Identification of Small-Molecule Regulators of Testicular Receptor 4 via a Drug Repurposing Screening

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ABSTRACT: The testicular receptor 4 (TR4) is a nuclear receptor implicated in multiple pathological processes, including cancer development, chemotherapy, and radiotherapy resistance. However, no effective TR4 small-molecule regulator is available to date. Here, we assessed a physical-interaction-based surface plasmon resonance imaging assay for discovery of TR4 regulators. We screened 1018 FDA-approved drugs and obtained 126 drugs with $K_D$ values below $10^{-6}$ M. The dual-luciferase-based biological assay verified four activatory compounds and two inhibitory compounds against TR4. Among them, nilotinib exhibited the most potent inhibitor, with an EC$_{50}$ of 1.05 $\mu$M, while genistein represented the most potent activator, with an EC$_{50}$ of 2.42 $\mu$M. Both drugs were predicted to bind in the ligand binding pocket of TR4. The circular dichroism spectroscopic assay revealed different conformation changes upon nilotinib or genistein binding. These results established our combined physical and biological approaches as a highly effective way to identify and develop new TR4 regulators.

INTRODUCTION

The testicular receptor 4 (TR4), also known as testicular orphan receptor 4, is a nuclear receptor that belongs to the nuclear hormone receptor subfamily 2 group C. Since it was first cloned from the testis in 1994, its specific tissue distribution, genomic organization, and chromosomal assignment have been well characterized. The TR4 expression profile revealed that TR4 was ubiquitously expressed throughout the body and was highly expressed in the brain, thyroid, testis, and skin. As a nuclear transcription factor, TR4 can form heterodimers that bind to AGGTCA repeats with 0–6 base intervals to transcriptionally regulate target genes involved in multiple physiological and pathological processes. Studies of TR4 knockout mice revealed that TR4 plays critical roles in physiological processes such as cerebellar development, fertility, glucose and lipid metabolism, responses to oxidative stress, muscle development, bone formation, and erythroid maturation. In recent years, it was reported that TR4 promoted the metastasis of several cancers, including prostate cancer, renal cell carcinoma, seminoma, and non-small-cell lung cancer, but suppressed the progression of hepatic cell carcinoma. Moreover, TR4 increased the docetaxel resistance of prostate cancer but sensitized the cis-platinum sensitivity of hepatic cell carcinoma. Besides, TR4 was proven to enhance the resistance to radiotherapy in both prostate cancer and cervical cancer. Since the critical roles of TR4 in human diseases have been revealed, there is a great need for TR4 activators and inhibitors for the purpose of TR4-targeted therapy. However, the information on specific TR4 small-molecule regulators is still quite limited. Several polyunsaturated fatty acids (PUFAs) and their metabolites may serve as the TR4 natural ligands. Meanwhile, a synthetic PPARγ agonist rosiglitazone was proven to activate TR4 transcriptional activity dose dependently. For the potential TR4 inhibitors, only several phosphorylation regulators, such as metformin, MEK-162,
and Parke-Davis (PD) 98059, have been reported to inhibit TR4 transactivation so far. Metformin probably activates AMPK and then induces phosphorylation of TR4 at the Ser351 site to inhibit TR4 transactivation. MEK-162 and PD98059 were ERK inhibitors, which can inhibit the ERK1/2-mediated phosphorylation activation of TR4. Collectively, inhibitors that directly target TR4 are still not available. Due to the 3D structure elucidation of the TR4 ligand binding domain in 2011, the retinol and its derivatives were identified as novel groups of TR4 activators. However, the solved TR4-LBD was in an autorepressed conformation, which hindered the application of high-throughput virtual screening of TR4 regulators.

Surface plasmon resonance imaging (SPRi) technology enables a rapid, real-time, label-free, sensitive, and high-throughput detection of biomolecular interactions. It has been successfully applied to detect compound–protein interaction, protein–protein interaction, DNA hybridization, lectin–glycan interaction, immune-interaction, and even exosome and cell adhesion. Luciferase-based assays have become a valuable tool to evaluate the activity of transcription factors including nuclear receptors. The system consists of a firefly luciferase driven by the cis-acting elements of specific transcription factors and a renilla luciferase as an internal control. In this study, the SPRi method was utilized to identify compounds that could directly bind to the TR4-LBD from the FDA-approved drug library. Next, a dual-luciferase report assay was applied to determine the effect of the binding compounds on TR4 transactivation. In total, 126 drugs from 1018 FDA-approved drugs were found to bind with TR4-LBD. Among them, four compounds could potentially promote TR4 transactivation, whereas two compounds showed inhibitory activity on TR4 transactivation. Nilotinib was found to be the most potent inhibitor, with an EC50 of 1.05 μM, while genistein was found to be the most potent activator, with an EC50 of 2.42 μM. In recent decades, three-dimensional (3D) structure-based protein modeling has helped to provide deeper insight into the understanding of ligand and protein interactions. In this study, a detailed binding network between nilotinib/genistein and TR4-LBD was further determined by the molecular docking method.

### RESULTS AND DISCUSSION

TR4 is a ligand-regulated nuclear receptor, wherein the ligand binding domain (LBD) is responsible for the ligand-induced regulation of TR4. The binding of ligand to the TR4-LBD is the first prerequisite for the ligand-induced regulation of TR4 transactivation. Thus, we applied a 3D small-molecule microarray in conjunction with high-throughput SPRi to identify compounds that can bind to TR4-LBD from the...
FDA-approved drug library. To this end, a pSumo-TR4-LBD plasmid was transfected into Escherichia coli strain BL21(DE3) to obtain the TR4-LBD protein. The purified TR4-LBD protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The distinct band of the TR4-LBD protein was observed at the molecular weight of 25 kDa (Figure 1A), which is consistent with its calculated molecular weight of 29 kDa. This purified TR4-LBD protein was then used in the SPRi assay. The real-time binding of TR4-LBD to the drugs immobilized on the chip is shown in Figure 1B. Based on the SPRi assay, 126 drugs were found to bind to TR4-LBD (Figure 1C), with the equilibrium dissociation constant ($K_D$) ranging from $5.36 \times 10^{-7}$ to $2.33 \times 10^{-13}$ M (Supporting Table 1). The binding curves of 600 nM TR4-LBD to all of the 126 drugs are shown in Figure 1D. Among these drugs, 105 drugs exhibit an associated constant ($K_A$) over $10^3$ (M s$^{-1}$), which indicates a relatively fast binding rate of these drugs to TR4-LBD (Figure 1E). The dissociation constant ($K_D$) of all of the 126 drugs is lower than $10^{-4}$ (s$^{-1}$), demonstrating stable binding of these drugs to TR4-LBD (Figure 1E). Together, results from Figure 1A–E demonstrated that 126 drugs were found to bind to TR4-LBD.

To visualize the effects of the above-identified compounds on TR4 transactivation activity, we use the dual-luciferase assay. TR4 binds to the TR4 response element (TR4RE) as a homodimer and activates target gene transcription. Thus, a dual-luciferase reporter plasmid (pGLO-TR4RE-Dluc) was constructed, which contains a TR4RE-dual-luciferase reporter plasmid (pGLO-TR4RE-Dluc) was transfected into 293T cells, the luciferase activity was enhanced by approximately 60 times upon TR4 transfection, indicating that the luciferase activity is TR4-responsive (Figure 2B). In addition, luciferase activity was elevated by all-trans-retinoic acid (ATRA), a previously reported TR4 activator, and was suppressed by newly identified inhibitor bexarotene (in press) (Figure 2B). Together, these results suggest that the established dual-luciferase reporter system can be used to evaluate the function of a compound on TR4 transactivation. Compound-induced enhanced luciferase activity is recognized as a potential TR4 activator, while suppressed luciferase activity is recognized as a potential TR4 inhibitor (Figure 2C).

Subsequently, the dual-luciferase reporter assay was applied to determine the potential effect of the TR4-LBD-bound drugs on TR4 transactivation. Specifically, after cotransfection with TR4 and the pGLO-TR4RE-Dluc plasmid for 24 h, the 293T cells were incubated with these drugs for another 24 h at a final concentration of 10 μM. Dimethyl sulfoxide (DMSO) was used as a negative control. In total, six compounds were recognized as TR4 transactivation regulators, two compounds showed inhibitory activity against TR4 transactivation, and four compounds stimulated TR4 transactivation to various extents (Figure 3A). Among them, nilotinib (S1342) is the most potent inhibitor, while genistein (S1342) is the most potent activator.

Further dose-dependency analysis revealed that nilotinib induced inhibition of TR4 transcription in a dose-dependent manner; the EC$_{50}$ is 1.05 μM (Figure 3B), which is much lower than that of bexarotene (37.5 μM) (Figure 3D). This observation is consistent with the results from the SPRi assay. According to the SPRi assay, the $K_D$ for nilotinib is 2.33 × 10$^{-3}$ M, while the $K_D$ for bexarotene is 8.48 × 10$^{-3}$ M, indicating a remarkably higher affinity of nilotinib to TR4 compared to that of bexarotene. Genistein also exhibited dose-dependent activation of the TR4 transcription; the EC$_{50}$ is 2.42 μM (Figure 3C), which is approximately 10 times more efficient than that of ATRA (23.8 μM). Moreover, nilotinib almost blocked the activation of TR4 by ATRA and the activatory effect of genistein in the presence of ATRA was equivalent to that of genistein single treatment. The estimated $K_D$ value for ATRA is 1.0 × 10$^{-13}$ M by SPRi analysis. Thus, we deduce that nilotinib and genistein, which showed higher binding affinity compared to ATRA, may compete for the binding pocket of TR4-LBD and block the binding of ATRA to TR4. Together, results from Figure 3A–E showed that nilotinib was identified as a TR4 inhibitor and genistein was identified as a TR4 activator, both of which are more efficient than the previously identified TR4 small-molecule regulators.

The interactions of TR4-LBD with the nilotinib or genistein are explored using the molecular docking method. The model of TR4-LBD in active conformation (with an open binding pocket) was built according to the RXRα (PDB code: 4K6I). A ligand binding pocket of 560 Å was observed with hydrophobic residues Ile405, Cys406, Trp442, Phe446, Ile576, Leu580, and Ile595 (Figure 4C). As predicted, the binding of nilotinib to TR4-LBD was almost blocked the activation of TR4 by ATRA and the ligand binding pocket of TR4-LBD (Figure 4A,B). The LigPLOT program was applied to visualize the detailed interaction network between the candidate ligand and TR4-LBD. As depicted in Figure 4A,B, both nilotinib and genistein settled in the ligand binding pocket of TR4-LBD (Figure 4A,B). The LigPLOT program was applied to visualize the detailed interaction network between the candidate ligand and TR4-LBD. As predicted, the binding of nilotinib to TR4-LBD was mainly achieved through hydrophobic interactions via several residues in the binding pocket of TR4-LBD: Val402, Ile405, Cys406, Ser408, Ala409, Leu412, Phe446, Thr447, Gly449, Leu450, Cys406, Ala409, Leu412, Phe446, Thr447, Gly449, Leu450, Cys453, Ile462, Leu463, Ala464, Leu479, Gln486, Ile491, Leu494, Ile576, Leu580, and Ile595 (Figure 4C).
Moreover, an additional hydrogen bond was observed between the Phe446 main chain and nilotinib (Figure 4C). For genistein, an additional hydrogen bond was formed between the side-chain hydroxyl group of Ser408 and the phenolic hydroxyl group of genistein, despite the fact that less hydrophobic interaction was predicted (Figure 4D). Among the surrounding residues, Trp442, Phe446, Ile576, and Leu580 have been proven to be critical for TR4 transactivation activity by point mutation.20

Resembling the activation of RXRα and COUP-TFII,34−36 TR4 switched the autorepressed LBD to active conformation upon ligand binding, which facilitated the coactivator recruitment and transcription activation by TR4. Hence, the circular dichroism (CD) spectroscopic assay was performed to determine the potential conformational change induced upon binding of nilotinib or genistein to TR4-LBD. The characteristic peak for the β-sheet at 216 nm was significantly reduced upon nilotinib binding (Figure 5A), while the characteristic peak for α-helix at 208 nm was significantly increased upon genistein binding (Figure 5B). These results indicated that the different conformation changes upon nilotinib or genistein binding contributed to the opposite function regulation of TR4 by nilotinib and genistein.

Drug repurposing, a strategy aimed at identifying new uses that are outside the scope of the original medical indication for approved or investigational drugs, has become an attractive proposition due to its advantages over developing an entirely new drug.37 One of the most famous drug repurposing examples is sildenafil. It was initially developed as an antihypertension drug. Retrospective clinical analysis revealed the potential of sildenafil for treatment of erectile dysfunction, and it was eventually repurposed as the wonder drug of erectile dysfunction.37 Other successful drug repurposing examples include zidovudine, minoxidil, thalidomide, celecoxib, atomoxetine, duloxetine, rituximab, raloxifene, topiramate, ketoconazole, aspirin, etc.38 Inspired by these successes, systematic target- and phenotype-based screening approaches have been developed to identify repurposable compounds. Here, we illustrate the identification of TR4 regulators using chips covalently bound with 1018

Figure 3. Functional validation of hit compounds against TR4 transactivation. (A) Regulatory activity of 24 selected FDA-approved drugs (10 μM) on TR4 transactivation. (B) Dose−response curve of the inhibitory activity of nilotinib. (C) Dose−response curve of the activatory activity of genistein. (D) Dose−response curve of the inhibitory activity of bexarotene. (E) Effect of nilotinib (10 μM) and genistein (10 μM) on TR4 transactivation in the presence or absence of TR4 activator ATRA (20 μM). Data are the means ± SD of three independent experiments. One-way ANOVA was used to test differences for statistical significance. *P < 0.05 and **P < 0.01.
FDA-approved drugs via the SPRi assay, providing a novel case of a target-based screening approach that could be readily applied to other disease-associated targets.

Nilotinib, a highly potent BCR-ABL inhibitor, has been approved to treat chronic myelogenous leukemia (CML) that is Philadelphia chromosome positive. In addition to ABL kinase, nilotinib was also reported to interact with and inhibit the receptor tyrosine kinase discoidin domain receptor 1 (DDR1), and targeted inhibition of DDR1 kinase activity with nilotinib may serve as a new therapeutic strategy for metastatic colorectal cancer. EphA4, another receptor tyrosine kinase, was also found to be potently inhibited by nilotinib via a virtual screening-guided approach combined with cellular assays. Besides, the oxidoreductase NQO2 has been revealed as the first non-kinase target of nilotinib recently. NQO2 was inhibited by nilotinib, with an IC50 of 1.8 μM, through a chemical proteomic profiling approach. Inhibition of NQO2 by nilotinib may contribute to the antiproliferative effect of nilotinib in CML cells. Here, we presented TR4 as the second non-kinase target of nilotinib, with an equilibrium dissociation constant (Kd) of 2.33 × 10^{-13} M.

Genistein is one of the most abundant isoflavones derived from soy. It has been used as a potent antineoplastic and...
antihelmintic agent. Genistein is a well-known nonspecific tyrosine kinase inhibitor that inhibits the activity of kinases including EGFR, CDK, PLK1, etc. Moreover, genistein can bind directly to nuclear receptors including estrogen receptor (ER) and androgen receptor (AR); however, whether it acts as an agonist or antagonist of these hormone receptors differs depending on the level of the endogenous hormone ligand. TR4 also belongs to the nuclear receptor superfamily, but the endogenous ligand of TR4 has not been defined yet. Our study demonstrated that genistein enables activation of the transcription activity of TR4 dose dependently, with an EC50 of 2.42 μM. In the presence of TR4 activator ATRA, genistein activated TR4 transcription to a similar extent to that of genistein single treatment.

■ CONCLUSIONS

Conclusively, we identified nilotinib as a potent TR4 inhibitor and genistein as a potent TR4 activator via an SPRi-based FDA-approved drug chip screening in combination with the dual-luciferase assay system. Molecular docking results indicate that nilotinib and genistein bind in the ligand binding site of TR4. The circular dichroism spectroscopic assay revealed a specific conformation change of TR4 upon nilotinib and genistein binding, respectively. As a pro-metastatic role of TR4 has been revealed in urogenital tumors, nilotinib may be applied as an anti-metastasis agent, whereas genistein may be utilized in the treatment of hepatic cell carcinoma to suppress metastasis as well as sensitize cis-platin efficacy. In the future, it is expected that more novel TR4 regulators will be found through SPRi-based screening with personalized compound chips.

■ EXPERIMENTAL SECTION

TR4-LBD Expression and Purification. The TR4-LBD expression plasmid (pSumo-TR4-LBD) was kindly provided by Dr. Zhou from the School of Medicine, Michigan University. TR4-LBD was heterologously expressed in BL21 (DE3). The resulting proteins tagged with a His6-sumo-motif at their N-termini were isolated and purified using a complete His-tag purification column (Roche, Mannheim, Germany). The His-sumo-motif was then removed using the RobustCutter Sumo protease (robustnique, Tianjin, China). Another complete His-tag purification was performed to collect the TR4-LBD protein in the flowthrough. The protein was changed to suitable buffers for the following SPRi (20 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.5) and Circular Dichroism (5 mM phosphate buffer) assay using the PD-10 desalting column (GE Healthcare, Freiburg, Germany). Protein concentrations were determined using the BCA method (ThermoFisher Scientific, Rockford).

SPRi Assay. Binding experiments were performed using the surface plasmon resonance (SPR)-based biosensor instrument PlexArray HT (Plexera Bioscience, Seattle, WA). The FDA-approved drug library (FDA1018) was immobilized on the sensor surface according to the standard procedure and the manufacturer’s instructions. Variable concentrations (150, 300, 600 nM) of TR4-LBD were injected at 2 μL/s, and binding to the small molecules immobilized on the chip was monitored in real time. Each sensorgram consists of an association phase (300 s), reflecting the binding of the injected protein to the drugs, followed by a dissociation phase (300 s), during which the running buffer was passed over the chip and the bound TR4-LBD was washed off the drug surface.

Construction of the pmirGLO-TR4RE Dual-Luciferase Plasmid. The dual-luciferase reporter plasmid pmirGLO was purchased from Promega (Beijing, China). The pGL3-(DR)3-TK was constructed as described previously [34]. The TR4RE-TK-Fluc motif was obtained by double digestion of the pGL3-(DR)3-TK plasmid with NheI/BamHI. The pGLO-TR4RE-Dluc plasmid was obtained by subcloning the TR4RE-TK-Fluc fragment into the NheI/BglII site of pmirGLO.

Cell Culture and Reagents. The COS-7 cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (ATCC, 30–2002) supplemented with 10% fetal bovine serum (Cellmax, Peking, China) and penicillin–streptomycin. The cells were incubated at 37 °C and a 5% CO2 atmosphere. The FDA-approved drugs were obtained from Selleck and stored at −80 °C.

Luciferase Assay. Upon reaching 30–40% confluence in 48-well plates, the COS-7 cells were transfected with 200 ng of the pGLO-TR4RE-Dluc plasmid and 50 ng of the pcDNA3.1-vector/TR4 plasmid using lipofectamine 3000 (Invitrogen, CA). After 24 h of transfection, the cells were treated with drugs for an additional 24 h. Luciferase activity was measured using a dual-luciferase assay kit (Yeasen, Shanghai, China) according to the manufacturer’s instructions.

Molecular Docking Analysis. The receptor model of the TR4-LBD domain in active confirmation was built on the crystal structure of RXR-α (PDB: 4K61) using Modeller. Water molecules, ligands, and other heteroatoms were removed from the protein molecule. The missing hydrogen atoms of the proteins were added and the energy minimization of protein was performed to remove bumps and correct the covalent geometry. The molecular structures of nilotinib and genistein were drawn using Chemical Draw. Thereafter, virtual screening was performed with AutoDock Vina under the default docking parameters [46] and point charges were initially assigned according to the AMBER03 force field.

CD Spectrum Analysis. Circular dichroism measurements were conducted using a Jasco J-815 spectropolarimeter (JASCO, Tokyo, Japan). The circular dichroism spectra of 5 mM phosphate buffer (pH 7.4) were obtained using a cell with a 0.5 cm path length. TR4-LBD protein at a concentration of 0.2 mg/mL with or without nilotinib or genistein (20 μM) was measured using a spectropolarimeter.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Data are presented as mean ± standard error of the mean (SEM) of three independent experiments, each performed at least in triplicate. Group differences were tested for statistical significance using Student’s t-test, one-way ANOVA, and two-way ANOVA as appropriate. P < 0.05 was considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04623.

SPRi screening results (xlsx)
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Author Contributions
L.X. performed protein purification and the circular dichroism spectroscopic assay. L.X., D.S., H.W., L.R. and Y.C. performed the drug activity test. L.X. and G.L. designed the study and wrote the manuscript.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
AMPK, AMP-activated protein kinase; AR, androgen receptor; ATRA, all-trans-retinoic acid; CDK, cyclin-dependent kinases; CML, chronic myelogenous leukemia; COUP-TFII, Chicken Ovalbumin Upstream Promoter-Transcription Factor II; DDR1, discoidin domain receptor 1; EGFR, epidermal growth factor receptor; ER, estrogen receptor; NQO2, N-rasobisulfidihydrinicotinamide: quinone reductase 2; PLK1, polo-like kinase 1; RXRα, retinoic X receptor α; TR4, reticular receptor 4; TR4-LBD, TR4 ligand binding domain; TR4RE, TR4 response element; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; SPRi, surface plasmon resonance imaging.

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