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Synthesis and cytotoxicity studies of steroid-functionalized titanocenes as potential anticancer drugs: sex steroids as potential vectors for titanocenes

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Abstract

Six titanocenyls functionalized with steroidal esters have been synthesized and characterized by infrared, ^{1}H, and ^{13}C NMR spectroscopy and elemental analysis. Among those steroids, dehydroepiandrosterone, *trans*-androsterone, and androsterone are androgens and pregnenolone is a progesterone precursor. Clionasterol is a natural steroid compound. These steroid-functionalized titanocenyls were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for in vitro cytotoxicity for MCF-7 breast cancer and HT-29 colon cancer cells. All complexes exhibited more cytotoxicity than titanocene dichloride. The titanocenyls containing androgen and progesterone derivatives as pendant groups had higher antiproliferative activities than those with cholesterol steroid compounds. Of particular significance is titanocenyl–dehydroepiandrosterone complex, which is 2 orders of magnitude more cytotoxic than titanocene dichloride and also shows much more sensitivity and selectivity for the MCF-7 cell line.

Keywords

Titanocene dichloride; Sex-steroid-functionalized titanocenyls; MCF-7 breast cancer cell; HT-29 colon cancer cell; Anticancer

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Introduction

The discovery of the anticancer activity of *cis*-diaminodichloroplatinum(II) (cisplatin) marked the beginning of a rich field of transition-metal-based medicinal chemistry. Despite its remarkable success, however, cisplatin has several disadvantages, including notable toxic side effects such as nephrotoxicity, neurotoxicity, and emesis [1]. In addition, some tumors exhibit inherent resistance to cisplatin, whereas others develop resistance after initial treatment, thereby limiting its clinical usefulness [1]. These particular disadvantages have driven the search for new compounds exhibiting high cytotoxic activity along with reduced side effects and no cross-resistance.

In 1979, Köpf and Köpf-Maier opened a new chapter in medicinal chemistry with the discovery of the first metal-locene-based organometallic anticancer agent, titanocene dichloride, Cp_2TiCl_2. The fact that it possesses significant antitumor properties in cancer cell lines that are insensitive to cisplatin as well as lower toxic effects motivated the scientific community to continue investigating this species [2–9]. Unfortunately, the efficacy of Cp_2TiCl_2 in phase II clinical trials in patients with some cancer types [10, 11] was too low for the use of Cp_2TiCl_2 to be pursued. To overcome the aforementioned efficacy problem and to increase the cytotoxic activity of titanocene dichloride derivatives, many titanocenes complexes have been synthesized [12–37].

Our research group has reported the structural modification of titanocene by either replacing chloride with hydrophilic or biologically important ligands or functionalizing the cyclopentadienyl (Cp) ring to study structure– activity relationships [38–42]. The structural modification of titanocene dichloride to enhance its anticancer properties requires a careful selection of the functional group to be appended to the Cp ring or replacement of the ancillary ligands by more active ones. Recently we reported the synthesis, structure, and biological activity of amide-functionalized titanocenyl complexes on the colon cancer cell line HT-29 [43]. We were able to achieve cytotoxic activities (IC_{50} values) on HT-29 in the micromolar range, which are 2 orders of magnitude greater than for titanocene dichloride. Motivated by these optimistic results, we pursued the synthesis of six steroid-functionalized titanocene complexes.

Sex steroids as pendant groups will provide more selectivity to specific cancer cell lines, potentially resulting in target-specific anticancer drugs for hormone-dependent cancers. The present study was undertaken to investigate the synthesis and biological activity of six novel steroidfunctionalized titanocenes. To determine the biomedical potential of the steroid complexes, cytotoxicity testing was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which led to improved cytotoxicities for MCF-7 and HT-29 cell lines, when compared with titanocene dichloride.

The selection of the steroids as pendant groups was based on the following criteria. Androsterone (**6**) and its epimer *trans*-androsterone (**5**; epiandrosterone) are natural androgens produced by the enzyme 5α-reductase from the adrenal hormone dehydroepiandrosterone (**4**; DHEA) [44]. Androsterone is a hormone with weak androgenic activity and can also be produced by the metabolism of testosterone, whereas *trans*-

androsterone is a more active species and has been used as a steroid hormone drug owing to its inhibitory effects on breast cancer [44]. DHEA is a natural steroid hormone produced by the adrenal glands, gonads, adipose tissue, and the brain. It is a precursor of androstenedione, testosterone, estradiol, and estrone and it is the most abundant hormone in the human body. It can act on the androgen receptor directly. DHEA has inhibitory effects on breast cancer [44, 45]. Chloresterol and dehydrochloresterol are precursors of steroid hormones and clionasterol (**1**) is a natural product analog of chloresterol. Pregnenolone (**2**) is a steroid hormone involved in the steroidogenesis of progesterone, mineralocorticoids, glucocorticoids, androgens, and estrogens [46]. It can be enzymatically converted to progesterone. Thus, all the sex steroid derivatives selected as pendant groups are biologically important compounds.

Results and discussion

Synthesis and characterization

The synthesis of the cholesterol-functionalized titanocene dichlorides was performed using a published procedure [47]. This involves the activation of the coordinated Cp ring by an acyl chloride, starting from fulvene. The reaction of the titanocene acyl chloride with the corresponding substituted steroids affords the steroid-functionalized titanocenyl dichloride (Fig. 1). The syntheses of the nine titanocenyl amide complexes presented has been reported previously by our group [43]. In this work, six novel steroid-functionalized titanocene complexes were synthesized to complete the series of titanocenyls with a wide variety of different steroid rings (see “Materials and methods”) (Fig. 2). These complexes are air- and moisture-stable and are soluble in dimethyl sulfoxide (DMSO) and water. They were characterized by IR and NMR spectroscopies and elemental analysis.

In the NMR spectra, these titanocenyl steroids showed –CH–O– signals between 4.52 and 4.96 ppm, corroborating the presence of the 3-αH or 3-βH protons, and the ester carbonyl carbons appeared at about 170 ppm. The IR spectral data of these species revealed characteristic absorption peak carbonyl bands at about 1,728–1,743 cm^{-1} corresponding to the ester groups. The ring signals of the substituted Cp ligand, in both ^{1}H and ^{13}C NMR spectra, are shifted downfield compared with the ring signals of the unsubstituted Cp ligands.

Structural density functional theory discussion

Despite our efforts to crystallize these six titanocenes, no crystal structures were obtained. To overcome this problem, density functional theory calculations were carried out for titanocenyls **2**, **5**, and **6** at the B3LYP level using the 6–31G** basis set. Selected bond lengths of the optimized structures of **2**, **5**, and **6** are listed in Table 1. The calculated structures are presented in Fig. 3.

The calculated structures for **2**, **5**, and **6** showed two η^5-Cp rings and two chlorides in a distorted tetrahedral geometry around the Ti(IV) center. In addition, the steroid pendant groups are positioned away from the chlorides. The Cl–Ti–Cl bond angle for the three complexes is approximately 97.5°. The Cp–Ti–Cp angle for **2** is 123.68°, for **5** is 123.63°,

and for **6** is 123.53°. Whereas the optimum calculated structures of **2** and **5** showed the pendant groups (steroids) to be pointing upward with repect to the Cp ligand plane, avoiding possible steric interactions with the chlorides, the Cp steroid of **6** was in the opposite direction, most likely as a result of its original steroid configuration on carbon 3. However, the steroid is rotated about 78° away from the chlorides, avoiding steric interactions.

The Ti–C(Cp) bond distances for the substituted Cp ring vary from 2.34898 to 2.6078 Å (**2**), from 2.3487 to 2.6136 Å (**5**), and from 2.3753 to 2.6141 Å (**6**), the longest Ti–C bond distance corresponding to the substituted carbon atom of the Cp ligand. The average Ti–C(unsubstituted Cp) bond distances for **2**, **5**, and **6** are very similar and fall within a very narrow range, 2.4403, 2.4398, and 2.4411 Å, respectively.

Although no X-ray structure has been reported for the titanocenyl–steroid complex, recent density functional theory calculations on alkenyl-, boryl-, and dimethylamino-substituted titanocenes have shown the pendant group on the Cp ring to be pointing upward, away from the chlorides, analogous to **2** and **5** [29, 36, 48]. Also, the Ti–C(Cp) bond distances and Ti–Cl bond distances and angles are almost identical to those for our calculated structures [12, 29, 36]. However, **6** showed the opposite direction of the pendant group and it was twisted about 78° to avoid steric interactions. Although the structure of **6** is completely different from that predicted for **2**, **5**, and other substituted titanocenes [12, 29, 36], the fact it is more stable (energetically) than when the pendant group (steroid) is pointing upward with respect to the Cp ligand plane strongly suggests that this is the preferred conformation for this steroid.

Cytotoxicity studies

The cytotoxicities of these complexes for breast cancer MCF-7 and colon cancer HT-29 cell lines were determined using a slightly modified MTT assay at 72 h [48, 49]. As a reference, the cytotoxic activity of Cp_2TiCl_2 was tested at 72 h and IC_{50} values of 413 ± 2.0 and 570 ± 5.0 μM were obtained for HT-29 and MCF-7, respectively. In addition, two control experiments were run in 100% medium and 5% DMSO/95% medium. Both control experiments behaved identically, demonstrating that 5% DMSO in the medium does not have any cytotoxic effect on these cells. Also the amount of CH_2Cl_2 (included as solvate) under the experimental conditions tested had no effect on the cytotoxicity of the complexes.

The IC_{50} data obtained on MCF-7 breast cancer and HT-29 colon cancer cell lines as determined by MTT assay are shown in Table 2 and the dose curves are depicted in Figs. 4 and 5. According to the analysis of the data in Table 2, first, we can observe that all the functionalized titanocenes are more cytotoxic than titanocene dichloride to breast cancer cell line MCF-7 (IC_{50} = 570 μM) and colon cancer cell line HT-29 (IC_{50} = 413 μM). Thus, the steroid functionalization enhances the cytotoxic activity of the titanocenes when compared with Cp_2TiCl_2. Additionally, two groups of titanocenyl complexes can be recognized: those containing cholesterol rings with IC_{50} values over 200 μM, with the exception of complex **1**, and highly active titanocenyls containing sex steroids with IC_{50} values below 50 μM for the MCF-7 cell line.

We should underline that the more highly cytotoxic complexes are those including sex steroid derivatives, whereas those including a cholesterol unit [titanocenyl–clionasterol, **1**; titanocenyl–dihydrocholesterol, **3**; titanocenyl– cholesterol, **7**] showed less cytotoxicity. Thus, there is great potential for the effect of the former species on hormone-dependent cancers such as prostate, testicular, and ovarian cancer to be studied. Also, we can foresee that a titanocenyl–estradiol complex (not yet synthesized) could exhibit high cytotoxicity for the MCF-7 breast cancer cell line.

From a structural point of view, **7** and its analog complexes (**1** and **3**) deserve special attention. These titanocenyls have very similar structures and all of them have very low cytotoxicity (IC_{50} > 200 μM) for the HT-29 cell line. On the other hand, **1** has significant cytotoxicity for the MCF-7 cell line. Since these three titanocenyls have very similar structures, the cytotoxic activity of **1** on MCF-7 may be explained easily in terms of lipophilicity, since **1** has an extra $–CH_2CH_3$ group. An increase in lipophilicity could facilitate the entry of the drug through the cell membrane [50].

Structural differences among the sex steroid derivatives must be also discussed. Titanocenyls **2** and **4** are similar in structure except for the substitution on carbon 17. Complex **4** is 1.5 times more cytotoxic to MCF-7 (IC_{50} 12.6 ± 2.3) than **2**, whereas the opposite behavior is observed for the HT-29 cell line. In addition, the cytotoxic activity of **2** is very similar in MCF-7 and HT-29 cell lines. In other words, since complex **4** is more cytotoxic to the breast cancer cell line it might be a better target-specific drug for the treatment of hormone-dependent cancers. There is a possible explanation for the observed cytotoxicity. DHEA is a naturally occurring steroid synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract, and it is known to have chemopreventive and antiproliferative actions on tumors, in particular on breast cancer [44, 45]. Also, it can act on the androgen receptor directly. Although the results of the investigation may suggest that a steroid containing a ring ketonic carbonyl appears to be more active than a steroid with a ring-substituted acetyl functional group, we believe that **4** is more cytotoxic owing to the inhibitory effects that DHEA itself has on breast cancer rather than because of the difference in the structures of **2** and **4**. Furthermore, when we analyze the IC_{50} values of **4**, **5**, and **6**, the possible structure–activity correlation must be used with caution.

Upon analysis of complex **4** and complex **5**, it is clear that the cytotoxic activity of complex **4** is higher than that of complex **5**. The only difference between **4** and **5** is the presence of a functional double bond in the internal steroid ring, perhaps increasing its hydrophobic character and making it easier to cross the cell membrane, particularly in the MCF-7 cancer cell line. For the pair of epimers, complexes **5** and **6**, the IC_{50} values suggest that the stereochemistry of the androgens has an effect on their cytotoxicity.

It can be envisaged that we will be able to construct some structure–activity relationship to determine the factors needed to enhance the anticancer activity. These observations indicate that further studies of these complexes for their activity in other cancer cell lines, in particular hormone-dependent cancers, is warranted.

Conclusion

The six new titanocenyl dichlorides **1–6** shown in Fig. 2 were prepared using the synthetic method developed previously by Gansäuer et al. [47]. This allowed us to study how the steroid pendant group influences the cytotoxic activity of titanocenyl complexes.

We have discussed some of the structure–activity parameters that may influence the cytoxicity of the new steroid titanocenyl complexes. Our results indicate that the sex steroid complexes have great potential as vectors for anticancer agents. In fact, titanocenyl–steroid complexes **2**, **4**, and **6** exhibit IC_{50} values for the MCF-7 cell line in the low micromolar range and deserve to be investigated in other cell lines. Evidently, besides estrogen receptors, MCF-7 expresses androgen and progesterone receptors [51–53]. Thus, species like **2**, **4**, and **6** could bind progesterone and androgen receptors before expressing their activity, serving the steroids as shuttles. Nevertheless, this possible mechanism needs to be investigated in more detail. We can foresee that these pendant groups could serve as vectors (shuttles) of the titanocenes to make chemotherapeutic agents for hormone-dependent and brain cancers.

Materials and methods

General procedure

All reactions were carried out under an atmosphere of dry nitrogen using Schlenk glassware or a glove box, unless otherwise stated. Reaction vessels were flame-dried under a stream of nitrogen, and anhydrous solvents were transferred by oven-dried syringes or cannula. Tetrahydrofuran was dried and deoxygenated by distillation over potassium benzophenone under nitrogen. Infrared (IR) spectra were obtained with dried KBr pellets. The NMR spectra were obtained with a Bruker DRX-500 spectrometer. For the samples prepared in $CDCl_3$, chemical shifts were referenced relative to $C\underline{H}Cl_3$ at 7.27 ppm (^{1}H NMR) and $\underline{C}HCl_3$ at 77.00 ppm (^{13}C NMR) as internal standards. Analytical data were obtained from Atlantic Microlab.

The breast adenocarcinoma cell line MCF-7 and the colon cancer cell line HT-29 were purchased from American Type Culture Collection (ATCC) and were maintained at 37 °C and 95% air/5%CO_2, as previously reported [43]. The growth medium for MCF7 was Eagle's minimum essential medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic/antimycotic, nonessential amino acids, and 0.01 mg/ml bovine insulin. MTT and Triton X-100 used for the cytotoxicity assay were obtained from Sigma. All MTT manipulations were performed in a dark room.

Synthesis and characterization

Titanocene acyl chloride and its precursor were prepared as described by Gansäuer et al. [47]. As reported by Gansäuer et al. and our group, these complexes crystallize as CH_2Cl_2 solvates [43, 47]. Apparently the inclusion of solvent is important for the stabilization of the complex in the solid state. A CH_2Cl_2 signal appears in all the spectra.

General procedure for the synthesis of complexes 1–6

Complex 1: Titanium carboxylate (0.25 mmol, 77.4 mg) was dissolved in $SOCl_2$ (1.0 ml) and stirred for 2 h at room temperature. Excess $SOCl_2$ was removed under a high vacuum, followed by drying for 24 h. The precipitate was dissolved in CH_2Cl_2 (2.0 ml), the resulting solution was added dropwise to a mixture of NaH (0.75 mmol, 18 mg) and the natural product **1** (0.25 mmol, 100 mg) in CH_2Cl_2 (2.0 ml), and the mixture was stirred for another 20 h. After filtration through Celite, the solvent was washed with a mixture of 1 N HCl and NaCl (1.0 g each 10 ml) (2 × 5.0 ml). The organic layer was dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was then chromatographed on Bio-Bead SX3 (200–400 mesh) (before use, Bio-Bead S-X3 was swollen in CH_2Cl_2 for 24 h) and was eluted with CH_2Cl_2 to give 0.14 g (75%) of viscous red oil. The product was resolved in CH_2Cl_2/hexane at −20 °C and a red solid was obtained. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 6.59 (s, 5H; Cp), 6.49 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 5.37 (dd, ^3J = 5.5 Hz, ^3J = 2.5 Hz, 1H; 6-H), 4.54 (dddd, ^3J = 10.8 Hz, ^3J = 10.7 Hz, ^3J = 6.4 Hz, ^3J = 4.4 Hz, 1H; 3-αH), 2.58 (s, 2H; –CH_2–COO–), 2.26–2.24 (m, 2H), 2.05 (ddd, ^2J = 12.5 Hz, ^3J = 3.6 Hz, ^3J = 3.6 Hz, 1H), 2.01 (dddd, ^2J = 17.4 Hz, ^3J = 5.2 Hz, ^3J = 5.3 Hz, ^5J = 2.1 Hz, 1H), 1.96 (ddd, ^2J = 9.6 Hz, ^3J = 9.6 Hz, ^3J = 6.0 Hz, 1H), 1.88 (ddd, ^3J = 9.5 Hz, ^3J = 9.5 Hz, ^3J = 6.0 Hz, 1H), 1.78 (dddd, ^2J = 12.5 Hz, ^3J = 9.0 Hz, ^3J = 3.7 Hz, ^3J = 3.7 Hz, 1H), 0.98–1.65 (m, 22H), 1.52 (s, 6H), 1.02 (s, 3H), 0.95 (d, ^3J = 6.5 Hz, 3H), 0.89 (d, ^3J = 6.5 Hz, 3H), 0.88 (d, ^3J = 6.5 Hz, 3H), 0.84 (t, ^3J = 6.5 Hz, 3H), 0.69 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 170.6, 146.5, 139.6, 122.7, 120.6, 120.2, 117.1, 117.0, 74.0, 56.8, 56.0, 50.0, 49.5, 46.1, 42.3, 40.4, 38.2, 36.9, 36.8, 36.6, 36.3, 33.9, 32.0, 31.9, 28.9, 28.2, 27.8, 27.8, 27.7, 26.4, 24.3, 23.0, 21.2, 19.6, 19.3, 18.9, 18.8, 12.0, 11.9. IR (KBr, cm^{-1}): 3,109, 3,092, 2,935, 2,867, 1,734, 1,466, 1,440, 1,368, 1,338, 1,175, 1,131, 1,008, 838, 819. Anal. calcd for $C_{44}H_{66}Cl_2O_2Ti$*3/4CH_2Cl_2: C, 66.51; H, 8.42. Found: C, 66.15; H, 8.40.

Complex 2: ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 6.59 (s, 5H; Cp), 6.50 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 5.38 (dd, ^3J = 5.5 Hz, ^3J = 2.5 Hz, 1H; 6-H), 4.52 (dddd, ^3J = 10.8 Hz, ^3J = 10.7 Hz, ^3J = 6.4 Hz, ^3J = 4.4 Hz, 1H; 3-αH), 2.58 (s, 2H; –CH_2–COO–), 2.56 (dd, ^3J = 9.0 Hz, ^3J = 6.5 Hz, 1H; 17-H), 2.26–2.25 (m, 2H), 2.15 (s, 3H; –$COCH_3$), 2.20 (ddd, ^2J = 12.5 Hz, ^3J = 3.6 Hz, ^3J = 3.6 Hz, 1H), 2.05 (m, 2H), 1.90–1.78 (m, 2H), 1.75–1.0 (m, 12H), 1.51 (s, 6H), 1.03 (s, 3H), 0.65 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 209.6, 170.7, 146.5, 139.5, 122.4, 120.6, 120.2, 117.0, 117.0, 73.8, 63.7, 56.8, 49.9, 49.4, 44.0, 38.8, 38.1, 36.9, 36.8, 36.6, 31.8, 31.7, 31.6, 29.7, 27.8, 27.8, 27.7, 24.5, 22.8, 21.0, 19.3, 13.2. IR (KBr, cm^{-1}): 3,111, 2,933, 2,850, 1,728, 1,702, 1,609, 1,439, 1,356, 1,330, 1,223, 1,195, 1,117, 1,014, 826. Anal. calcd for $C_{36}H_{48}Cl_2O_3Ti$*1/8 CH_2Cl_2: C, 65.92; H, 7.39. Found: C, 65.50; H, 7.65.

Complex 3: ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 6.59 (s, 5H; Cp), 6.49 (dd, ^3J = 2.5 Hz, ^4J = 2.5 Hz, 2H; Cp), 4.63 (dddd, ^3J = 10.8 Hz, ^3J = 10.7 Hz, ^3J = 6.4 Hz, ^3J = 4.4 Hz, 1H; 3-αH), 2.56 (s, 2H; –CH_2–COO–), 2.00–1.96 (dddd, ^3J = 9.6 Hz, ^3J = 9.6 Hz, ^3J = 9.0 Hz, ^3J = 3.7 Hz, 1H), 1.87–1.79 (m, 2H), 1.75–1.70 (dddd, ^3J = 9.0 Hz, ^3J = 9.0 Hz, ^3J = 3.7 Hz, ^3J = 3.7 Hz, 1H), 1.70–1.65 (ddd, ^3J = 9.6 Hz, ^3J = 9.6 Hz, ^3J = 3.7 Hz, 1H), 1.60–1.47 (m, 4H), 1.45–0.6 (m, 23H), 1.53 (s, 6H),

0.92 (d, ^{3}J = 6.5 Hz, 3H), 0.89 (d, ^{3}J = 6.5 Hz, 3H), 0.88 (d, ^{3}J = 6.5 Hz, 3H), 0.82 (s, 3H), 0.65 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 170.7, 146.6, 120.6, 120.5, 120.2, 119.5, 117.2, 73.7, 56.4, 56.3, 54.2, 49.6, 44.7, 42.6, 39.9, 39.5, 36.7, 36.3, 35.8, 35.5, 34.1, 32.0, 29.7, 28.6, 28.2, 28.0, 27.8, 27.5, 24.2, 23.8, 22.8, 22.6, 21.2, 18.7, 12.2, 12.1. IR (KBr, cm^{-1}): 3,111, 2,926, 2,867, 1,732, 1,467, 1,443, 1,349, 1,178, 1,128, 1,010, 820. Anal. calcd for $C_{42}H_{64}Cl_2O_2Ti$: C, 70.08; H, 8.96. Found: C, 69.89; H, 9.17.

Complex 4: ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 6.59 (s, 5H; Cp), 6.49 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 5.40 (dd, ^{3}J = 5.5 Hz, ^{3}J = 2.5 Hz, 1H; 6-H), 4.54 (dddd, ^{3}J = 10.8 Hz, ^{3}J = 10.7 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.4 Hz, 1H; 3-αH), 2.59 (s, 2H; –CH_2–COO–), 2.47 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-βH), 2.36–2.21 (m, 2H), 2.15–2.05 (m, 1H), 2.10 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-αH), 1.94 (dddd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 5.7 Hz, ^{3}J = 0.9 Hz, 1H; 15-αH), 1.90–1.83 (m, 3H), 1.72–1.62 (m, 2H), 1.59–1.45 (m, 3H), 1.52 (dd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, 1H; 15-βH), 1.51 (s, 6H), 1.32–1.25 (m, 3H), 1.14–0.90 (m, 2H), 1.04 (s, 3H), 0.89(s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 221.0, 170.6, 146.5, 139.8, 121.9, 120.6, 120.6, 120.3, 117.1, 117.0, 73.7, 51.8, 50.1, 49.3, 47.5, 38.1, 36.9, 36.8, 36.7, 35.9, 31.5, 31.4, 30.8, 29.7, 27.8, 27.9, 27.7, 21.9, 20.3, 19.4, 13.6. IR (KBr, cm^{-1}): 3,110, 2,936, 2,865, 1,736 (br s), 1,438, 1,371, 1,330, 1,197, 1,117, 1,023, 824. Anal. calcd for $C_{34}H_{44}Cl_2O_3Ti$*1/8CH_2Cl_2: C, 65.04; H, 7.10. Found: C, 65.14; H, 7.39.

Complex 5: ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 6.59 (s, 5H; Cp), 6.50 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 4.63 (dddd, ^{3}J = 10.8 Hz, ^{3}J = 10.7 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.4 Hz, 1H; 3-αH), 2.57 (s, 2H; –CH_2–COO–), 2.45 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-βH), 2.09 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-αH), 1.94 (dddd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 5.7 Hz, ^{3}J = 0.9 Hz, 1H; 15-αH), 1.81–1.80 (m, 2H), 1.74–1.19 (m, 13H), 1.52 (dd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, 1H; 15-βH), 1.50 (s, 6H), 1.15–0.97 (m, 2H), 0.89 (s, 3H), 0.85 (s, 3H), 0.75–0.69(m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 221.3, 170.8, 146.5, 120.6, 120.5, 120.2, 117.1, 117.1, 73.5, 54.3, 51.4, 49.5, 47.8, 44.7, 36.7, 36.6, 35.9, 35.6, 35.0, 33.9, 31.5, 30.8, 29.7, 28.2, 27.7, 27.5, 21.8, 20.5, 13.8, 12.2. IR (KBr, cm^{-1}): 3,109, 2,922, 2,852, 1,743, 1,727, 1,465, 1,447, 1,364, 1,339, 1,201, 1,121, 1,014, 821. Anal. calcd for $C_{34}H_{46}Cl_2O_3Ti$: C, 65.69; H, 7.46. Found: C, 65.44; H, 7.79.

Complex 6: ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.65 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 6.60 (s, 5H; Cp), 6.48 (dd, ^{3}J = 2.5 Hz, ^{4}J = 2.5 Hz, 2H; Cp), 4.96 (dd, ^{3}J = 3.0 Hz, ^{3}J = 2.5 Hz, 1H; 3-βH), 2.60 (s, 2H; –CH_2– COO–), 2.45 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-βH), 2.09 (ddd, ^{2}J = 19.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 0.9 Hz, 1H; 16-αH), 1.94 (dddd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, ^{3}J = 5.7 Hz, ^{3}J = 0.9 Hz, 1H; 15-αH), 1.81–1.80 (m, 2H), 1.74–1.19 (m, 14H), 1.52 (dd, ^{2}J = 12.0 Hz, ^{3}J = 9.0 Hz, 1H; 15-βH), 1.51 (s, 6H), 1.08–0.99 (m, 1H), 0.89 (s, 3H), 0.82 (s, 3H), 0.80–0.84(m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 221.5, 170.8, 146.5, 120.5, 120.4, 120.3, 117.2, 117.1, 69.9, 54.3, 53.5, 51.4, 49.5, 47.8, 40.1, 36.7, 35.9, 35.8, 35.0, 32.9, 32.8, 31.5, 30.8, 28.1, 27.8, 27.7, 26.1, 21.8, 20.1, 13.8, 11.4. IR (KBr, cm^{-1}): 3,110, 2,931, 2,855, 1,734 (br s), 1,597, 1,447, 1,386, 1,368,

1,354, 1,250, 1,199, 1,117, 1,057, 1,015, 822. Anal. calcd for $C_{34}H_{46}Cl_2O_3Ti*1/4CH_2Cl_2$: C, 63.99; H, 7.30. Found: C, 63.60; H, 7.48.

Cytotoxicity assay

Biological activity was determined using the MTT assay originally described by Mossman [49] but using 10% Triton X-100 in 2-propanol as a solvent for the MTT formazan crystals [50]. HT29 and MCF7 cells were maintained at 37 °C and 95% air/5% CO_2 in McCoy's 5A (ATCC) complete medium, which had been supplemented with 10% (v/v) fetal bovine serum (ATCC) and 1% (v/v) antibiotic/antimycotic (Sigma). Asynchronously growing cells were seeded at 1.5×10^4 cells per well in 96-well plates containing 100 μl of complete growth medium, and were allowed to recover overnight. Various concentrations of the complexes (1–1,300 μM) dissolved in 5% DMSO/95% medium were added to the wells (eight wells per concentration; experiments performed in quadruplicate plates). The solutions of the complexes were prepared by first dissolving the corresponding titanocenyl in DMSO and then medium was added to a final composition of 5% DMSO/95% medium. In addition to experiments on the cells treated with the titanocenyls, two control experiments were performed: one without any addition of solvent mixture (5% DMSO/95% medium) and one with addition of 5% DMSO/95% medium to the cells. Both control experiments behaved identically, showing that 5% DMSO in the medium was not toxic to these types of cells. Although CH_2Cl_2 may have an effect on the cytotoxicity, under our experimental conditions at concentrations of 10^{-4}–10^{-7} M, CH_2Cl_2 has no effect at all in terms of cytotoxicity. Actually, we estimated that the IC_{50} of CH_2Cl_2 for MCF-7 is 30 mM (3.0×10^{-2} M) and for HT-29 is higher than 50 mM (5.0×10^{-2} M).

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 and the mixture was incubated for an additional 2h. After this period, all MTT-containing medium was removed, the cells were washed with cold phosphate-buffered saline, and were dissolved in 200 μl of a 10% (v/v) Triton X-100 solution in 2-propanol. After complete dissolution of the formazan crystals, well absorbances were recorded in triplicate with a 340 ATTC microplate reader (SLT Lab Instruments) at 570 nm with background subtraction at 630 nm. The concentrations of the compounds required to inhibit cell proliferation by 50% (IC_{50}) were calculated by fitting the data to a four-parameter logistic plot by means of SigmaPlot from SPSS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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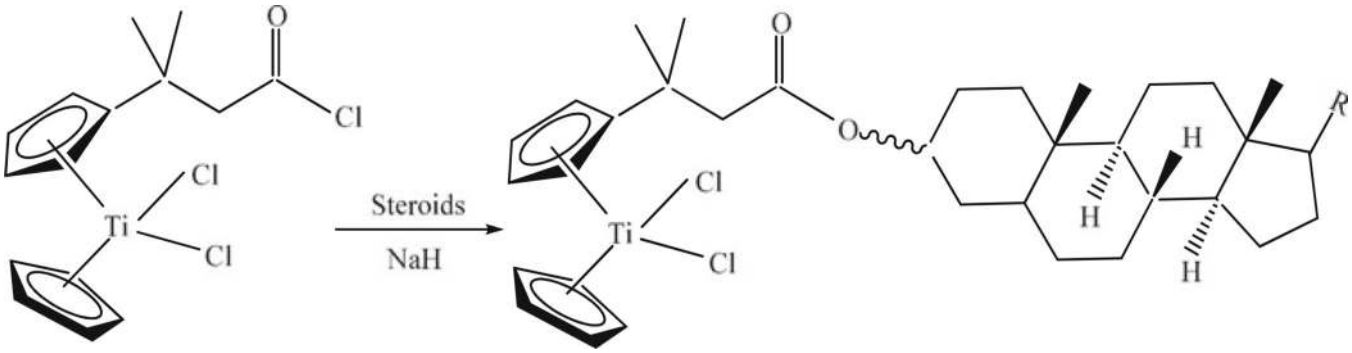

Fig. 1.
Esterification of titanocenyl chloride

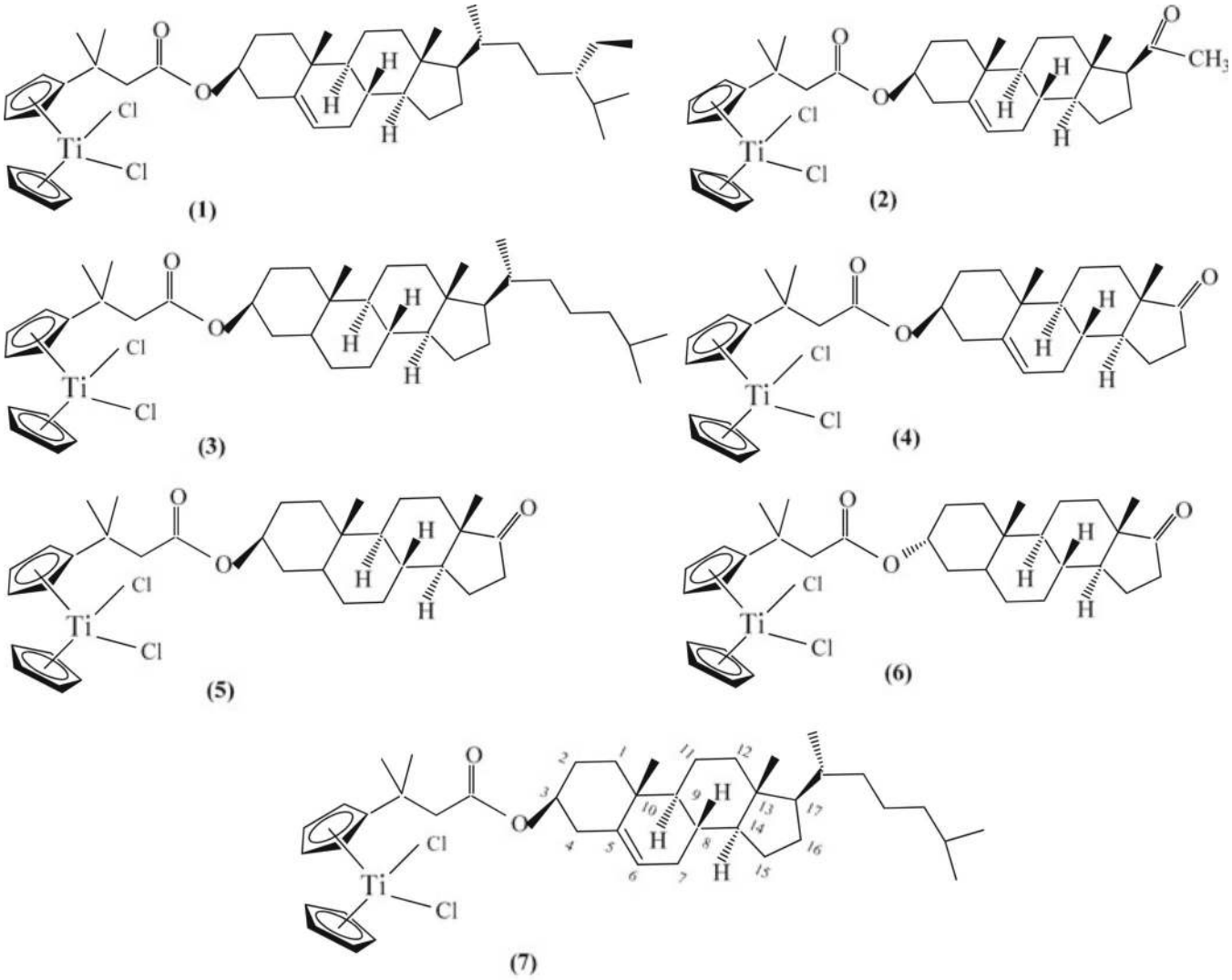

Fig. 2.
The structures of steroid-functionalized titanocenyl dichlorides **1–7**

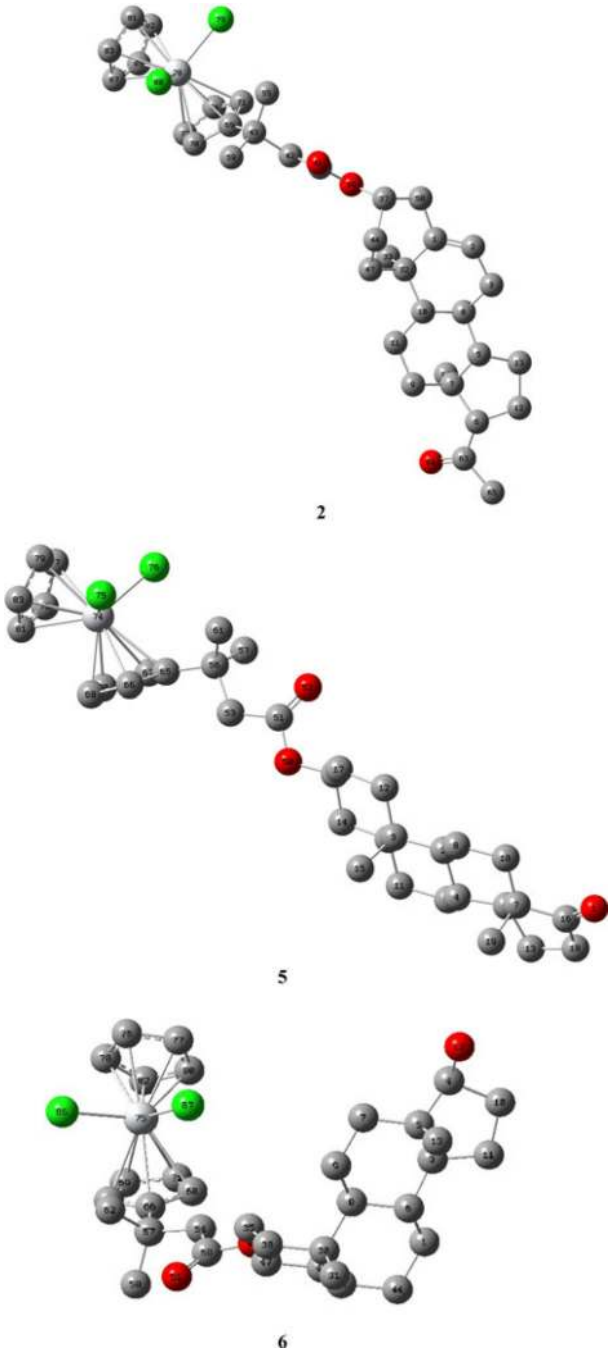

Fig. 3.
Density functional theory calculated structures of **2**, **5**, and **6**

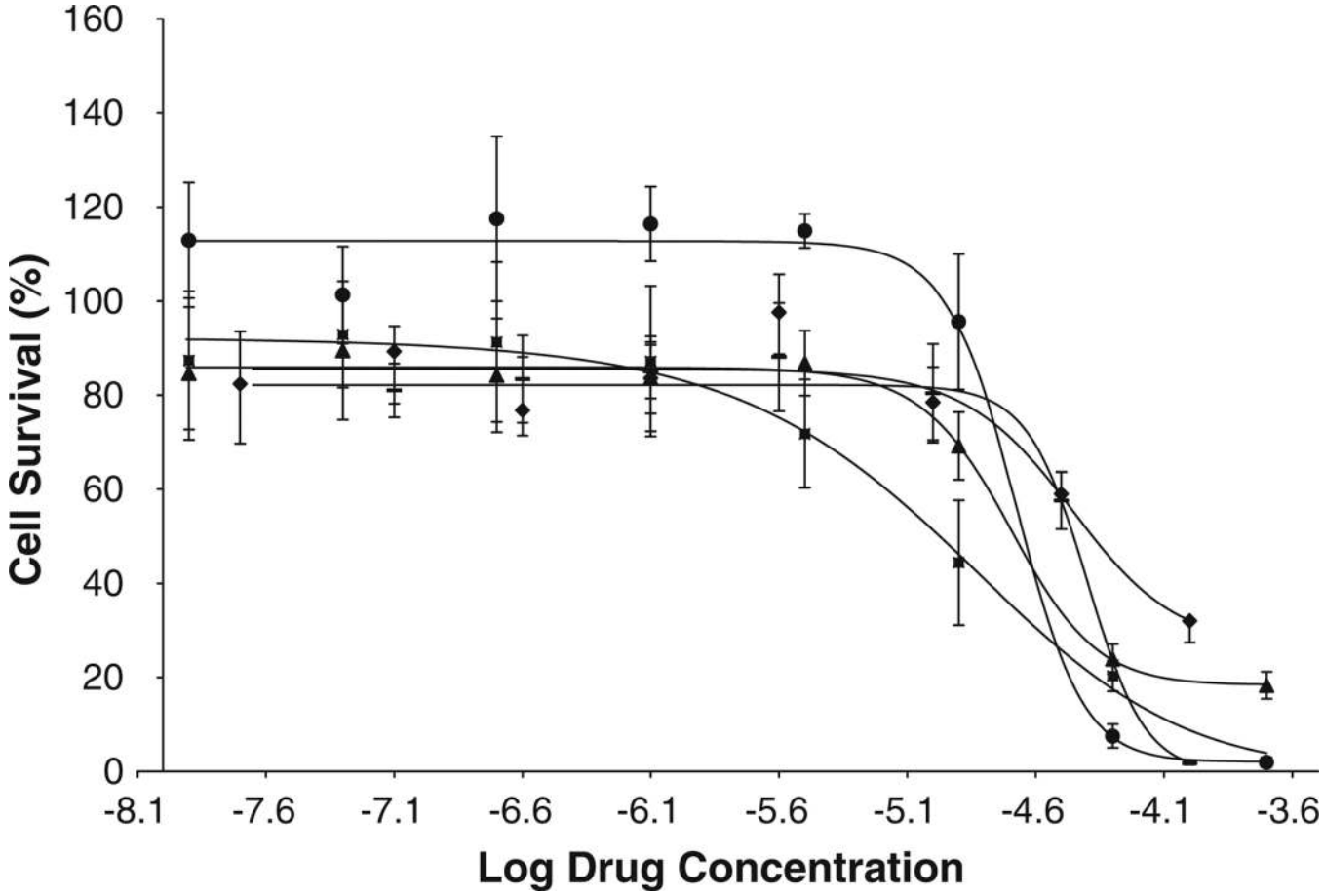

Fig. 4.

Dose–response curves for the effect of selected steroid-functionalized titanocenyl complexes on MCF-7 colon cells at 72 h of drug exposure. Complex **1** *diamonds*, complex **2** *triangles*, complex **4** *squares*, complex **5** *line segments*, and complex **6** *sunlamps*. Each value represents the mean ± one standard error of four experiments

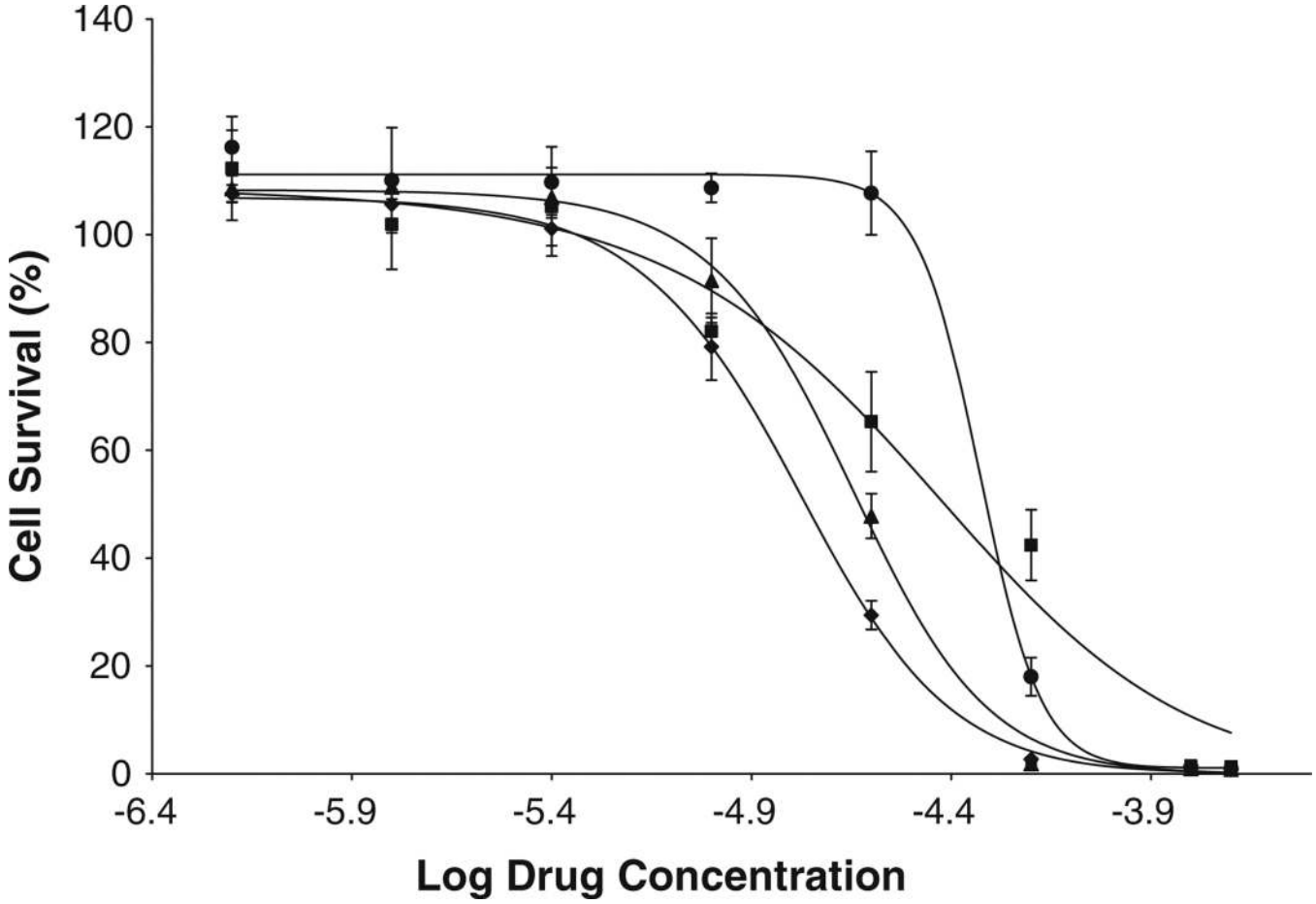

Fig. 5.

Dose–response curves for the effect selected steroid-functionalized titanocenyl complexes on HT-29 colon cells at 72 h of drug exposure. Complex **2** *diamonds*, complex **4** *triangles*, complex **5** *squares* and complex **6** *sunlamps*. Each value represents the mean ± one standard error of four experiments

Table 1

Selected bond distances and angles for pregnenolone (**2**), *trans*-androsterone (**5**), and androsterone (**6**)

Complex **2**			
Cp(unsubstituted)–Ti centroid	2.0716 Å	Cp–Ti–Cp bent	123.67°
Cp*–Ti centroid	2.1596 Å	Cl–Ti–Cl	97.58°
Average Ti–Cp(unsubstituted)	2.4409 Å		
Ti–Cp(substituted)			
Ti–C(69)*	2.6114 Å		
Ti–C(70)	2.5057 Å		
Ti–C(71)	2.4939 Å		
Ti–C(72)	2.3759 Å		
Ti–C(74)	2.3480 Å		
Ti–Cl(79)	2.3780 Å		
Ti–Cl(80)	2.3708 Å		
Complex **5**			
Cp(unsubstituted)–Ti centroid	2.1056 Å	Cp–Ti–Cp bent	123.63°
Cp*–Ti centroid	2.1791 Å	Cl–Ti–Cl	97.51°
Average Ti–Cp(unsubstituted)	2.4441 Å		
Ti–Cp(substituted)			
Ti–C(65)*	2.6141 Å		
Ti–C(66)	2.4963 Å		
Ti–C(67)	2.5064 Å		
Ti–C(68)	2.3479 Å		
Ti–C(70)	2.3753 Å		
Ti–Cl(85)	2.3772 Å		
Ti–Cl(86)	2.3713 Å		
Complex **6**			
Cp(unsubstituted)–Ti centroid	2.0925 Å	Cp–Ti–Cp bent	123.67°
Cp*–Ti centroid	2.1953 Å	Cl–Ti–Cl	97.62°
Average Ti–Cp(unsubstituted)	2.4403 Å		
Ti–Cp(substituted)			
Ti–C(66)*	2.6078 Å		
Ti–C(67)	2.4936 Å		
Ti–C(68)	2.4987 Å		
Ti–C(69)	2.3498 Å		
Ti–C(71)	2.3761 Å		
Ti–Cl(86)	2.3754 Å		
Ti–Cl(87)	2.3792 Å		

Cp–Ti* indicates the substituted carbon atom of the cyclopentadienyl (*Cp*) ring

Table 2

Cytotoxic activities of complexes **1–7** (see Fig. 2) on HT-29 and MCF-7 cancer cell lines

Cell line	IC_{50} (μM/L)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Cp_2TiCl_2**
HT-29	>200	16.2 ± 0.3	>200	22 ± 1	34 ± 11	47 ± 3	>200	413 ± 2
MCF-7	34 ± 17*	20 ± 2	>200	13 ± 2	40 ± 25*	21 ± 5	>200	570 ± 5

* The high standard deviations for **1** and **5** reflect the limited solubility in dimethyl sulfoxide/medium, in particular at concentrations higher than 10^{-4} M