences in their binding interactions (Figure 4). The strong agonist 17β-estradiol is hydrogen bonded to the side chains of three residues (Glu353, Arg394, and His524); the strong antagonist 4-OH TAM is hydrogen bonded to two residues (Glu353 and Arg394). In each case, the protonated guanidinium group of Arg394 forms multiple hydrogen bonds with the ligand. MS_1105 forms a hydrogen bond with the side chain of Glu353, but the key interaction with Arg394 is replaced by a weaker hydrogen bond with the backbone NH of Met388. Compound 3257–4404 forms a single hydrogen bond with Glu353. All compounds form a hydrogen bond with the buried water molecule. In summary, the weaker ERα binding affinities of MS_1105 and 3257–4404 compared with that of 4-OH TAM can be explained in terms of weaker and/or fewer hydrogen bonds. The number of hydrogen bonds and their relative strengths is in general agreement with the order of binding affinities determined by experiment: 4-OH TAM ≥ 17β-estradiol > MS_1105 > 3257–4404 (Figure 3).

An examination of the chemical structures of our eight test compounds (Chart 1) suggests that minor structural modification would improve their (anti)estrogenic activity. For example, conversion of the OMe group in 3T-0238 and 8R-0263, and likewise the NO_2 group in 5386105, to OH would yield the characteristic phenolic OH group associated with virtually all (anti) estrogenic molecules. In other cases, insertion of OH (e.g., SP-00944) or removal of blocking groups (MS_1105) would likewise enhance binding affinity. These examples illustrate a major strength of *Shape Signatures*: its ability to retrieve interesting structures from large, structurally diverse chemical libraries that serve as building blocks for initiating drug discovery programs. In the present study, *Shape Signatures* distinguished itself by identifying previously

unrecognized antiestrogenic compounds (MS_1105 and 3257_4404) from structurally diverse chemical databases.

Discussion

In this study, a multistep virtual screening procedure incorporating *Shape Signatures* succeeded in identifying at least two previously unrecognized (anti)estrogenic compounds from a structurally diverse database of compounds. It is worth noting that these hits are structurally dissimilar to the query compounds and, consequently, might be missed by searching based on (sub)-structure similarity or pharmacophores alone.

MS_1105 and 3257–4404 were predicted as potential antiestrogens by our computational approach, a result that was subsequently confirmed by experiment. These encouraging findings instill confidence that *Shape Signatures* represents a powerful new virtual screening tool for toxicology-related applications. It is worth noting that these or closely related compounds have been studied for various commercial applications as herbicides or pharmaceuticals (21,22).

Shape Signatures shows promise as a tool for virtual screening of chemical databases in applications as diverse as drug discovery and toxicology (17–19). The method streamlines the screening process and significantly reduces time, labor, and cost. One can envision various ways to employ *Shape Signatures* for the virtual screening of potential (anti)estrogenic compounds. For example, the Shape Signature of a known ERα agonist or antagonist might serve as a query to screen collections of untested compounds in search of (anti)estrogenic compounds such as that done in this study. Alternatively, one could screen one or more query chemicals against the Shape Signatures' exclusive collections of (anti)estrogenic compounds. Hits from these procedures would undergo further experimental evaluation. This same general protocol would be applicable to other EDCs (viz., (anti)androgens) and beyond to any chemicals that may pose a threat to humans or wildlife.

We have assembled *Shape Signatures* databases of >300 (anti)estrogenic and >250 (anti) androgenic chemicals. In addition, our general Shape Signatures small-molecule database of commercially available compounds is quite large (2+ million chemicals) and continues to grow. The screening process is exceptionally rapid, and the databases are expandable virtually without limit. Once the profiles are generated, these Shape Signatures databases can be screened time and time again without need for reformulation as required by other methods (e.g., QSAR).

Shape Signatures is fundamentally distinct from both receptor-based (e.g., docking & scoring) and ligand-based (e.g., QSAR, pharmacophore, and substructure similarity) virtual screening methods. In contrast to docking & scoring methods, *Shape Signatures* involves no physical docking of the ligand to the receptor. Instead, the method generates compact representations of molecular shape and surface charge for the ligands. In contrast to QSAR and related ligand-based approaches that compute hundreds of descriptors, the Shape Signature can be viewed as a compact descriptor that encodes molecular shape and electrostatics in a single entity. At the same time, the *Shape Signatures* tool is highly complementary to these traditional *in silico* screening techniques; hence, it can be used alone or together with existing *in silico* approaches related to drug discovery, predictive toxicology, and virtual screening.

A major concern about all predictive models for regulatory purposes is the need to minimize, if not eliminate, false negatives. No single computational method will likely approach, let alone achieve, this holy grail. New and more reliable computational strategies continue to be devised to meet the stringent requirements for regulatory applications. Given the vast numbers of chemicals that may require screening, hierarchical frameworks and consensus prediction models are particularly well-suited to this task. The hierarchical framework organizes

individual computational models starting with the computationally fast but less accurate to the computationally intensive but more accurate. Likewise, consensus prediction models more fully capture the SAR relationship of bioactive molecules by using multiple, independent models rather than a single model. The present study demonstrates the potential utility of the *Shape Signatures* method within such a hierarchical framework-consensus modeling architecture. *Shape Signatures* is ideally suited for rapid screening and prioritizing of potential hazardous chemicals. For example, it would be possible to screen vast libraries of chemical compounds against a database of Shape Signatures corresponding to known and/or suspected toxicants.

We anticipate that the utility of *Shape Signatures* will increase given the recent proliferation of massive, freely available small-molecule databases such as PubChem (http://pubchem.ncbi.nlm.nih.gov/) and eMolecules (http://www.emolecules.com/). Likewise, tremendous progress has been achieved in the development of unified, accessible, and searchable databases devoted to toxicology-related issues (23,24), notably DSSTox (http://www.epa.gov/ncct/dsstox/index.html), the Carcinogenic Potency Project (CPDB: http://potency.berkeley.edu/cpdb.html), the Comparative Toxicogenomics Database (http://ctd.mdibl.org/), and most recently the USEPA's ToxCast project.

Abbreviations

ERα, estrogen receptor α; EDCs, endocrine disrupting chemicals; DES, diethylstilbestrol; 4-OH TAM, 4-hydroxy tamoxifen; E2, 17β-estradiol.

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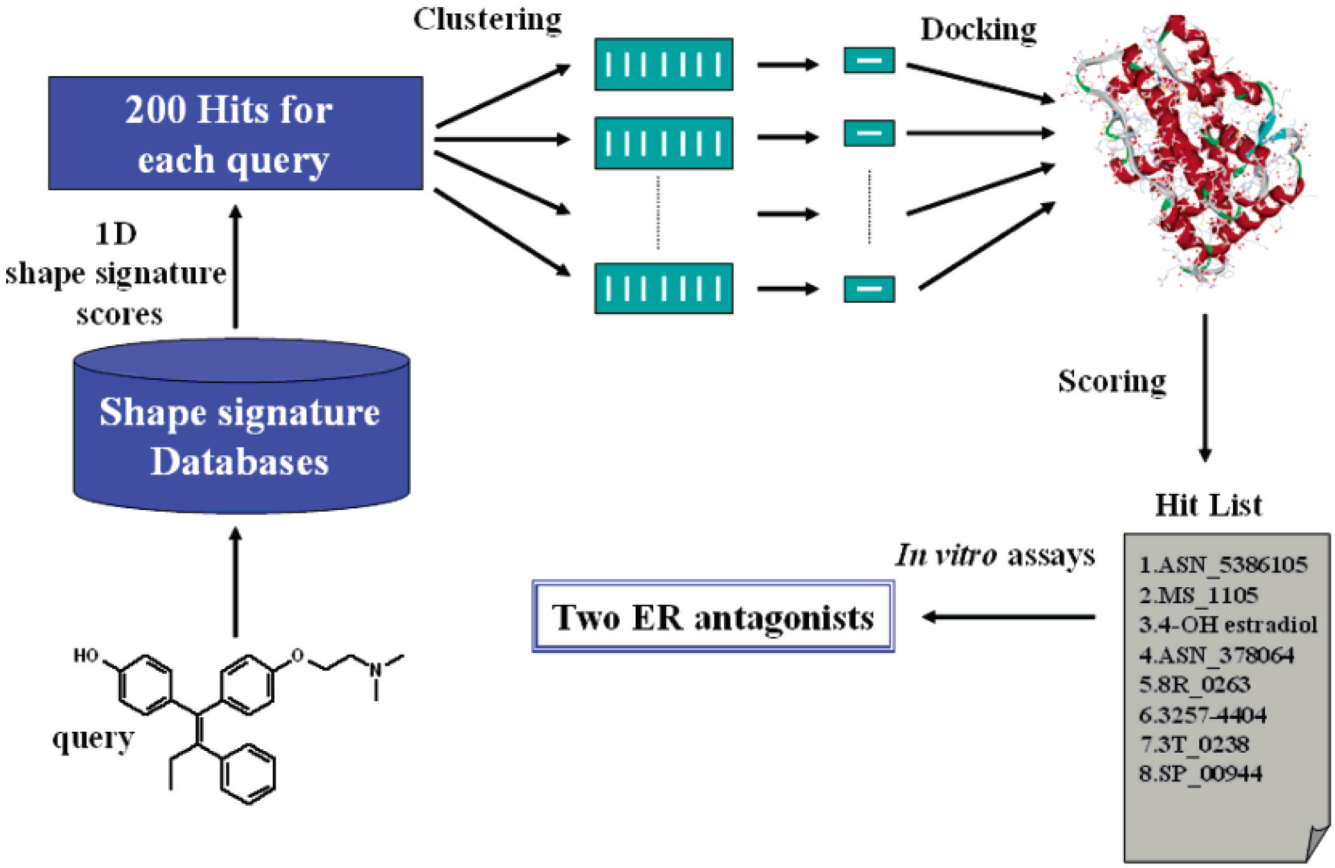

Figure 1.
Overview of computational approach employed to screen our database for the identification of estrogen receptor active compounds. The antiestrogen 4-OH tamoxifen (4-OH TAM) is shown as the query.

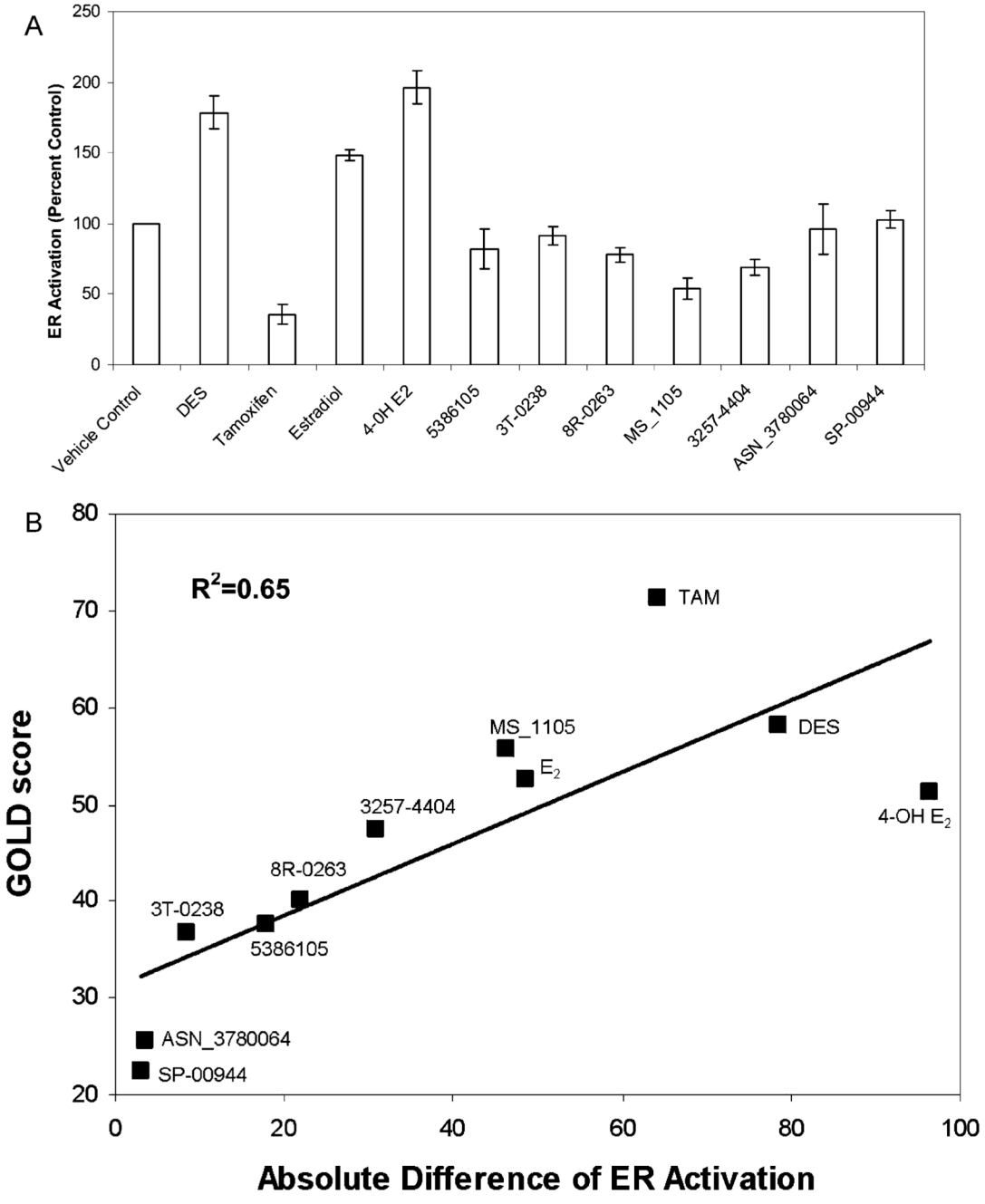

Figure 2.

(A) Results of estrogenic activity assays on hits retrieved by virtual screening. (B) Correlation of GOLD score and absolute difference of % ERα activation between the tested compounds and the vehicle control.

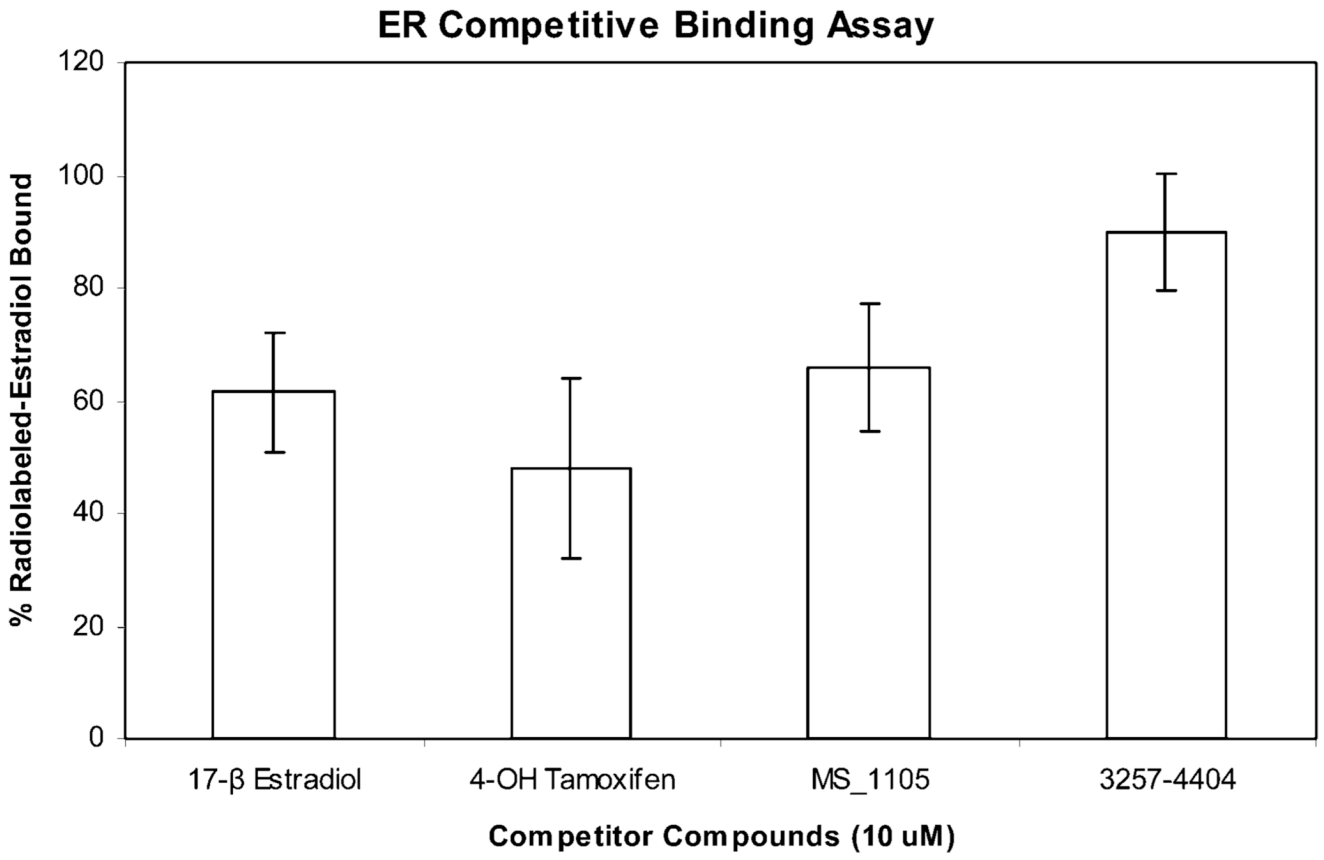

Figure 3.

ERα competitive binding assays on test compounds MS_1105 and 3257–4403. 17β-estradiol and 4-OH tamoxifen were the positive controls (strong ERα ligands). Stronger ERα binding corresponds to a lower percentage of bound radiolabeled estradiol.

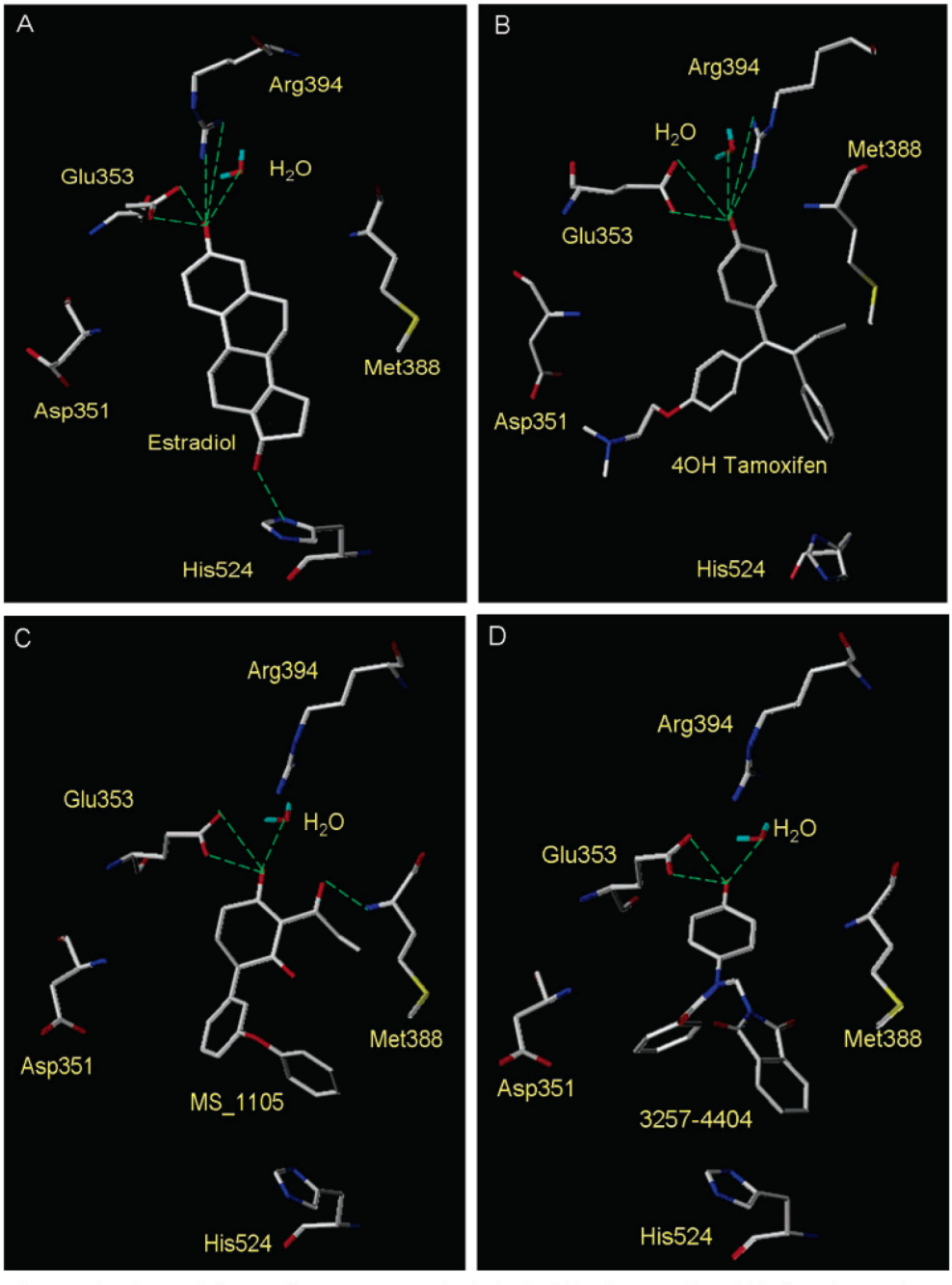

Figure 4.

Snapshots from molecular modeling studies on compounds docked within the ERα ligand binding site. Hydrogen bonds (depicted by green dashed lines) are shown between binding-site residues and the following ligands: (A) 17β-estradiol, (B) 4-hydroxy tamoxifen, (C) MS_1105, and (D) 3257-4404.

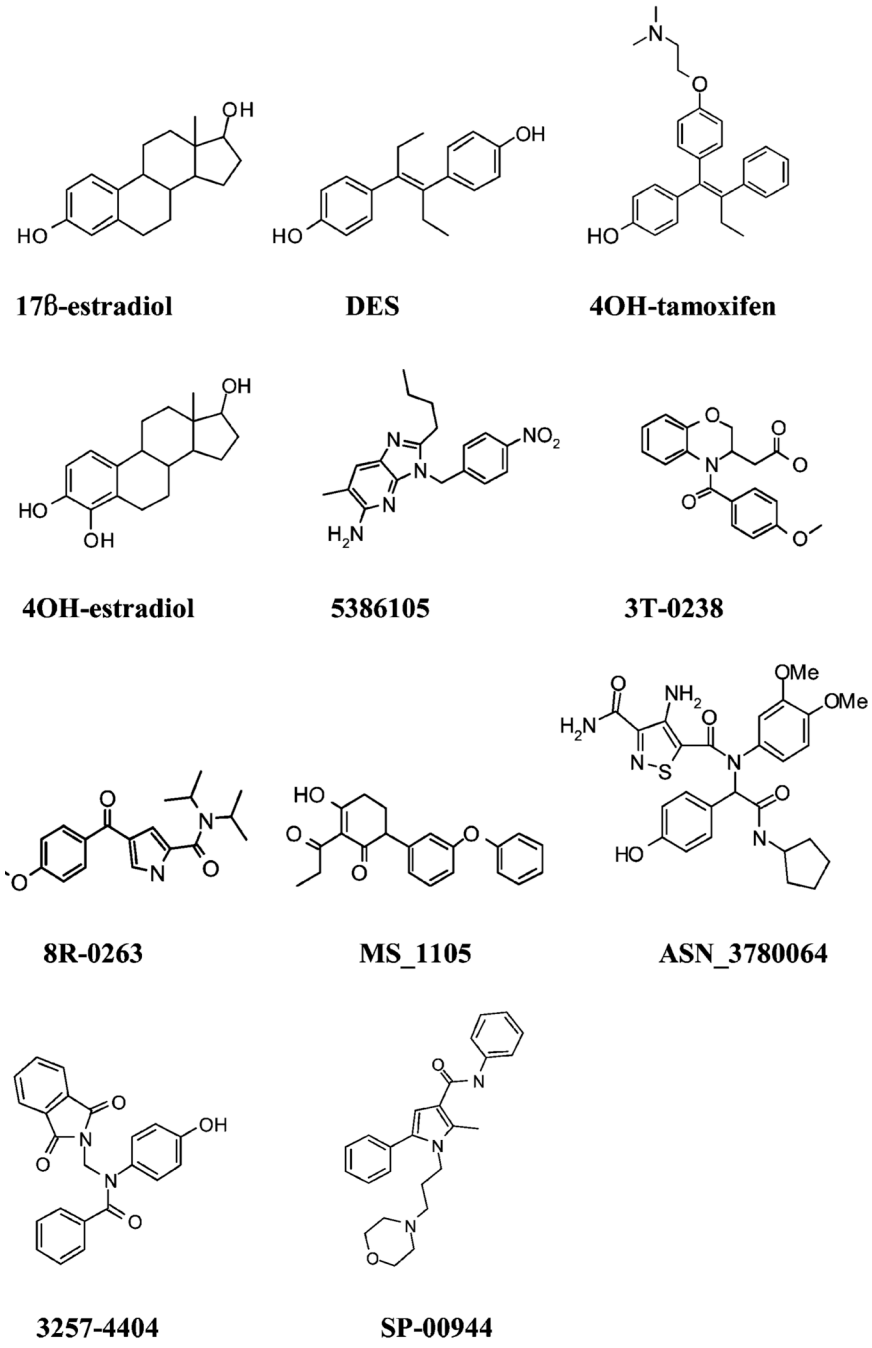

Chart 1.

Chemical Structures of Top-Scoring Hits from the Virtual Screening Procedure[a]

[a] The 17β-estradiol was chosen as a positive control in biological assays. Additional controls DES and 4-OH tamoxifen were employed as queries to screen chemical databases.