inorganics

MDPI

Article

Steroid-Functionalized Titanocenes: Docking Studies with Estrogen Receptor Alpha

Li Ming Gao [1], Wilson Maldonado [2], Xiomara Narváez-Pita [1], José A. Carmona-Negrón [1], Jesus Olivero-Verbel [2] and Enrique Meléndez [1,*]

[1] Department of Chemistry, University of Puerto Rico, P.O. Box 9019, Mayagüez 00681, Puerto Rico; gaoliming512@gmail.com (L.M.G.); xiomara.narvaez@upr.edu (X.N.-P.); jose.carmona@upr.edu (J.A.C.-N.)

[2] Environmental and Computational Chemistry Group, School of Pharmaceutical Sciences, University of Cartagena, Cartagena 130014, Colombia; wmaldonador@unicartagena.edu.co (W.M.); joliverov@unicartagena.edu.c (J.O.-V.)

* Correspondence: enrique.melendez@upr.edu, Tel: +1-787-832-4040

Academic Editors: Luigi Messori and Wolfgang Weigand
Received: 15 October 2016; Accepted: 25 November 2016; Published: 30 November 2016

Abstract: Estrogen receptor alpha (ERα) is a transcription factor that is activated by hormones, with 17β-estradiol being its most active agonist endogenous ligand. ERα is also activated or inactivated by exogenous ligands. ER is overexpressed in hormone-dependent breast cancer, and one of the treatments for this type of cancer is the use of an ER antagonist to halt cell proliferation. We have previously reported four steroid-functionalized titanocenes: pregnenolone, dehydroepiandrosterone (DHEA), *trans*-androsterone, and androsterone. These steroids have hormonal activity as well as moderate antiproliferative activity, thus these steroids could act as vectors for the titanocene dichloride to target hormone-dependent cancers. Also, these steroids could increase the antiproliferative activity of the resulting titanocenes based on synergism. In order to elucidate which factors contribute to the enhanced antiproliferative activity of these steroid-functionalized titanocenes, we performed docking studies between ERα and the titanocenes and the steroids. The binding affinities and type of bonding interactions of the steroid-functionalized titanocenes with ERα are herein discussed.

Keywords: steroid-functionalized titanocene; estrogen receptor; hormones; docking study; anticancer drug

1. Introduction

The discovery of the anticancer activity of cisplatin opened the door to metal-based agents as cancer therapeutics. The clinical utility of cisplatin is, however, hindered by severe toxic side effects, including neuro- and hepatotoxicity as well as development of drug resistance [1–5]. The power of cisplatin in cancer treatment has raised interest in other transition metal-based drugs as potential chemotherapeutics with lower detrimental health effects and different mechanisms of action. Among them, the antitumor activity of titanocene dichloride was explored for the first time in 1979 by Köpf and Köpf-Maier [6]. This compound reached clinical trials phase I and II, but due to its low response on metastatic renal and breast cancers further clinical trials were discontinued [7,8].

Several strategies were pursued to improve the anticancer activity and efficacy of titanocene dichloride. In particular, our group has functionalized titanocene by appending biologically important groups on the cyclopentadienyl (Cp) ring [9]. Our previously reported sex steroid-functionalized titanocenes showed substantial enhancement on their antiproliferative activity relative to titanocene dichloride on MCF-7 and HT-29 cancer cell lines [9]. MCF-7 is a hormone-dependent breast cancer cell line which overexpresses estrogen receptor alpha (ERα), and the pendant groups on these functionalized titanocenes have hormonal activity on ERα and inhibitory effects on breast

cancer [10,11]. In hormone-dependent cancers, estrogen receptor has an important role in the proliferation, development, and migration of breast cancer. Current chemotherapy for this type of cancer is the administration of antiestrogens (antagonist) to halt the rapid cell proliferation induced by β-estradiol. Thus, we initially speculated that the antiproliferative activity of the subject compounds were correlated to ERα recognition [9].

One of the most remarkable organometallic compounds that have encountered application in hormone-dependent breast cancer is ferrocifen, the ferrocene analog of tamoxifen [12]. It is important to point out that tamoxifen is a selective estrogen receptor modulator (SERM) serving as an antagonist, which is commonly used in hormone-dependent breast cancer therapy [13]. Using the known properties of tamoxifen as a basis, ferrocifen was evaluated in a hormone-dependent breast cancer MCF-7 cell line. Ferrocifen showed excellent antiproliferative activity on MCF-7, and docking studies on ERα revealed that its activity is due to the antiestrogenic properties it elicits from the receptor [12]. There are more recent precedents of titanocenes and half-titanocenes with enhanced antiproliferative activity on the MCF-7 cell line, particularly some of them with IC_{50} values in the micromolar range [14–16]. However, the mechanistic aspects of these species have not been thoroughly explored. Given that our functionalized titanocenes (vectorized titanocenes) have steroids with hormonal activity, we envisioned these organometallic species could be transported to the ERα by the vectors (steroids), the later behaving as shuttles. Once the titanocene is inside the cell and bound to the receptor, it may or may not dissociate from the steroid, eliciting its antiproliferative activity.

In order to understand and explain the type of binding interactions in which the subject titanocenes engage, we performed docking studies with the estrogen receptor alpha. The binding affinities and type of bonding interactions the steroid-functionalized titanocenes have in the ERα are presented herein.

2. Results and Discussion

We have previously reported the synthesis and biological activity of seven steroid-functionalized titanocene dichlorides [9]. Among them, **1**, **2**, and **4** (Figure 1) showed significant cytotoxic activity on MCF-7 hormone-dependent breast cancer cell line, with IC_{50} values of 20, 13, and 21 μM, respectively, while the value for titanocene dichloride was 570 μM [9]. These remarkable differences between the parent compound and the steroid-functionalized titanocene motivated us to perform docking studies between the estrogen receptor alpha and titanocene compounds and the pendant vectors (steroids).

The geometry optimization of the steroid-functionalized titanocenes was performed using DFT with B3LYP/Lanl2dz basis set with Gaussian 09 program. These titanocenes are the ligands to be docked on the ERα. Figure 2 shows the optimized structures of the steroid-functionalized titanocenes and Table 1 lists some of the bonding parameters. The optimized structures of **1**–**4** showed the two Cps and the two chlorides engaged in bonding to Ti(IV) in a pseudo-tetrahedral coordination geometry. The Cl–Ti–Cl and Cp–Ti–Cp bent angles range from 97.40°–97.62° and 131.84°–132.76°, respectively. The average Ti–C(Cp) bond distances of unsubstituted rings are 2.44 Å and the average Ti–C(Cp*) bond distances are 2.46–2.47 Å. These bonding parameters are very similar to those reported by X-ray diffraction techniques [17]. The Ti–C(Cp*) bond distances corresponding to the substituted carbon atoms of the Cp ligands (2.61 Å) are substantially longer than the remaining Ti–C distances. **1**, **2**, and **3** have the steroid oriented outward (away) with respect to the chlorides, whereas **4** has the steroid oriented outward with respect to the chlorides as a result of its cis-stereochemistry at C-3 of the androsterone.

Docking calculations of the steroid-functionalized titanocenes were carried out on a previously optimized ERα ligand-binding domain (using Powell's conjugate gradient method) using Surflex-Dock (Sybyl-X 2.0 Program Package, Tripos, St. Louis, MO, USA). Docking calculations were performed using dynamic docking. For comparison, docking studies of the steroids (vectors = pregnenolone, androsterone, *trans*-androsterone, and dehydroepiandrosterone) were performed on the ERα (PDB: 1A52). Table 2 summarizes the docking calculations together with the IC_{50} values on the MCF-7 cell line. The total

score value represents the pKd (−log Kd), which is correlated to binding affinity. As a reference, docking analysis on β-estradiol was performed.

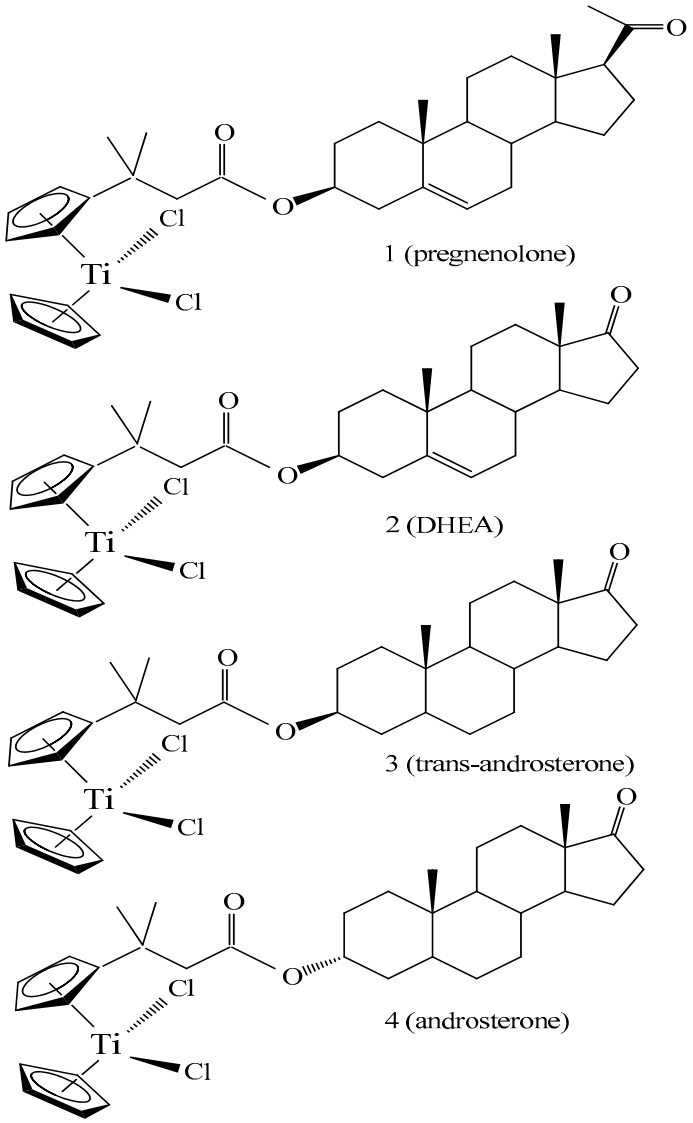

Figure 1. Steroid-functionalized titanocene dichlorides.

Table 1. Selected bond distances and angles of steroid-functionalized titanocenes.

Compound	Ti-Androsterone	Ti-Pregnenolone	Ti-*Trans*-Androsterone	Ti-DHEA
Distances (Å)				
Ti–C(Cp) ave.	2.438 (22)	2.441 (21)	2.441 (21)	2.439 (19)
Ti–C(Cp*) ave.	2.46 (11)	2.47 (10)	2.47 (11)	2.46 (11)
Ti–Cp centroid	2.113	2.119	2.119	2.114
Ti–Cp* centroid	2.139	2.150	2.15	2.140
Ti–Cl	2.376, 2.380	2.378, 2.371	2.377, 2.371	2.379, 2.370
Cp ave.	1.431 (8)	1.424 (8)	1.424 (8)	1.431 (8)
Cp* ave.	1.433 (7)	1.427 (7)	1.427 (7)	1.427 (10)
Angles (°)				
Bent angle	132.76	131.92	131.84	132.72
Cl–Ti–Cl	97.40	97.58	97.62	97.62

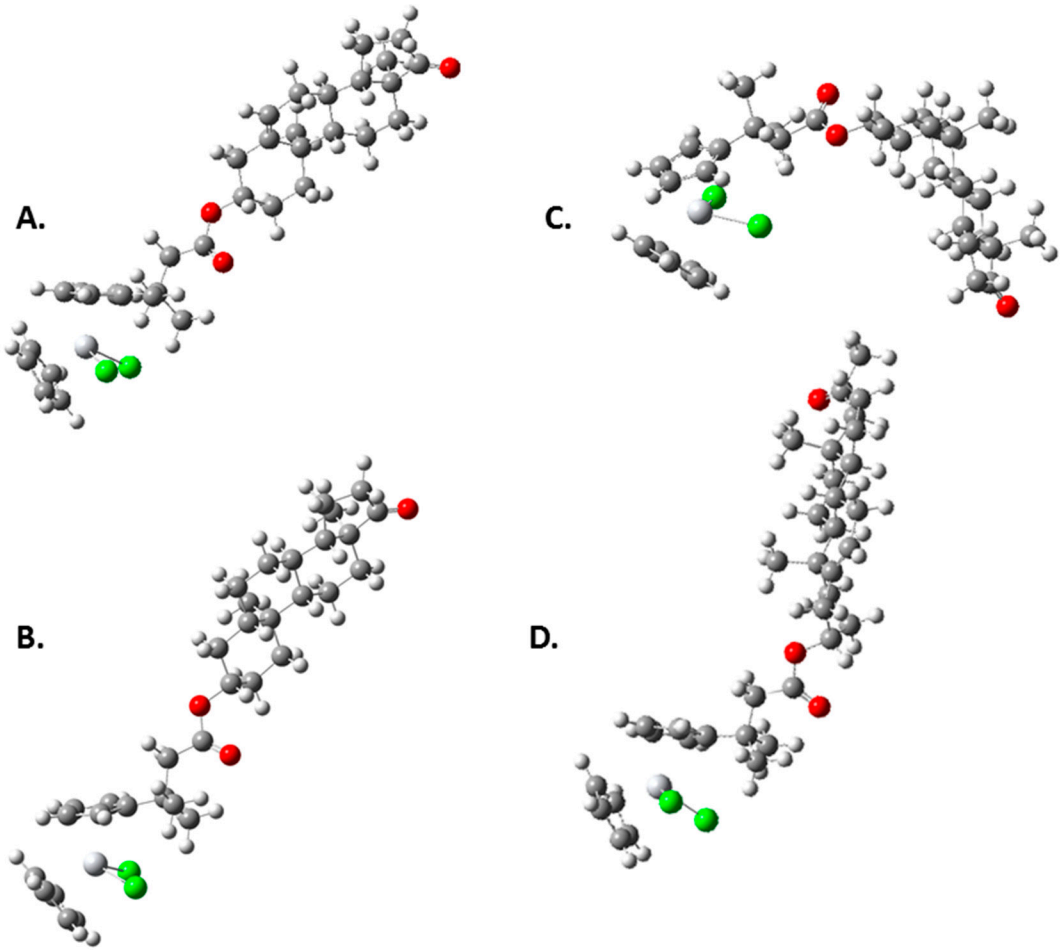

Figure 2. Density functional theory calculated structures of (**A**) titanocene-dehydroepiandrosterone (Ti-DHEA); (**B**) titanocene-*trans*-androsterone (Ti-*trans*-androsterone); (**C**) titanocene-androsterone (Ti-androsterone); (**D**) titanocene-pregnenolone (Ti-pregnenolone).

Due to the size and flexibility of the cavity of the ERα ligand-binding domain (ERα-LBD), it has the ability to embrace a wide variety of nonsteroidal compounds [18]. The cavity size of the LBD (about 450 $Å^3$) is nearly twice the volume occupied by their natural endogenous ligand, β-estradiol (245 $Å^3$). The calculated volume for the four steroid-functionalized titanocene dichlorides is 546 $Å^3$.

Two sets of docking modeling were performed: using PDB 1A52 with titanocene-vectorized complexes and steroids at ProtoMol Grid of 23 × 24 × 23 and ProtoMol Grid of 24 × 29 × 27. The best data was obtained at 23 × 24 × 23, and our discussion will be based on this grid. Upon examination of Table 2 and Figure 3, it is clearly shown that all the steroids (pregnenolone, androsterone, *trans*-androsterone, and dehydroepiandrosterone (DHEA)) dock in the same location and in almost the same orientation as β-estradiol (E2), sharing common amino acid residues such as Glu-353, Arg-394, His-524, Leu-387, and Met-388.

Table 2. Total score for Ti-derivatives, steroids, and β-estradiol on ligand-binding domains estrogen receptor (PDB: 1A52) and cytotoxic data of compounds on hormone-dependent breast cancer cell line MCF-7. "-": no inhibitory activity under conditions tested. [a] Contact residues/type of interaction obtained from crystallographic structure, PDB: 1A52. [b] Contact residues/type of interaction predicted by LigandScout 3.1. std = standard deviation of four independent experiments; * = Values taken from [9].

Ligand	Total Score (Surflex-Dock–log (Kd))	Contact Residues/Type of Interaction	IC_{50} μM (Std) MCF-7 Cell Line
β-estradiol (Native ligand)	7.4641	Glu-353, Arg 394, His-524 (Hydrogen bonds); Leu-387, Met-388, Leu-391, Phe-404 (hydrophobic interactions) [a]	-
Ti-pregnenolone	5.4054	Met-388, Ile-424, Met-421, Leu-384, Leu-525, Ala-350, Trp-383, and Leu-354 (hydrophobic interactions) [b]	20 (2) *
Ti-*trans*-androsterone	8.1246	Leu-354, Trp-383, Met-343, Thr-347, Ala-350, Leu-525, Leu-384, Met-421, Met-388, and Ile-424 (hydrophobic interactions) [b]	40 (25) *
Ti-dehydroepiandrosterone	9.5712	Trp-383, Leu-525, Leu-384, Leu-387, Ala-350, Met-343, Met-421, and Leu-346 (hydrophobic interactions) [b]	13 (2) *
Ti-androsterone	5.7370	Leu-428, Met-421,Ile-424, Leu-384, Met-388, Leu-346, Ala-350, Trp-383, and Thr-347 (hydrophobic interactions) [b]	21 (5) *
Titanocene dichloride	5.0383	Leu-387, Leu-346, Leu-387, Leu-525,Phe-404, Met 421, and Met-388 (hydrophobic interactions) [b]	570 (40) *
Pregnenolone	5.3205	Glu-353, Leu-387, Leu-525 (Hydrogen bonds); Leu-384, Ala-350, Leu-387, Leu-525, Leu-384, Met-421, Met-388 (hydrophobic interactions) [b]	323 (29) *
Trans-androsterone	8.0030	His-524, Met-343, Glu-353, Leu-387 (Hydrogen bonds); Ala-350, Met-343, Leu-525, Leu-384, Leu-387, Met-388, Met-421 (hydrophobic interactions) [b]	119 (9)
Dehydroepiandrosterone (DHEA)	8.9881	His-524, Met-343, Glu-353, Arg-394, Leu-387 (Hydrogen bonds); Leu-525, Ala-350, Leu-384, Leu-387, Met-388, Met-421 (hydrophobic interactions) [b]	105 (5)
Androsterone	7.2547	His-524, Met-343 (Hydrogen bonds); Ala-350, Met-421, Leu-525, Leu-384, Met-343, Leu-387, Met-388 (hydrophobic interactions) [b]	121 (10)

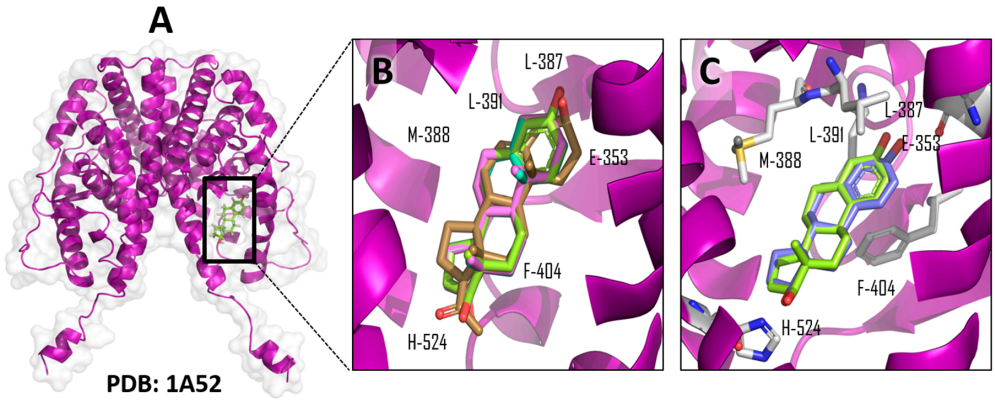

Figure 3. 3D-view estrogen receptor ligand-binding domain (ERα-LBD)-β-estradiol (native ligand) complex (**A**); ERα-LBD-β-estradiol with pregnenolone, dehydroepiandrosterone, androsterone, and *trans*-androsterone on binding site (**B**). Superimposing of β-estradiol predicted using molecular docking protocols (Surflex-Dock), green and experimental β-estradiol on Protein Data Bank (PDB), blue: 1A52 (**C**).

An empirical scoring function was previously used to select the likely binding modes and conformations of the ERα-LBD [19]. The scoring function includes steric, polar, entropic, and solvation terms to estimate the binding affinity of each docked ligand bound to the protein. According to Spyrakis et al. [20], a positive score suggests favorable contributions to the overall binding affinity, while a negative score suggests unfavorable contributions in protein–ligand interactions. The calculated total scores for each docking correspond to their pKd, and these values were used to select the optimal binding geometries. pKd < 4 indicates low affinity, whereas pKd > 9 indicates high affinity. Table 2 shows that DHEA has the highest affinity (8.9881) to ERα, while *trans*-androsterone (8.003) and androsterone (7.255) have similar affinities to the receptor as β-estradiol (7.4641). Remarkably lower affinity is observed for pregnenolone (5.3205).

As mentioned previously, these steroids have hormonal activity and inhibitory effects on breast cancer. Thus, we have determined their antiproliferative activity on hormone-dependent breast cancer cell line MCF-7, which is included in Table 2. DHEA, *trans*-androsterone, and androsterone have similar IC_{50} values (105, 119, 121 μM, respectively), which are different from pregnenolone, IC_{50} = 323 μM. Accordingly, the first three steroids have similar pKd values, which are larger than pregnenolone. As it can be observed, there is a good correlation between binding affinity to ERα and the cytotoxic activity of the steroids. In light of this observation, we examined the data of the functionalized titanocenes with these steroids to pinpoint the role of the vectors.

Figures 4 and 5 show the results of the docking study performed on 1A52 with vectorized-titanocene complexes at ProtoMol Grid: 23 × 24 × 23. While the titanocenes dock in the LBD, the position of the steroids cannot be superimposed with β-estradiol. The titanocene moiety of Ti-DHEA and Ti-*trans*-androsterone are positioned in the β-estradiol binding pocket with the steroid moiety pointing outward from the binding pocket, whereas the Ti-pregnenolone and Ti-androsterone are positioned with the steroid in the β-estradiol binding pocket but in a different orientation with respect to β-estradiol. In all the docking configurations, the titanocene compounds are engaged with different amino-acid residues compared to β-estradiol, and their interactions are hydrophobic in nature.

As mentioned above, Ti-pregnenolone and Ti-androsterone adopt similar docking orientations and both share Met-388, Ile-424, Met-421, Leu-384, Ala-350, and Trp-383 amino-acid residues via hydrophobic interactions. Likewise, Ti-*trans*-androsterone and Ti-DHEA adopt similar docking orientations (but different orientation from Ti-pregnenolone and Ti-androsterone), sharing Trp-383, Leu-525, Leu-384, Ala-350, Met-343, and Met-421 amino-acid residues via hydrophobic interactions, (see Table 2 and Figure 5).

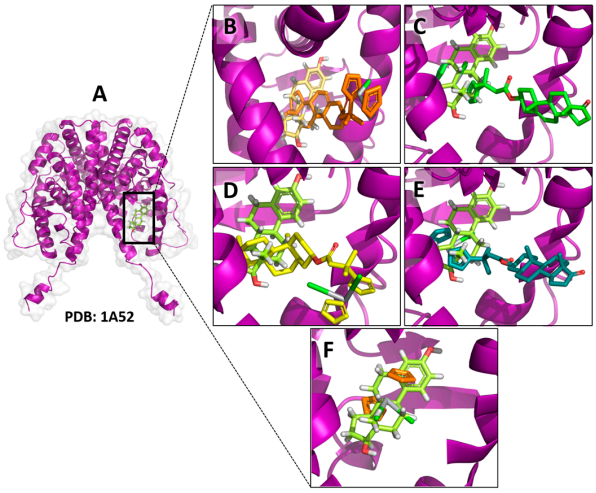

Figure 4. 3D-view ERα-LBD-β-estradiol (native ligand) complex (**A**); ERα-LBD-β-estradiol with Ti-pregnenolone (**B**); Ti-dehydroepiandrosterone (**C**); Ti-androsterone (**D**); Ti-*trans*-androsterone (**E**); and titanocene dichloride (**F**) on binding site.

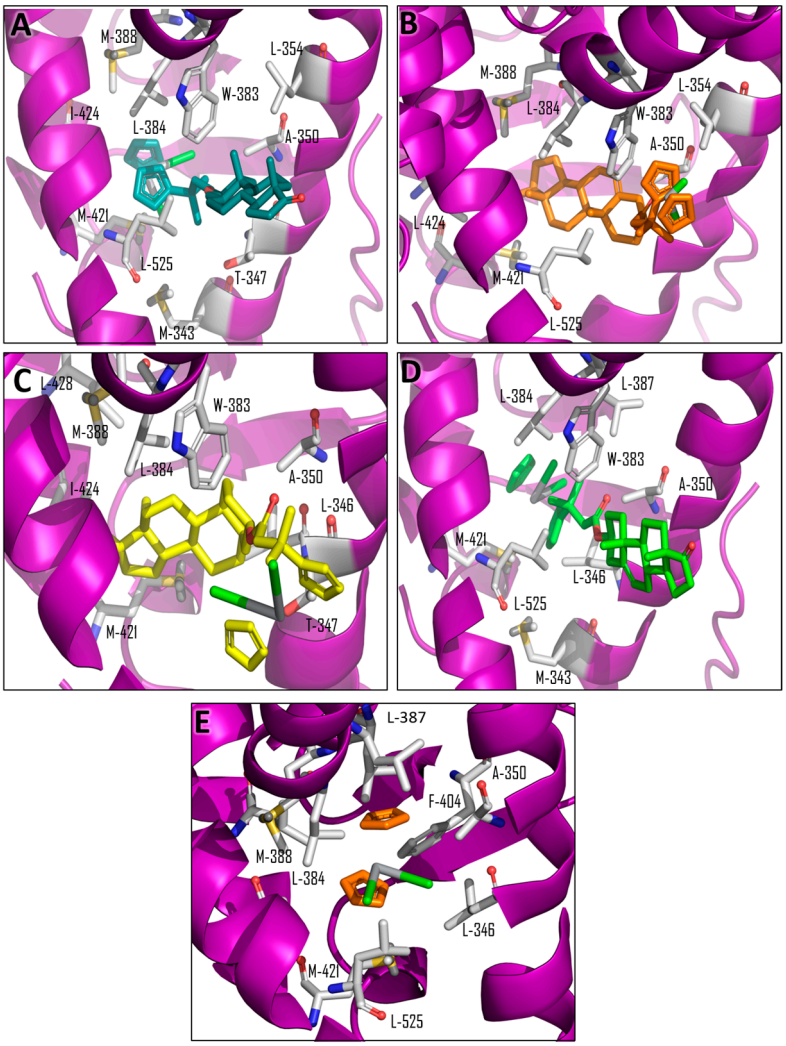

Figure 5. (**A**) ERα-LBD-Ti-*trans*-androsterone; (**B**) ERα-LBD-Ti-pregnenolone; (**C**) ERα-LBD-Ti-androsterone; (**D**) ERα-LBD-Ti-dehydroepiandrosterone; (**E**) ERα-Titanocene dichloride. Interacting residues predicted by LigandScout 3.1 program on binding site.

Given that the cavity size of the LBD is about 450 $Å^3$, in order for the steroid-functionalized titanocene species (V = 546 $Å^3$) to fit in the LBD, helixes 5, 6, and 7 have to be repositioned, leading to a more open conformation (Figure 6).

With respect to the binding affinity to the ERα-LBD (pK_d), titanocene-dehydroepiandrosterone (pK_d = 9.5712) has the higher stability and affinity followed by titanocene-*trans*-androsterone (8.1246) > titanocene-androsterone (5.7370) > titanocene-pregnenolone (5.4054) > titanocene dichloride (5.0383), which displayed moderate affinity to the receptor. However, these binding affinities to the ERα-LBD do not correlate with the IC_{50} values on the MCF-7 cell line. While titanocene-dehydroepiandrosterone has the highest affinity (9.5712) and the best IC_{50} (13 μM), titanocene-androsterone (IC_{50} = 21 μM) and titanocene-pregnenolone (IC_{50} = 20 μM) have high cytotoxic activities on the MCF-7 cell line, but they have low binding affinity to the ERα-LBD, with pKd values of 5.7370 and 5.4054, respectively. Particularly important, with the exception of Ti-DHEA, the incorporation of titanocene dichloride to the steroid does not enhance the binding affinity (pKd) to ERα, but improves the cytotoxic activity of the resulting complex.

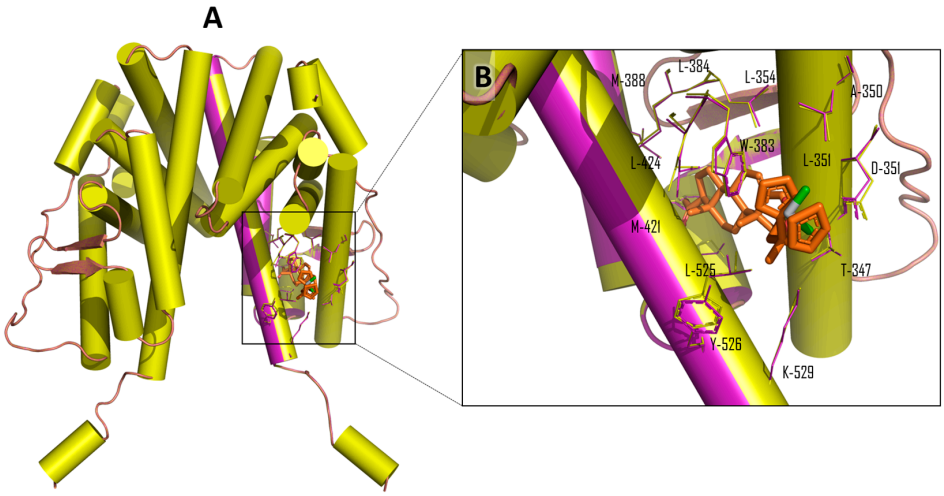

Figure 6. Superposition of ERα-LBD before docking with Ti-pregnenolone (green) and after docking with Ti-pregnenolone complex (violet) *demonstrating the repositioning* and opening of the binding site, (**A**) full structure (**B**) ligand-binding site.

Our docking studies revealed several important points. First, the steroid-functionalized titanocenes engage in hydrophobic interactions within the ERα ligand-binding domain, but the pendant group (steroid) does not adopt the estradiol binding position as pregnenolone, DHEA, androsterone, and *trans*-androsterone do. This indicates that the steroids and the steroid-functionalized titanocenes do not have the same target position. One possible explanation is that the steroid-functionalized titanocenes release the steroid via ester hydrolysis and they become two independent moieties, each one expressing their respective biological activities and targeting. In this regard, synergism between the titanocene and the steroid could be invoked, but receptor recognition does not have a role in their antiproliferative activities. It is also known that this type of compound partially decomposes in an aqueous environment in about 6 h. Another possibility is the titanocene undergoes a stripping process and "Ti^{4+}" binds the ER, as previously proposed by Tacke et al. [21].

3. Materials and Methods

3.1. Theoretical Calculations

A method for predicting ligand-binding sites in estrogen receptor alpha (ERα) for the titanocenes under study was developed using ligand-protein docking computations. The study included 10 ligands: β-estradiol, pregnenolone, dehydroepiandrosterone (DHEA), *trans*-androsterone, androsterone, Cp_2TiCl_2, titanocene-pregnenolone, titanocene-DHEA, titanocene-*trans*-androsterone and titanocene-androsterone.

3.2. Ligand Structure Optimization

The three-dimensional structures of the steroid-functionalized titanocenes forming the data set were drawn using the GaussView 5.0 [22]. All ab initio calculations of the steroid-functionalized titanocenes were performed using GAUSSIAN 09 program [23]. Geometry optimizations were performed using density functional theory (DFT) calculations with B3LYP/Lanl2dz basis set. Repeated cycles of 1000 minimization steps were performed, and the final structure was taken in its lowest energy conformation. Once convergence was reached, these titanocene structures were used for the subsequent docking experiments.

3.3. Molecular Docking Protocols

Docking studies between steroid-functionalized titanocene derivatives and the estrogen receptor alpha ligand binding domain (ERα-LBD) were carried out by using Surflex-Dock (Sybyl-X 2.0 program package, Tripos, St. Louis, MO, USA) [24]. Optimized structures of β-estradiol, pregnenolone, dehydroepiandrosterone, *trans*-androsterone, and androsterone were evaluated using the same docking parameters for comparative purposes.

3.3.1. Protein Preparation

The 3D structure ERα-LDB (Protein Data Bank (PDB) code: 1A52) was previously downloaded from Protein Data Bank (http://www.rcsb.org/pdb/) and prepared for docking calculations by removing all water molecules and others ligands, and repairing and fixing amides of side chains. The resulting structure was optimized using Powell's conjugate gradient method with a distance-dependent dielectric constant value of 1.0, and a gradient convergence value of 0.005 kcal·mol^{-1}, 1000 Max Iterations, using a force field Kollman United and subsequently Kollman all-atoms with AMBER charges.

3.3.2. ProtoMol Generation and Docking Calculations

ProtoMol construction was based on the proximal residues to the native ligand present in the crystallographic structure (ERα, PDB: 1A52) fixing the parameter settings: threshold 0.01 Å and a bloat 10. Additionally, Surflex-Dock details were modified: the options "Include Self Scoring" and "Results Optimization" were activated, allowing protein movement, "Covalent Force Field Weighting" of 1 and 0.6 for ligand and protein, respectively, and "Molecule Fragmentation" option was turned off. All dockings for evaluated ligands performed by Surflex-Dock (maximum of 10 for each) used the same parameters. For all results, only the top-ranked pose returned by Surflex-Dock was used.

3.4. Antiproliferative Studies

The antiproliferative activity of the titanocene-steroids have been previously reported using MTT assay [9]. The stability of the complexes in the 5% DMSO-d_6/water is about 6 h with ~25% decomposition by ^{1}H-NMR. Antiproliferative activity of the steroids was determined using the MTT assay originally described by Mossman, but using 10% Triton X-100 in isopropanol as a solvent for the MTT formazan crystals [25,26] in a manner identical to the titanocenes (dissolved in in 5% DMSO–95% medium). The breast adenocarcinoma cell line MCF-7 was purchased from American Type Culture Collection (Manassas, VA, USA, ATCC HTB22) and was grown under sterile conditions in a culture chamber at 37 °C and 95% air/5% CO_2 (USP grade). MTT and Triton X-100 used for the cytotoxic assay were obtained from Sigma-Aldrich (Milwaukee, WI, USA). All MTT manipulations were performed in a dark room.

Breast adenocarcinoma cell line MCF-7 was grown in Eagle's Minimum Essential Medium supplemented with 10% (*v*/*v*) fetal bovine serum, 1% (*v*/*v*) penicillin/streptomycin, and 0.01 mg/mL bovine insulin. Asynchronously growing cells were seeded at 1.0 × 10^5 cells per well in 96-well plates containing 100 µL of complete growth medium, and allowed to recover overnight. Twenty concentrations of the steroids (0.01 M to 2.9 × 10^{-5} M) dissolved in 5% DMSO–95% medium were added to the wells (eight wells per concentration; experiments performed in quadruplicate plates). The steroid solutions were prepared first by dissolving the corresponding steroid in DMSO, and then medium was added to a final composition of 5% DMSO–95% medium. In addition to the cells treated with the steroids, two control experiments were run: one with 100% medium and one adding 5% DMSO–95% medium to the cells. Both control experiments behaved identically, showing that 5% of DMSO in the medium did not render toxicity to this type of cell. The cells were incubated for an additional 70 h. After this time, MTT dissolved in complete growth medium was added to each well to a final concentration of 1.0 mg·mL^{-1} and incubated for two additional hours. After this period of time, all MTT-containing medium was removed, cells were washed with cold phosphate-buffered saline (PBS) and dissolved with

200 mL of a 10% (*v*/*v*) Triton X-100 solution in isopropanol. After complete dissolution of the formazan crystals, absorbances were recorded in triplicate on a 340ATTC microplate reader (SLTLab Instruments, Sunnyvale, CA, USA) at 570 nm with background subtraction at 630 nm. Concentrations of compounds required to inhibit cell proliferation by 50% (IC_{50}) were calculated by fitting data to a four-parameter logistic plot by means of SigmaPlot software from SPSS (SPSS; Chicago, IL, USA).

4. Concluding Remarks

The present docking studies suggest that the antiproliferative activity of the subject titanocene compounds is not fully associated to estrogen receptor binding affinity, or at least does not correlate with the IC_{50} values. This raises a question about the cytotoxic character of these steroid-functionalized titanocenes. First, *trans*-androsterone, androsterone, and DHEA groups have moderate cytotoxic activity. It is evident that the incorporation of the titanocene dichloride into the steroid scaffold likely enhances the antiproliferative activity by synergism between titanocene dichloride and the steroid, but it is not correlated to the ERα binding affinity. Second, the MCF-7 cell line primarily expresses ERα, but also ERβ to a lesser degree. ERβ is responsive to estrogens and it has antiproliferative and tumor growth inhibition properties on breast cancer [27–29]. Perhaps more important, the MCF-7 cell line also expresses G-protein coupled estrogen receptor (GPER), which is activated by estrogens and steroids [30–33]. Crosstalk between GPER and ERs has been suggested in uterine and ovarian cancers [34–36]. Therefore, this makes the scenario more complex than anticipated but offers the opportunity for new targeted breast cancer therapy. At this point we can only propose that the cytotoxic activity of the subject compounds proceeds mainly from synergism between the appended steroid and titanocene as well as the increased hydrophobic character of the resulting steroid-functionalized titanocenes, facilitating cell entry and permeability to reach the target location.

Acknowledgments: Enrique Meléndez is grateful to the NIH-SCORE Program, NSF-CREST II 000743-00002 and the Department of Chemistry for financial support. We also thank Héctor Collazo, Esq., for the funds provided through the International Health Games.

Author Contributions: Li Ming Gao synthesized the titanocenes and performed the cytotoxic activity on MCF-7. Wilson Maldonado performed the docking on estrogen receptor and Jesus Olivero-Verbel analyzed the receptor binding affinities. Xiomara Narváez-Pita performed the cytotoxic activity on the hormones. José A. Carmona-Negrón calculated the binding site size and geometric constrains. Enrique Meléndez calculated the titanocene structures using Gaussian 09 Program.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lorusso, D.; Petrelli, F.; Coinu, A.; Raspagliesi, F.; Barni, S. A systematic review comparing cisplatin and carboplatin plus paclitaxel-based chemotherapy for recurrent or metastatic cervical cancer. *Gynecol. Oncol.* **2014**, *133*, 117–123. [CrossRef] [PubMed]
2. Sandler, A.; Graham, C.; Baggstrom, M.; Herbst, R.; Zergebel, C.; Saito, K.; Jones, D. An open-label, multicenter, three-stage, phase II study of s-1 in combination with cisplatin as first-line therapy for patients with advanced non-small cell lung cancer. *J. Thorac. Oncol.* **2011**, *6*, 1400–1406. [CrossRef] [PubMed]
3. Pabla, N.; Dong, Z. Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. *Kidney Int.* **2008**, *73*, 994–1007. [CrossRef] [PubMed]
4. Galanski, M.; Jakupec, M.A.; Keppler, B.K. Update of the preclinical situation of anticancer platinum complexes: Novel design strategies and innovative analytical approaches. *Curr. Med. Chem.* **2005**, *12*, 2075–2094. [CrossRef] [PubMed]
5. Dempke, W.; Voigt, W.; Grothey, A.; Hill, B.T.; Schmoll, H.J. Cisplatin resistance and oncogenes—A review. *Anticancer Drugs* **2000**, *11*, 225–236. [CrossRef] [PubMed]
6. Köpf, H.; Köpf-Maier, P. Titanocene Dichloride—The First Metallocene with Cancerostatic Activity. *Angew. Chem. Int. Ed. Engl.* **1979**, *18*, 477–478. [CrossRef] [PubMed]
7. Luemmen, G.; Sperling, H.; Luboldt, H.; Otto, T.; Ruebben, H. Phase II trial of titanocene dichloride in advanced renal-cell carcinoma. *Cancer Chemother. Pharmacol.* **1998**, *42*, 415–417. [CrossRef] [PubMed]

8. Kröger, N.; Kleeberg, U.R.; Mross, K.; Sass, G.; Hossfeld, D.K. Phase II clinical trial of titanocene dichloride in patients with metastatic breast cancer. *Onkologie* **2000**, *23*, 60–62. [CrossRef]
9. Gao, L.M.; Vera, J.L.; Matta, J.; Meléndez, E. Synthesis and Cytotoxicity Studies of Steroid Functionalized Titanocenes as Potential Anticancer Drugs: Sex Steroids as Potential Vectors for Titanocenes. *J. Biol. Inorg. Chem.* **2010**, *15*, 851–859. [CrossRef] [PubMed]
10. Labrie, F. Dehydroepiandrosterone, androgens and the mammary gland. *Gynecol. Endocrinol.* **2006**, *22*, 118–130. [CrossRef] [PubMed]
11. Yoshida, S.; Honda, A.; Matsuzak, Y.; Fukushima, S.; Tanaka, N.; Takagiwa, A.; Fujimoto, Y.; Miyazaki, H.; Salen, G. Anti-proliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines. *Steroids* **2003**, *68*, 73–83. [CrossRef]
12. Hillard, E.A.; Vessières, A.; Jaouen, G. Ferrocene Functionalized Endrocrine Modulators as Anticancer Agents. *Top. Organomet. Chem.* **2010**, *32*, 81–117.
13. Jordan, V.C. The role of tamoxifen in the treatment and prevention of breast cancer. *Curr. Probl. Cancer* **1992**, *16*, 129–176. [PubMed]
14. Saturnino, C.; Sirignano, E.; Botta, A.; Sinicropi, M.S.; Caruso, A.; Pisano, A.; Lappano, R.; Maggiolini, M. Pasquale Longo, New titanocene derivatives with high antiproliferative activity against breast cancer cells. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 136–140. [CrossRef] [PubMed]
15. Sirignano, E.; Saturnino, C.; Botta, A.; Sinicropi, M.S.; Caruso, A.; Pisano, A.; Lappano, R.; Maggiolini, M.; Longo, P. Synthesis, characterization and cytotoxic activity on breast cancer cells of new half-titanocene derivatives. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 3458–3462. [CrossRef] [PubMed]
16. Le Bideau, F.; Dagorne, S. Synthesis of Transition-Metal Steroid Derivatives. *Chem. Rev.* **2013**, *113*, 7793–7850. [CrossRef] [PubMed]
17. Clearfield, A.; Warner, D.K.; Saltarriaga-Molina, C.H.; Ropal, R.; Bernal, I. Structural studies of complexes and their derivatives. The structure of bis(-cyclopentadienyl)titanium dichloride. *Can. J. Chem.* **1975**, *53*, 1622–1629. [CrossRef]
18. Brzozowski, A.M.; Pike, A.C.; Dauter, Z.; Hubbard, R.E.; Bonn, T.; Engström, O.; Ohman, L.; Greene, G.L.; Gustafsson, J.A.; Carlquist, M. Molecular Basis of Agonism and Antagonism in the Oestrogen Receptor. *Nature* **1997**, *389*, 753–758. [CrossRef] [PubMed]
19. Jain, A.N. Surflex: Fully automatic flexible molecular docking using a molecular similarity-based search engine. *J. Med. Chem.* **2003**, *46*, 499–511. [CrossRef] [PubMed]
20. Spyrakis, F.; Cozzini, P.; Bertoli, C.; Marabotti, A.; Kellogg, G.E.; Mozzarelli, A. Energetics of the protein-DNA-water interaction. *BMC Struct. Biol.* **2007**, *7*, 1–18. [CrossRef] [PubMed]
21. Vessieres, A.; Cabestaing, M.-A.P.C.; Claffey, J.; Dieckmann, S.; Hogan, M.; Müller-Bunz, H.; Strohfeldt, K.; Tacke, M. Proliferative and anti-proliferative effects of titanium- and iron-based metallocene anti-cancer drugs. *J. Organomet. Chem.* **2009**, *694*, 874–879. [CrossRef]
22. Dennington, R.; Keith, T.; Millam, J. *GaussView*, version 5; Semichem Inc.: Shawnee Mission, KS, USA, 2009.
23. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G.A.; et al. *Gaussian 09*, revision D.01; Gaussian, Inc.: Wallingford, CT, USA, 2009.
24. *Sybyl-X Molecular Modeling Software Packages*, version 2.0. TRIPOS Associates, Inc.: St. Louis, MO, USA, 2012.
25. Mossman, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxycity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]
26. Denizot, F.; Lang, R. Rapid Colorimetric Assay for Cell Growth and Survival. Modification to the Tetrazolium Dye Procedure Giving Improved Sensitivity and Reliability. *J. Immunol. Methods* **1986**, *89*, 271–277. [CrossRef]
27. Shankle, E.K.; Xu, W. Selectivity targeting estrogen receptors for cancer treatment. *Adv. Drug Deliv. Rev.* **2010**, *62*, 1265–1276. [CrossRef] [PubMed]
28. Palmeri, C.; Cheng, G.J.; Zelada-Hedman, M.; Wärri, A.; Weihua, Z.; van Noorden, S.; Wahlstrom, T.; Coombes, R.C.; Warner, M.; Gustafsson, J. Estrogen receptor beta in breast cancer. *End. Rel. Cancer* **2002**, *9*, 1–13. [CrossRef]
29. Helguero, L.A.; Faulds, M.H.; Gustafsson, J.Å.; Haldosén, L.-A. Estrogen receptors alfa (ERα) and beta (ERβ) differentially regulate proliferation and apoptosis of normal murine epithelial cell line HC11. *Oncogene* **2005**, *24*, 6605–6616. [CrossRef] [PubMed]
30. Prossnitz, E.R.; Barton, M. Estrogen biology: New insights into GPER function and clinical opportunities. *Mol. Cell. Endocrinol.* **2014**, *389*, 71–83. [CrossRef] [PubMed]

31. Rosano, C.; Lappano, R.; Santolla, M.F.; Ponassi, M.; Donadini, A.; Maggiolini, M. Recent Advances in the Rationale Design of GPER Ligands. *Curr. Med. Chem.* **2012**, *19*, 6199–6206. [CrossRef] [PubMed]
32. Arnatt, C.K.; Zhang, Y.G. G Protein-Coupled Estrogen Receptor (GPER) Agonist Dual Binding Mode Analyses Toward Understanding of its Activation Mechanism: A Comparative Modeling Approach. *Mol. Inf.* **2013**, *32*, 647–658. [CrossRef] [PubMed]
33. Barton, M. The membrane estrogen receptor GPER—Clues and questions. *Steroids* **2012**, *77*, 935–942. [CrossRef] [PubMed]
34. Albanito, L.; Madeo, A.; Lappano, R.; Vivacqua, A.; Rago, V.; Carpino, A.; Oprea, T.I.; Prossnitz, E.R.; Musti, A.M.; Ando, S.; et al. G Protein-Coupled Receptor 30 (GPR30) Mediates Gene Expression Changes and Growth Response to 17B-Estradiol and Selective GPR30 Ligand G-1 in Ovarian Cancer Cells. *Cancer Res.* **2007**, *67*, 1859–1866. [CrossRef] [PubMed]
35. Huang, G.S.; Gunter, M.J.; Arend, R.C.; Li, M.; Arias-Pulido, H.; Prossnitz, E.R.; Goldberg, G.L.; Smith, H.O. Co-expression of GPR30 and ERbeta and their association with disease progression in uterine carcinosarcoma. *Am. J. Obstet. Gynecol.* **2010**, *203*, 242e1–242e5. [CrossRef] [PubMed]
36. Gao, F.; Ma, X.; Ostmann, A.B.; Das, S.K. GPER30 Activation Opposes Estrogen-Dependent Uterine Growth via Inhibition of Stromal ERK1/2 and Estrogen Receptor Alpha Phospholyration Signals. *Endocrinology* **2011**, *152*, 1434–1447. [CrossRef] [PubMed]