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Identification of androgen receptor antagonists: *In vitro* investigation and classification methodology for flavonoid

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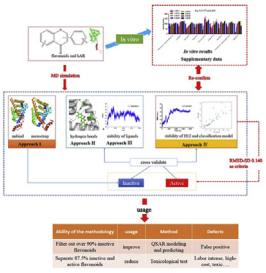
HIGHLIGHTS

- AR-antagonist of different flavonoids were analyzed *in vitro* and *in silico*.
- A methodology based on four approaches that saves labor-intense assays was generated.
- The methodology could filter out over 90% inactive AR-antagonists.

GRAPHICAL ABSTRACT



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ABSTRACT

A tremendous gap exists between the number of potential endocrine disrupting chemicals (EDCs) possibly in the environment and the limitation of traditional regulatory testing. In this study, the anti-androgenic potencies of 21 flavonoids were analyzed *in vitro*, and another 32 flavonoids from the literature were selected as additional chemicals. Molecular dynamic simulations were employed to obtain four different separation approaches based on the different behaviors of ligands and receptors during the process of interaction. Specifically, ligand-receptor complex which highlighted the discriminating features of ligand escape or retention via "mousetrap" mechanism, hydrogen bonds formed during simulation times, ligand stability and the stability of the helix-12 of the receptor were investigated. Together, a methodology was generated that 87.5% of flavonoids could be discriminated as active versus inactive antagonists, and over 90% inactive antagonists could be filtered out before QSAR study. This methodology could be used as a "proof of concept" to identify inactive anti-androgenic flavonoids, as well could be beneficial for rapid risk assessment and regulation of multiple new chemicals for androgenicity.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are natural or synthetic compounds present in the environment which tend to bind to steroid hormone receptors, including estrogen receptor (ER) and androgen receptor (AR) (Li and Gramatica, 2010), causing

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dysfunction of reproductive development and sexual behavior which lead to detrimental effects in animals and humans (Lee et al., 2013). Over the past 20 years, EDCs have attracted both scientific and public concern with major attention being given to identifying chemicals and a wide range of substances in commerce that could cause endocrine disruption (Braun et al., 2014; Strong et al., 2015). However, to date, more than 108 million chemicals are registered in the Chemical Abstracts Service (CAS), which indicates a tremendous gap between the number of potential EDCs possibly in the environment and the limited ability of traditional regulatory testing (Brack, 2005). Thus, Member countries of the Organization for Economic Cooperation and Development (OECD) agreed on the principles for validating models of Quantitative Structure Activity Relationships ((Q)SARs) to be used in the regulatory assessments of chemical safety (de García et al., 2013).

Unlike the traditional process of assessing the risks of EDCs, QSAR studies can be conducted without using large numbers of animals and dangerous highly-purified toxic chemicals, as well as avoid labor intensive protocols. These attributes of QSAR studies are desirable from an economic, ethical, and cultural perspective. The foundation of traditional QSAR techniques is the similarity between chemical structures. By comparing the similarities between tested and untested compounds, the activities of untested chemicals can be predicted. However, as structural similarity does not necessarily equate with functional similarity, EDCs might exhibit wildly different effects while sharing similar structures; therefore, some inactive compounds could be falsely predicted as being active by QSAR models. Consequently, in QSAR studies, almost all authors only choose known active chemicals and ignore inactive chemicals in model building (Wang et al., 2005; Xu et al., 2007), which is the biggest obstacle of practical use of QSAR technology.

In recent years, some researchers such as Yang et al (Yang et al., 2010). and Mouchlis et al (Mouchlis et al., 2012). attempted to filter out inactive chemicals before QSAR analysis by generating classification models. However, all such models were conducted based on criteria set by the authors themselves, which established a kind of static data discrimination rather than based on the fact that the determinant of active or inactive chemicals is their interactions with receptors. As these classification methods were based on the same calculation system used to build the models themselves, the results appear to represent explanations of chemical inactive and active states rather than attempt to separate them. Thus, facing with the continuously increasing number of potential EDCs in commerce, it is extremely important to find a fast, accurate and QSAR-independent method to separate inactive and active compounds, and act as a forerunner to make the QSAR prediction more reliable.

Previous studies have shown that the repositioning of Helix12 (H12) in the ligand binding domain (LBD) of nuclear receptors (NRs) plays a vital role in pharmacologic NR function (Watanabe et al., 2014). In addition, some studies have reported that transcriptional activity might be influenced not only by binding site interactions but also by the process of binding or unbinding (Winn et al., 2002; Li et al., 2007). AR, whose function is essential for male phenotype, male sexual behavior and fertility (Smith and Walker, 2014), is a member of the estrogen-receptor like subfamily of nuclear receptors (Laudet, 1997; Committee, 1999; Kohno et al., 2008). Exploration of interaction processes between ligands and receptor can be measured for screening AR-antagonists, which could be conducted by Molecular dynamic (MD) simulations (Wang et al., 2015).

Flavonoids, which are one of the largest classes of plant secondary metabolites with over 8 000 varieties and have been considered as potential EDCs, were investigated in present study. Therefore, the interactions of flavonoids with the human androgen receptor (hAR) were thoroughly studied to obtain a fast and accurate method to separate chemicals as active and inactive AR-antagonists. Three research steps were conducted as follows: 1) 21 flavonoids were chosen to detect the anti-androgenic activity by a cell-based assay to demonstrate the difficulty to discriminate inactive chemicals by the traditional method; 2) Another 32 flavonoids with or without anti-androgenic activities from the literature were chosen as additional chemicals for traditional 3D-CoMSIA model building and classification testing, to illustrate the disadvantages of the traditional QSAR method; 3) An unrestrained all-atom MD simulation and other computational methods were combined to provide insights into the different behaviors of ligands upon their interactions with receptors, upon which different separation approaches that could discriminate inactive chemicals were generated. This research, with both *in silico* and *in vitro* investigations, generates a "proof of concept" to identify inactive anti-androgenic flavonoids and has potential benefits for identifying anti-androgenic chemicals prior to or without toxicity testing, and might guide more efficient and objective assessments of risks of EDCs that interact with hAR.

2. Materials and methods

2.1. Chemicals

The 21 tested chemicals (>98% pure) were purchased from J&K Scientific (J&K Scientific Ltd., Beijing, China). The 5α-dihydrotestosterone (DHT; >99.5% pure) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO; Tedia Co. Ltd., Fairfield, OH, USA) and stored at −20 °C. Moreover, another set of 32 flavonoids was selected from published literature (Nishizaki et al., 2009), 5 of them (apigenin, 3-hydroxy-6-methoxyflavone, prunetin, naringenin and 6-methoxyflavanone) were also tested for cross-validation. The common structure of all flavonoids and structural features of all chemicals are listed in Fig. S1 and Table S1 (Supporting Information).

2.2. MDA-kb2 cell culture and reporter gene assay

MDA-kb2 cells (catalog number CRL-2713; American Tissue Culture Collection, Manassas, VA, USA) are stably transfected with a luciferase reporter gene, which is driven by an androgen response element-containing promoter. The cells were maintained as the previous study (Liu et al., 2011; Wang et al., 2013), then seeded at a density of 1×10^5 cells/mL in 384-well white opaque plate (Corning Inc., Corning, NY, USA) with 80 μL of assay media per well and incubated for 24 h. The cells were then exposed to seven dilutions (from 1×10^{-8} mol/L to 1×10^{-5} mol/L) of tested chemicals with or without DHT (1 nM). A blank control and a solvent control were presented in each plate. Various concentrations of DHT (from 1×10^{-13} mol/L to 1×10^{-6} mol/L) were included in each plate for quality control. Luciferase activity was measured immediately with Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Each chemical was assayed independently at least 3 times (3 replicate assays) with a minimum of 3 wells per each replicate assay.

2.3. Structural models preparation

The 3D-structures of the compounds were initially constructed using the sketch molecular module of the Sybyl 7.3 molecular modeling package (Tripos Inc., St. Louis, MO, USA). All hydrogen atoms were added, and the compound geometries were subsequently optimized using a Tripos force field with Gasteiger-Hückel