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Original article

New platinum(II) complexes conjugated at position 7α of 17β-acetyl-testosterone as new combi-molecules against prostate cancer: Design, synthesis, structure–activity relationships and biological evaluation

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ABSTRACT

Prostate cancer is a major public health problem worldwide and, more specifically, new treatments for hormone-refractory cancers are highly sought by several research groups. Although platinum(II)-based chemotherapy and other strategies grow in interest to treat castration-resistant prostate cancer (CRPC), they still exhibit modest activity on CRPC and overall patient survival. In this study, we designed and prepared new combi-molecules using 17β-acetyl-testosterone and amino acid platinum(II) complexes linked at the position 7α to target and to improve the antiproliferative activity of platinum(II)-based chemotherapy on prostate cancer cells. Twelve chemical intermediates and six new combi-molecules were prepared and characterized. Structure–activity relationships studies show that the platinum complex moiety is essential for an optimal cytocidal activity. Moreover, stereochemistry of the amino acid involved in the platinum complexes had only minor effects on the antiproliferative activity whereas pyridinyl (**10a** and **b**) and thiazolyl (**10f**) complexes exhibited the highest cytocidal activities that are significantly superior to that of cisplatin used as control on human prostate adenocarcinoma LNCaP (AR^+), PC3 (AR^-) and DU145 (AR^-). Compounds **10a**, **b** and **f** arrested the cell cycle progression in S-phase and induced double strand breaks as confirmed by the phosphorylation of histone H2AX into γH2AX. Compounds **10a** and **f** showed 33 and 30% inhibition, respectively of the growth of HT-1080 tumors grafted onto chick chorioallantoic membranes. Finally, compounds **10a** and **10f** exhibited low toxicity on the chick embryos (18 and 21% of death, respectively), indicating that these new combi-molecules might be a promising new class of anticancer agents for prostate cancer.

1. Introduction

Prostate cancer is a major public health problem worldwide. In the United-States, prostate cancer is ranked first amongst all cancer cases diagnosed in men and it is the second most lethal cancer. It is estimated that in 2013, 238 590 new cases of prostate cancer will be diagnosed in the US and that 29 720 Americans will die from that disease [1]. Androgen receptor (AR) is a cytoplasmic receptor that mediates gene expression. AR is also a member of the superfamily of steroid and nuclear receptors [2]. In adult men, androgens and AR are responsible for secondary sexual characteristics such as spermatogenesis, muscle mass, bone mineral density, erythropoiesis and the maintenance of libido [3,4]. AR activation by endogenous androgens such as testosterone (**1**, Fig. 1) and dihydr otestosterone (DHT, **2**) leads to sequential conformations of the receptor (required by the dissociation of chaperone proteins), dimerization, phosphorylation and translocation into the nucleus for transcription of the target genes [2]. AR and endogenous androgens play key roles in the initiation of the differentiation, progression and the maintenance of prostate cancer [5–7].

Anti-hormone therapies using either luteinizing hormone-releasing analogs (e.g. leuprolide [8]), anti-androgens (bicalutamide (**3**) [9], cyproterone acetate (**4**) [10]) and orchiectomy are effective against prostate cancer at their early stages. However, it

Abbreviations: AR, androgen receptor; CRPC, castration-resistant prostate cancer; CDDP, cisplatin; PSA, prostate-specific antigen; CAM, chick chorioallantoic membrane.

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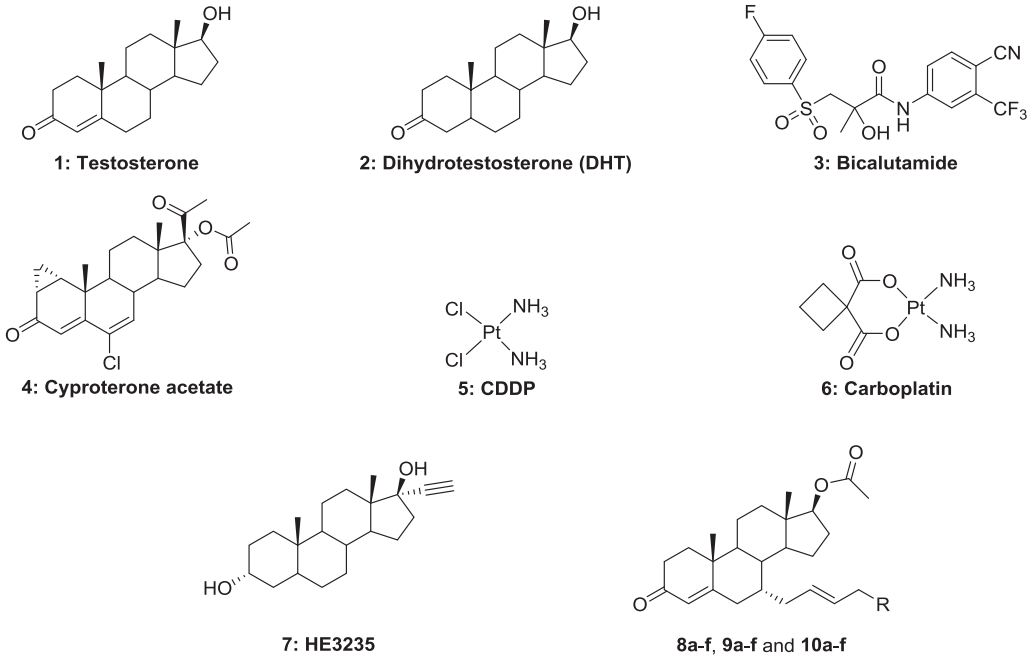


Fig. 1. Molecular structures of testosterone (**1**), dihydrotestosterone (**2**), bicalutamide (**3**), cyproterone acetate (**4**), CDDP (**5**), carboplatin (**6**) HE3235 (**7**) compounds assessed in this study (compounds **8a**–**f**, **9a**–**f** and **10a**–**f**).

must be kept in mind that anti-hormone therapies exhibit several significant side effects such as incontinence, erectile dysfunction, depression, muscle loss, anemia, osteoporosis and many others [11]. Although many patients benefit from the therapy and experience prolonged remission, 10–20% of patients are still developing castration-resistant prostate cancer (CRPC) within 5 years of the follow-up and 84% of those CRPC are metastatic [12]. At this point, the first-line chemotherapy regimen approved by the FDA includes first docetaxel and prednisone for three weeks [13]. The positive response to the treatment does not exceed 6 month with a median survival time of 18.9 months [14]. The second-line chemotherapy involves several other antineoplastics such as abiraterone acetate, cabozantinib, ipilimumab, TAK-700 and their therapeutic combinations that are under clinical investigation to assess their clinical efficacy and potential benefits [15]. Among those, platinum(II)-based chemotherapeutics such as cisplatin (CDDP, **5**) and carboplatin (**6**) are of growing interest [16] despite the belief that they exhibit only modest activity against prostate cancers and CRPC [17]. However, the clinical benefits achieved so far remains low and increasing the survival by only 3–6 months [15,17]. Therefore, the development of more effective platinum(II)-based therapies to improve both life expectancy and the quality of life of CRPC patients is still urgently needed.

Although anti-hormone therapies are ineffective against CRPC, several groups have shown that ARs are still expressed [18] and they still play important roles in tumor growth of CRPC as demonstrated by the expression of the AR regulated gene, prostate-specific antigen (PSA) [5]. On the other hand, chemoresistance can also be related to multifactorial causes such as increased expression of AR, AR gene mutations and altered ligand specificity, downstream signaling receptor for androgens and other bypass pathways. The latter can result in activation of AR requiring very low androgen concentration, activation by ligands that are not normally agonistic or ligand-independent [19–21].

In such a context, treatment of CRPC with androgen supplementation seems counterintuitive. However, preclinical studies showed that testosterone inhibits tumor cell growth both *in vitro* [22] and *in vivo* [23]. Moreover, it has been shown that high-doses of exogenous testosterone can be safely administered to patients with CRPC [24,25]. The authors have evidenced the antitumor activity of the treatment and the long-term stabilization of the disease in patients exhibiting low PSA concentrations. Another example of androgen supplementation approach is the use of HE3235 (**7**), a synthetic androgen analog. HE3235 exhibited a potent antiproliferative and proapoptotic activity both *in vitro* and *in vivo* [26,27]. Moreover, phase I studies showed that it was safe and well tolerated [28]. Thus, androgen therapy targeting AR-mediated signaling seems a possible and an innovative new approach for treatment of selected types of CRPC [29,30].

In the aim to improve the cytocidal activity, the selectivity and the biopharmaceutical properties of anticancer drugs toward prostate cancer tumors, we designed new combi-molecules merging 17β-acetyl-testosterone and different new platinum(II) complexes to target simultaneously the AR-mediated signaling pathway and DNA. In this study, we described the design, the preparation and the biological evaluation of three series of combi-molecules namely: Boc amino acids, amino acids and platinum(II) complexes that are conjugated at position 7α of the 17β-acetyl-testosterone (17β-acetyl-testosterone-7α-Boc amino acids (compounds **8a**–**f**), 17β-acetyl-testosterone-7α-amino acids (compounds **9a**–**f**) and 17β-acetyl-testosterone-7α-platinum(II) com plexes (compounds **10a**–**f**), respectively). We assessed the antiproliferative activity of the Boc, amine and platinum(II) complex moieties, respectively on androgen-sensitive human prostate adenocarcinoma LNCaP cells and androgen-insensitive human prostate adenocarcinoma PC3 and DU145 cells and that in regard of the nature of the amino acid side-chain. The most potent combi-molecules were assessed also for: (1) antiproliferative activity on a panel of 8 cancer cell lines unrelated to prostate cancer comprising HT-1080, HT-29, M21, MCF-7, MDA-MB-231, MDA-MB-468, CEM and CEM-VLB cells, (2) effect on cell cycle progression, (3) potential induction of histone H2AX phosphorylation and (4) antitumoral activities and toxicity onto the chick chorioallantoic membrane (CAM) tumor assays.

2. Design and chemistry

First, 17β-acetyl-testosterone was selected as the carrier for combi-molecules based on its natural tropism for prostate tissue, to optimize the pharmacokinetics and the pharmacodynamics of testosterone towards AR and to activate AR signaling pathways. Second, position 7α of testosterone is located away from the 3-carbonyl and 17-hydroxyl groups that are involved in key hydrogen interactions with AR. These two crucial groups must be free of steric hindrances to favor optimal interactions with AR and to trigger the signaling pathways. Finally, L- and D-2-pyridylalanine, L- and D-methionine, L-histidine and L-4-thiazolylalanine amino acids were selected because they easily form platinum(II) complexes and that they are inexpensive templates to optimize our synthetic approaches [31,32]. We also observed in our recent studies on estrogen combi-molecules that amino pyridine group easily form platinum(II) complexes and show interesting biological activities [33–36].

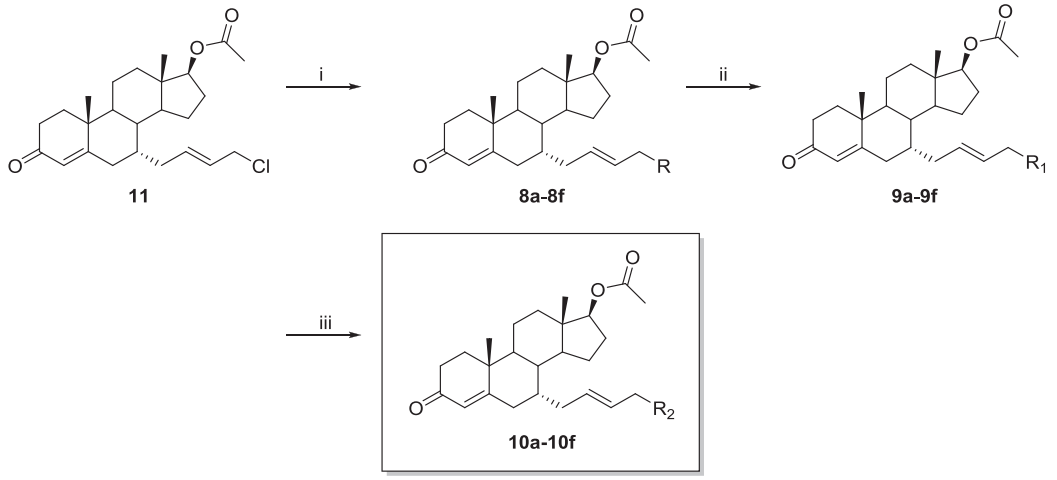
The methods used for the preparation of 17β-acetyl-testosterone-7α-Boc amino acids (compounds **8a**–**f**), 17β-acetyl-testosterone-7α-amino acids (compounds **9a**–**f**) and 17β-acetyl-testosterone-7α-platinum(II) complexes (compounds **10a**–**f**) are described in Scheme 1. Compound **11** was prepared in excellent yields as published elsewhere using the method described by Nickisch et al. [37] and Bastien et al. [38–41]. Then, 17β-acetyl-testosterone-7α-Boc amino acids (compounds **8a**–**f**) were obtained after treatment of compound **11** and the selected amino acid in the presence of cesium carbonate in methyl ethyl ketone. Afterward, 17β-acetyl-testosterone-7α-amino acids (compounds **9a**–**f**) were achieved by treatment of compounds **8a**–**f** with trifluoroacetic acid in methylene chloride. Finally, compounds **9a**–**f** were treated with potassium tetrachloroplatinate(II) in a mixture of dimethylformamide and water to afford 17β-acetyl-testosterone-7α-platinum(II) complexes (compounds **10a**–**f**).

3. Results and discussion

3.1. Antiproliferative activity on prostate cancer cell lines and structure–activity relationships

The antiproliferative activity of compounds **8a**–**f**, **9a**–**f** and **10a**–**f** was assessed at first on LNCaP, an androgen-sensitive human prostate adenocarcinoma cells, and on PC3 and DU145, that are both androgen-insensitive human prostate adenocarcinoma cells. The antiproliferative activity and the median lethal concentration were evaluated *in vitro* using the MTT cell proliferation assay with minor modifications [42,43]. The results are summarized in Table 1 and show antiproliferative activity (IC_{50}) and the median lethal concentration (LC_{50}) of **8a**–**f**, **9a**–**f** and **10a**–**f**. IC_{50} is defined, as the concentration of drug inhibiting cell growth by 50% and the LC_{50} is concentration required to kill 50% of the cells. CDDP was used as positive control.

The antiproliferative activity of all three series of compounds was slightly higher on PC3 cells followed by LNCaP and DU145 cells. The antiproliferative activity of 17β-acetyl-testosterone-7α-Boc amino acids (compounds **8a**–**f**) and 17β-acetyl-testosterone-7α-amino acids (compounds **9a**–**f**) were evaluated to assess the contribution of the platinum complex to the cytocidal activity. Compounds **8a**–**f** and **9a**–**f** were cytostatic and exhibiting IC_{50} between 12 and >100 μM while their LC_{50} were higher than 100 μM. Boc and amine series were clearly less active than their platinum complex counterparts showing IC_{50} in the low μM range (1.4–28 μM) indicating that the platinum complex moiety is crucial for the activity. Interestingly, the stereochemistry of the amino acids used has minor effects on the activity as exemplified by the R-pyridinyl (series **a**) and the S-pyridinyl (series **b**) as well as the R-methionyl (series **c**) and the S-methionyl (series **d**). The 17β-acetyl-testosterone-7α-platinum(II) complexes bearing a pyr



Scheme 1. Reagents and conditions: (i) Boc-amino acids, Cs_2CO_3, MEK, 110 °C, 72 h; (ii) TFA, DCM, 22 °C, 4 h and (iii) K_2PtCl_4, DMF:H_2O, 22 °C, 5 days.

Table 1
Antiproliferative activity and median lethal concentration of novel 17β-acetyl-testosterone-7α-Boc amino acids (compounds **8a**–**f**), 17β-acetyl-testosterone-7α-amino acids (compounds **9a**–**f**) and 17β-acetyl-testosterone-7α-platinum(II) complexes (compounds **10a**–**f**) and CDDP on androgen-sensitive (LNCaP) and androgen-insensitive (PC3 and DU145) human prostate adenocarcinoma cell lines.

Compd	IC_{50} (μM)[a]			LC_{50} (μM)[b]		
	LNCaP (AR^+)	PC3 (AR^-)	DU145 (AR^-)	LNCaP (AR^+)	PC3 (AR^-)	DU145 (AR^-)
8a	25	14	34	57	>100	>100
8b	21	12	29	>100	>100	>100
8c	>100	19	>100	>100	>100	>100
8d	12.5	18.1	>100	>100	>100	>100
8e	42	24	54	70	43	>100
8f	32	17	>100	>100	>100	>100
9a	>100	40	>100	>100	>100	>100
9b	46	33	46	>100	>100	>100
9c	>100	58	12	>100	>100	>100
9d	>100	43	89	>100	>100	>100
9e	45	29	49	67	>100	96
9f	>100	58	>100	>100	>100	>100
10a	2.5	1.9	3.6	6.4	3.5	9.4
10b	3.1	2.3	3.5	8.0	4.0	11
10c	28	14	23	64	31	41
10d	21	11	16	44	25	31
10e	20	12	13	80	34	41
10f	1.8	1.4	1.9	4.7	2.6	6.4
CDDP	6.7	7.4	2.1	44	>100	>100

[a] Inhibitory concentration (IC_{50}) is concentration of drug inhibiting cell growth by 50%.
[b] Median lethal concentration (LC_{50}) is the concentration required to kill 50% of the cells.

idinyl (compounds **10a** and **b**) or a thiazolyl (compound **10f**) side chain exhibited the highest IC_{50} between 1.4 and 3.6 μM. The substitution of a pyridinyl (**10a** and **b**) and thiazolyl (**10f**) chelating moiety by a thiomethyl (**10c** and **d**) or by an imidazolyl moiety (**10e**) decreased the IC_{50} by up to 10-fold. Interestingly, the LC_{50} for compounds **10a**, **b** and **f** (2.6–8 μM) were several times lower than CDDP (44 to >100 μM) indicating that **10a**, **b** and **f** were much more cytocidal than CDDP. On one hand, compound **10f** bearing a thiazolyl moiety exhibited the highest antiproliferative activity with IC_{50} of 1.8, 1.4 and 1.9 μM on LNCaP, PC3 and DU145 cells which are up to five times more potent than CDDP alone that exhibited IC_{50} of 6.7, 7.4 and 2.1 μM, respectively. On the other hand, the LC_{50} of **10f** on the same cell lines were from 9 to over 38-times more potent than CDDP (4.7, 2.6 and 6.4 μM for **10f** vs. 44, >100 and >100 μM for CDDP, respectively). Moreover, the general aspects of the antiproliferative curves for 17β-acetyl-testosterone-7α-platinum(II) complexes and CDDP are quite different. For example, when the concentration of CDDP alone increases, the cell growth decreases slowly while when using 17β-acetyl-testosterone-7α-platinum(II) complexes the cell growth decreases dramatically within a short range of concentrations suggesting that CDDP and 17β-acetyl-testosterone-7α-platinum(II) complexes do not act completely through the same mechanism of action.

3.2. Antiproliferative activity of compounds on a panel of cancer cell lines unrelated to prostate cancer

AR is not only expressed in male reproductive organs such as prostate, testis and seminal vesicle but it is also expressed in a variety of normal and cancer tissues including hepatic, thyroid, pancreatic, gastrointestinal, renal, bone marrow, breast and many others [44–51]. Thus, in the aim to verify that the new combi-molecules concept can be useful to the chemotherapy of other cancers, compounds **10a**, **b** and **f** exhibiting IC_{50} and LC_{50} lower than 12 μM on LNCaP, PC3 and DU145 tumor cell lines were assessed on eight cell lines unrelated to prostate tissues. The IC_{50} and the LC_{50} are summarized in Tables 2 and 3, respectively and were evaluated using the MTT cell proliferation assay as described above on human fibrosarcoma HT-1080, colon carcinoma HT-29, skin melanoma M21, breast carcinoma MCF-7, breast adenocarcinoma MDA-MB-231, breast adenocarcinoma MDA-MB-468, T cell leukemia CEM and CEM-VLB cells, respectively. CEM-VLB cells are overexpressing P-glycoprotein [52], which is responsible for the cellular efflux of several anticancer drugs such as doxorubicin, paclitaxel, and vinblastine. In addition P-glycoprotein is part of a potent mechanism of resistance encountered in clinical settings [53–55]. CDDP, colchicine and vinblastine are used as positive controls.

Compounds **10a**, **b** and **f** showed antiproliferative activities in the same order of magnitude as previously determined on LnCAP, PC3 and DU145 prostate cancer cell lines (0.39–3.1 μM). Interestingly, the IC_{50} of 17β-acetyl-testosterone-7α-platinum(II) complexes were higher than CDDP alone on MDA-MB-468, CEM and CEM-VLB cells while they were lower on other cancer cell lines. CEM-VLB cells exhibited low resistance to **10a**, **b** and **f** (5.7-, 6.7- and 7.2-fold) which are more than 10-times lower than for colchicine (87-fold) and several logs lower than vinblastine (980-fold) while CDDP was clearly unaffected by the overexpression of P-glycoprotein. This suggests that overexpression of P-glycoprotein does not affect the antiproliferative activity of 17β-acetyl-testosterone-7α-platinum(II) complexes. In addition, except for CEM-VLB cells, the LC_{50} of 17β-acetyl-testosterone-7α-platinum(II) complexes **10a**, **b** and **f** (0.64–4.0 μM) were several times lower than that of CDDP (1.3–50 μM), which were similar to the results obtain on prostate cancer cell lines. However, the IC_{50} and the LC_{50} of the most potent 17β-acetyl-testosterone-7α-platinum(II) complexes **10a**, **b** and **f** were several times higher than colchicine and vinblastine.

Table 2
Antiproliferative activity (IC_{50}) of compounds **10a**, **b** and **f**, CDDP, colchicine and vinblastine on selected cancer cell lines.

Compd	IC_{50} (μM)[a]								Ratio resistant/wild-type
	HT-1080	HT-29	M21	MCF-7	MDA-MB-231	MDA-MB-468	CEM	CEM-VLB[b]	CEM-VLB/CEM
10a	0.77	1.8	1.3	0.88	1.3	0.59	0.57	3.0	5.2
10b	0.72	1.6	1.0	0.78	1.0	0.46	0.56	3.1	5.5
10f	0.52	1.1	0.83	0.58	0.83	0.39	0.39	3.0	7.9
CDDP	1.8	2.8	3.8	1.6	5.0	0.32	1.0	0.89	0.87
Col[c]	0.014	0.011	0.018	0.010	0.018	0.0068	0.0071	0.30	42
Vbl[d]	0.00046	0.00055	0.00081	0.00047	0.0016	0.00033	0.00054	0.35	640

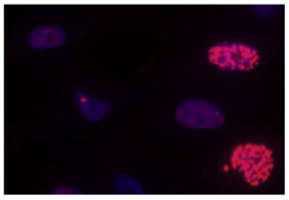
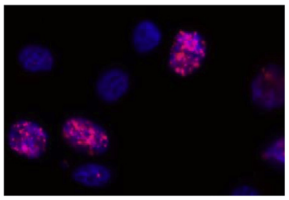
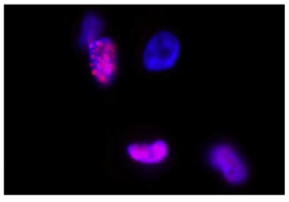
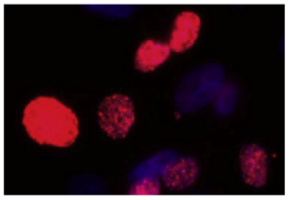
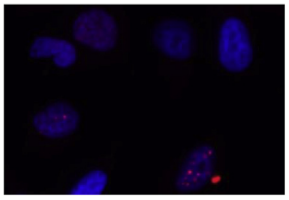
[a] IC_{50} (μM) is the concentration of the tested drug inhibiting cell growth by 50%.
[b] Multidrug-resistant leukemia CEM-VLB cells.
[c] Col, colchicine.
[d] Vbl, vinblastine.

Table 3
Median lethal concentration of compounds **10a**, **b** and **f**, CDDP, colchicine and vinblastine on selected cancer cell lines.

Compd	LC_{50} (μM)[a]								Ratio resistant/wild-type
	HT-1080	HT-29	M21	MCF-7	MDA-MB-231	MDA-MB-468	CEM	CEM-VLB[b]	CEM-VLB/CEM
10a	1.4	4.0	2.2	2.4	2.0	0.94	1.2	6.6	5.7
10b	1.3	3.2	1.5	2.2	1.5	0.76	1.0	6.5	6.7
10f	1.1	2.5	1.5	1.7	1.3	0.64	0.90	6.5	7.2
CDDP	8.8	49	11	50	27	1.3	14	3.0	0.21
Col[c]	0.084	>0.10	>0.10	>0.10	>0.10	0.021	0.018	1.6	87
Vbl[d]	0.0052	>0.10	>0.10	>0.10	>0.10	0.0089	0.0030	2.9	980

[a] LC_{50} (μM) is the concentration of the drug required to kill 50% of the cells.
[b] Multidrug-resistant leukemia CEM-VLB cells.
[c] Col, colchicine.
[d] Vbl, vinblastine.

Table 4
Effects of compounds **10a**, **b** and **f**, and CDDP on cell cycle progression and on the phosphorylation of histone H2AX into γH2AX.

Compd	Cell cycle progression					Phosphorylation of histone H2AX[a]
	Conc (μM)	Sub-G_1	G_0/G_1	S	G_2/M	
10a	9.1	9	46	18	27	
10b	12	2	53	15	30	
10f	6.9	12	45	19	24	
CDDP	19	1	69	15	15	
DMF	0.1%	0	68	8	24	

[a] For phosphorylation of histone H2AX experiments, PC3 cells were incubated for 24 h in the presence of the selected 17β-acetyl-testosterone-7α-platinum(II) complexes at 1.5-fold of IC_{50}.

3.3. Effect on cell cycle progression

Table 4 shows the percentage of PC3 cells that are in Sub-G1, G0/G1, S and G2/M phases, respectively, after treatment with compounds **10a**, **b** and **f**, and CDDP for 24 h at optimal concentrations regarding the arrest of cell cycle progression in S-phase. Control cells treated with the excipient (0.1% DMF) were found in sub-G1, G0/G1, S and G2/M phases at 0, 68, 8 and 24%, respectively. Compounds **10a**, **b** and **f** arrested mainly the cell cycle progression in the S-phase as observed with CDDP where the percentage of S-phase cells increased by 7–11%.

3.4. Phosphorylation of histone H2AX

DNA replication is the main event occurring during the S-phase and platinum(II)-based anticancer agents are well known to form reactive species such as cationic aqua ligand that bind to DNA, resulting in intrastrand and interstrand cross-links [56,57], triggering the formation of DNA single- and double-strand breaks that lead to apoptosis [58,59]. Moreover, phosphorylation of Ser-139 at the C-terminus of histone H2AX (γH2AX) occurs upon induction of DNA double-strand breaks [60,61]. Consequently, we used the phosphorylation of H2AX into of γH2AX to assess the effect of the platinum complex moiety in the mechanism of action and in the activity of compounds **10a**, **b** and **f**. As depicted in Table 4, compounds **10a**, **b** and **f** and CDDP induced H2AX phosphorylation in PC3 cells. γH2AX was detected as nuclear red spots in nuclei stained in blue by 4′,6-diamidino-2-phenylindole (DAPI). As shown in Table 4, compounds **10a**, **b** and **f** induced lower level of γH2AX than CDDP and therefore less double-strand breaks.

The induction of DNA double-strand breaks confirmed by the phosphorylation of H2AX (γH2AX) explains at least in part the mechanism of action of 17β-acetyl-testosterone-7α-platinum(II) complexes. In fact, the phosphorylation of H2AX into γH2AX does not correlate to the cytotoxicity of CDDP and 17β-acetyl-testosterone-7α-platinum(II) complexes; 17β-acetyl-testosterone-7α-platinum(II) complexes induced a lower number of double-strand breaks than CDDP while they were significantly more cytotoxic than CDDP. This suggests that the platinum(II) complexes are responsible for only a portion of the cytocidal activity. Research is in progress to determine the contribution of each constituent of the combi-molecules (testosterone and the various platinum complexes) to the cytocidal activity.

3.5. Chick chorioallantoic membrane (CAM) tumor assay

The CAM tumor assay is a simple and inexpensive animal model providing a large amount of information related to the systemic toxicity, the antineoplastic and the antiangiogenic potential of a new drug on a human tumor grafted onto the chorioallantoic membrane. In addition, it contributes to validate the drug formulations developed for intravenous administration of the new drugs and their future biopharmaceutical studies. However, it must be noted that only a limited number of human cell lines can form sizable tumors on the CAM. In that context, the human HT-1080 fibrosarcoma cell lines were selected instead of PC3 cells. HT-1080 cells are sensitive to most anticancer agents and form solid tumors easy to handle [62–64]. As depicted in Fig. 2 the results were obtained from two independent experiments using 10 to 12 eggs per experiments. No systemic toxicity was observed on chick embryos treated with the excipient alone. Sham-treated embryos were used as negative controls and for normalization of the results. Forty micrograms of compounds **10a**, **b** and **f** freshly dissolved in DMF were added to a mixture of cremophor EL™, 99% ethanol and phosphate buffered saline (PBS) (1/1/14 v/v) were administered *iv* (100 μL) in the CAM. The concentration of DMF was kept at 0.3% to avoid embryo's toxicity. CDDP (10 μg) was used as positive control.

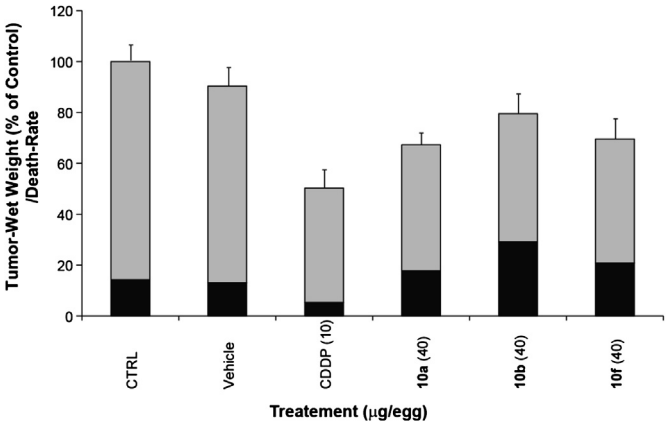


Fig. 2. Effects of compounds **10a**, **b** and **f** as well as CDDP on the growth of HT-1080 tumors and their toxicity on chick embryos in the CAM assay. Gray bars represent the percentage of wet weight of tumors treated with or without excipient. Black bars represent the percentage of chick embryo mortality.

As depicted in Fig. 2, compounds **10a**, **b** and **f** significantly inhibited tumor growth by 33, 21 and 30%, respectively and exhibited toxicity in 18, 29, and 21% of the chick embryos, respectively. CDDP inhibited tumor growth by 50% and exhibited toxicity in 5% of the chick embryos. Thus, we conclude that the excipient is not toxic for the embryos, the compounds **10a**, **b** and **f** show antitumoral activities on HT-1080 cells and low toxicity toward embryos suggesting that this new compounds have interesting biological activities and biopharmaceutical properties to continue biological and *in vivo* investigations.

4. Conclusion

In this study, we designed, prepared and characterized six new combi-molecules using 17β-acetyl-testosterone and L- and D-2-pyridylalanine, L- and D-methionine, L-histidine and L-4-thiazolylalanine amino acid platinum(II) complexes linked at position 7α to target prostate cancer cells (17β-acetyl-testosterone-7α-platinum(II) complexes **10a**–**f**). To the best of our knowledge, it is the first time such testosterone-7α-Pt(II) combi-molecules are reported in the literature. They exhibited antiproliferative activity (IC_{50}) and median lethal concentration (LC_{50}) at the micromolar level on panel of prostate cancer cell lines and cancer cell lines unrelated to prostate cancer. Structure–activity relationships showed that platinum complex is essential for optimal activity. The stereochemistry of the amino acids used had only minor effects on the cytocidal activity. Moreover, L- and D-2-pyridylalanine, and L-4-thiazolylalanine platinum(II) complexes (compounds **10a**, **b** and **f**, respectively) exhibited the most potent antiproliferative activity and are unaffected by the overexpression of P-glycoprotein. In addition, compounds **10a**, **b** and **f** arrest the cell cycle progression in S-phase and induce DNA double-strand breaks as confirmed by the phosphorylation of H2AX into γH2AX. Finally, compounds **10a** and **f** showed antitumoral activity on HT-1080 tumors grafted onto CAM and exhibited low toxicity on the chick embryos. The strategic location of the platinum(II) moiety and the efficient chemical transformations of testosterone represent an attractive strategy to develop that type of anticancer agents. Hence, the innovative design and the significant biological activity of 17β-acetyl-testosterone-7α-platinum(II) complexes toward AR^+ and AR^- prostate cancer cells suggest that they might be members of a promising new class of anticancer agents for prostate cancers.

5. Experimental protocols

5.1. Biological methods

LNCaP androgen-sensitive human prostate adenocarcinoma, PC3 androgen-insensitive human prostate adenocarcinoma, DU145 androgen-insensitive human prostate adenocarcinoma, HT-1080 human fibrosarcoma, HT-29 human colon carcinoma, MCF7 human breast carcinoma, MDA-MB-231 human breast carcinoma and MDA-MB-468 human breast adenocarcinoma were purchased from the American Type Culture Collection (Manassas, VA). M21 human skin melanoma cells were kindly provided by Dr. David Cheresh (University of California, San Diego School of Medicine, CA). T cell leukemia CEM and multidrug-resistant leukemia CEM-VLB were generously provided by Dr. William T. Beck (University of Illinois at Chicago, College of Pharmacy, IL) [53]. Cells were cultured in RPMI medium (Hyclone, Logan, UT) supplemented with 10% of calf serum and Penicillin–Streptomycin–Glutamine. The cells were maintained at 37 °C in a moisture saturated atmosphere containing 5% CO_2.

5.1.1. Antiproliferative activity assay

The antiproliferative activity of compounds **8a**–**f**, **9a**–**f**, **10a**–**f**, CDDP, colchicine and vinblastine was assessed using the MTT assay [42,43]. Briefly, the 96-well microtiter plates were seeded with 75 μL of a tumor cells in suspension (for LNCaP, 12 000; PC3, 6000; DU145, 6000; HT-1080, 2500; HT-29, 3000; M21, 3000; MCF-7, 3000; MDA-MB-231, 5000; MDA-MB-468, 5000; CEM, 10 000; CEM-VLB, 10 000) in medium. Plates were incubated at 37 °C, 5% CO_2 for 24 h. Freshly solubilized drugs in DMF were diluted in fresh medium, and 50 μL aliquots containing escalating concentrations of the drug were added and the plates were incubated for 48 h. The final concentration of DMF in the culture media was 0.1% and was kept constant in all experiment. After 48 h, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL in PBS) were added to the wells. Four hours later, 100 μL of the solubilization solution (10% sodium dodecyl sulfate (SDS) in 0.01 M HCl) was added and the plate was incubated overnight (37 °C, 5% CO_2). The optical density was read using a Fluostar optima BMG (BMG Labtech inc., Durham, NC) at 565 nm. Readings obtained from treated cells were compared with measurements from control cells plates fixed on the treatment day, and the percentage of cell growth inhibition was then calculated for each drug. The experiments were performed at least twice in triplicate. The assays were considered valid when the coefficient of variation for a given set of conditions and within the same experiment was <10%.

5.1.2. Cell cycle analysis

PC3 cells (2.5×10^5) were seeded into six-well plates and incubated for 24 h. Cells were then incubated with the test compound at 1.5 and 3-times their respective IC_{50} for 24 h at 37 °C. The control solution consisted of DMF dissolved in culture medium at 0.1% v/v. After incubation, the cells were trypsinized, washed with PBS and resuspended in 250 μL of PBS. Cells were fixed by the addition of 750 μL of ice-cold EtOH and stored at −20 °C until analysis. Prior to FACS, the cells were washed with PBS and resuspended in 1 mL of PBS containing 2 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Sigma, Oakville, Ontario, Canada). Fixed cell suspensions were incubated on ice for 1 h, and the cell cycle distribution was then analyzed using a BD SORP LSR II cytometer (BD Biosciences, Franklin Lakes, NJ).

5.1.3. Immunofluorescence

Cover slides (22 mm × 22 mm) sterilized with 70% (v/v) EtOH were placed in six-well plates. To promote cell adhesion, cover slides were treated with 1.5 mL of a fibronectin solution in PBS (10 μg/mL) for 1 h at 37 °C. Slides were then rinsed twice with PBS. PC3 cells (1.5×10^5) were seeded into the plates and incubated for 24 h. Cells were then incubated with the test compound at 1.5 and 3-times their respective IC_{50} for 24 h at 37 °C. The control solution consisted of DMF dissolved in culture medium at 0.1% (v/v). Cells were fixed using 1 mL of formaldehyde at 3.7% and permeabilized by addition of a saponin solution (0.1% in PBS) containing 3% (w/v) bovine serum albumin (saponin-BSA). Cells were incubated with mouse anti-H2AX pS139 antibody (Millipore, Billerica, MA). Cover slides were next incubated for 3 h at room temperature and then washed twice with PBS supplemented with 0.05% (v/v) Tween 20 (PBS-T). Saponin-BSA containing goat anti-mouse IgG conjugated to AlexaFluor 594 (Invitrogen, Burlington, Ontario, Canada), and DAPI (0.3 μg/mL) was then added. The cover slides were incubated for 2 h at room temperature and then washed twice with PBS-T and twice with PBS. The cover slides were mounted in darkness on a microscope slide overnight with Fluoromount-G™ (Southern Biotech no: 0100-01) before analysis using an Olympus BX51 epifluorescence microscope. Images were captured as 8-bit tagged image format files with a Q imaging RETIGA EXI digital camera driven by Image Pro Express software.

5.1.4. CAM tumor assay

Human HT-1080 fibrosarcoma cells were used to assess the antitumoral activity of compound **10a**, **b**, and **f** in the CAM assay [63,65,66]. Briefly, fertilized chicken eggs purchased from Couvoirs Victoriaville (Victoriaville, Québec, Canada) were incubated for 10 days in a Pro-FI egg incubator fitted with an automatic egg turner before being transferred to a Roll-X static incubator for the rest of the incubation time (incubators were purchased from Lyon Electric, Chula Vista, San Diego, CA). The eggs were kept at 37 °C in a 60% humidity atmosphere for the entire incubation period. On day 10, by use of a hobby drill (Dremel, Racine, WI), a hole was drilled on the side of the egg and a negative pressure was applied to create a new air sac. A window was opened on this new air sac and was covered with transparent adhesive tape to prevent contamination. A freshly prepared cell suspension (40 μL) of HT-1080 (3.5×10^5 cells/egg) cells in the culture media was applied directly onto the freshly exposed CAM tissue through the window. On day 11, the drugs dissolved in DMF were extemporaneously diluted in the excipient (Cremophor™/ethanol 99%/PBS, 6.25/6.25/87.5 v/v). The concentration of DMF in the excipient was kept at 0.3% to avoid any potential toxicity. 40 μg for 17β-acetyl-testosterone-7α-platinum(II) complexes and 10 μg for CDDP dissolved in 100 μL of excipient were injected iv into 10–12 eggs. The eggs were incubated until day-17, at which time the embryos were euthanized at 4 °C followed by decapitation. Tumors were collected, and the tumor-wet weights were recorded. The number of dead embryos and signs of toxicity from the different groups were recorded.

5.2. Chemistry

Proton NMR spectra were recorded on a Varian 200 MHz NMR or a Bruker AM-300 spectrometer. Chemical shifts (δ) are reported in parts per million. IR spectra were recorded on a Magna FT-IR spectrometer (Nicolet Instrument Corporation, Madison, WI, U.S.). Chemicals were supplied by Aldrich Chemicals (Milwaukee, WI, USA) or VWR International (Mont-Royal, Qc, Canada) and used as received. Testosterone was supplied by Steraloids Inc. (Newport, RI) and Boc-amino acids were supplied by Peptech Corporation (Bedford, MA). Uncorrected melting points were determined on an Electrothermal melting point apparatus. Mass spectral were obtained from NanoQAM (Université du Québec à Montréal) using a Time-of-Flight LC/MS (LC/MS-TOF), Agilent Technologie, LC 1200

Series/6210 TOF-LCMS with electrospray ionization and positive mode (ESI+). Liquid flash chromatography was performed on silica gel F60, 60 Å, 40–63 μm supplied by Silicycle (Québec, Canada) using a FPX flash purification system (Biotage, Charlottesville, VA) and using solvent mixtures expressed as v/v ratios. Solvents and reagents were used without purification unless specified otherwise. The completion of all reactions was monitored by TLC on precoated silica gel 60 F_{254} TLC plates (VWR). The chromatograms were viewed under UV light at 254 and/or 265 nm.

5.3. General procedure for the synthesis of Boc amino acid conjugates at position 7α of 17β-acetyl-testosterone (compounds **8a**–**f**)

The appropriate Boc-amino acid (0.16 mmol, 1 eq.) and cesium carbonate (0.16 mmol, 1 eq.) were added to a solution of the 7α-(*E*)-4-chlorobut-2-enyl-4-androsten-17β-ol-3-one acetate (**11**) (0.16 mmol, 1 eq.) in butan-2-one (2 mL). The key intermediate 7α-(*E*)-4-chlorobut-2-enyl-4-androsten-17β-ol-3-one acetate (**11**) was efficiently prepared as described in an earlier study [37–41]. The reaction mixture was heated to 110 °C under pressure in sealed tube and stirred for 3 days. Afterward the mixture was cooled to room temperature, the solvent was evaporated under reduced pressure. The residue was solubilized by methylene chloride (15 mL) and washed with a solution of sodium hydroxide 1 N (15 mL). The aqueous phase was extracted thrice with methylene chloride (15 mL). The combined organic phases were washed with brine (25 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum. The crude compound was purified by flash chromatography on silica gel to afford compounds **8a**–**f**.

5.3.1. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (2S)-2-[(tert-butoxycarbonyl) amino]-3-(pyridin-2-yl)propanoate (**8a**)

Compound **8a** was synthesized from Boc-L-2-pyridylalanine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50)). Yield, 86%; White solid; mp: 66–68 °C. IR *ν*: 2940 (Ar), 1712 (C=O), 1672 (C=O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 8.46–8.44 (m, 1H, pyr), 7.61–7.52 (m, 1H, pyr), 7.14–7.08 (m, 2H, pyr), 5.92 (d, 1H, *J* = 8.3 Hz, NH), 5.65–5.43 (m, 3H, 3× =CH), 4.68–4.48 and 4.17–3.99 (m, 4H, CH, CHOAc and CH_2), 3.27–3.25 (m, 2H, CH_2), 2.41–1.08 (m, 35H, 4× CH, 8× CH_2, and 5× CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 198.6, 171.3, 170.7, 168.5, 156.8, 155.2, 148.5, 136.4, 133.7, 125.8, 125.5, 123.5, 121.5, 82.0, 79.2, 65.2, 52.8, 46.6, 45.6, 42.2, 38.9, 38.2, 37.9, 36.0, 35.8, 35.6, 35.6, 33.6, 28.2, 27.9, 26.9, 22.5, 20.8, 20.4, 17.6, 11.5. HRMS (ESI+) *m*/*z* found 649.3825; $C_{38}H_{53}N_2O_7$ (M^+ + H) requires 649.3854.

5.3.2. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (2R)-2-[(tert-butoxycarbonyl) amino]-3-(pyridin-2-yl)propanoate (**8b**)

Compound **8b** was synthesized from Boc-D-2-pyridylalanine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50)). Yield, 68%; White solid; mp: 60–62 °C. IR *ν*: 2928 (Ar), 1714 (C=O), 1671 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.45–8.44 (m, 1H, Pyr), 7.59–7.54 (m, 1H, pyr), 7.14–7.08 (m, 2H, pyr), 5.92 (d, 1H, *J* = 8.1 Hz, NH), 5.63–5.39 (m, 3H, 3× =CH), 4.66–4.46 and 4.09–4.01 (m, 4H, CH, CHOAc and CH_2), 3.27–3.20 (m, 2H, CH_2), 2.37–1.05 (m, 35H, 4× CH, 8× CH_2, and 5× CH_3), 0.79 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.8, 171.6, 171.0, 168.8, 157.2, 155.5, 148.9, 136.7, 134.0, 126.2, 125.8, 123.9, 121.8, 82.3, 79.5, 65.4, 53.1, 47.0, 46.0, 42.5, 39.2, 38.6, 38.2, 36.4, 36.1, 36.0, 35.9, 33.9, 28.6, 28.3, 27.3, 22.9, 21.1, 20.7, 17.9, 11.9. HRMS (ESI+) *m*/*z* found 649.3835; $C_{38}H_{53}N_2O_7$ (M^+ + H) requires 649.3854.

5.3.3. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (tert-butoxycarbonyl)-L-methioninate (**8c**)

Compound **8c** was synthesized from *N*-Boc-L-methionine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50)). Yield, 68%; White solid; mp: 54–57 °C. IR *ν*: 2920 (Ar), 1713 (C=O), 1672 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.67–5.44 (m, 3H, 3× =CH), 5.30 (d, 1H, *J* = 7.5 Hz, NH), 4.60–4.54 and 4.40–34.37 (m, 4H, CH, CHOAc and CH_2), 2.52–1.07 (m, 42H, 4× CH, 10× CH_2, and 6× CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.9, 172.1, 171.1, 168.7, 134.7, 126.2, 125.7, 82.3, 79.8, 65.7, 52.9, 47.0, 46.0, 42.6, 38.6, 38.3, 36.4, 36.2, 35.9, 34.0, 32.2, 30.0, 28.7, 28.3, 27.3, 22.9, 21.1, 20.7, 18.0, 15.4, 11.9. HRMS (ESI+) *m*/*z* found 632.3592; $C_{35}H_{54}NO_7S$ (M^+ + H) requires 632.3622.

5.3.4. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (tert-butoxycarbonyl)-D-methioninate (**8d**)

Compound **8d** was synthesized from *N*-Boc-D-methionine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50)). Yield, 74%; White solid; mp: 58–61 °C. IR *ν*: 2940 (Ar), 1713 (C=O), 1671 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.62–5.42 (m, 3H, 3× =CH), 5.22 (d, 1H, *J* = 7.6 Hz, NH), 4.57–4.51 and 4.38–34.34 (m, 4H, CH, CHOAc and CH_2), 2.48–1.04 (m, 42H, 4× CH, 10× CH_2, and 6× CH_3), 0.79 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.8, 172.0, 171.0, 168.8, 134.7, 126.2, 125.6, 82.3, 79.8, 65.7, 52.8, 47.0, 45.9, 42.5, 38.6, 38.2, 36.4, 36.2, 36.0, 35.9, 33.9, 32.1, 30.0, 28.6, 28.3, 27.3, 22.9, 21.1, 20.7, 17.9, 15.4, 11.9. HRMS (ESI+) *m*/*z* found 632.3607; $C_{35}H_{54}NO_7S$ (M^+ + H) requires 632.3622.

5.3.5. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (tert-butoxycarbonyl)-L-histidinate (**8e**)

Compound **8e** was synthesized from *N*-Boc-L-histidine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50) to methylene chloride to methylene chloride/methanol (90:10)). Yield, 51%; White solid; mp: 100–104 °C. IR *ν*: 2937 (Ar), 1713 (C=O), 1671 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.69 (s, 1H, imidazole), 6.82 (s, 1H, imidazole), 6.51 (brs, 1H, NH of imidazole), 5.95 (d, 1H, *J* = 7.7 Hz, NH), 5.68–5.37 (m, 3H, 3× =CH), 4.62–4.53 (m, 4H, CH, CHOAc and CH_2), 3.11–3.05 (m, 2H, CH_2), 2.49–1.13 (m, 35H, 4× CH, 8× CH_2, and 5× CH_3), 0.83 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 200.0, 171.8, 171.2, 170.4, 155.6, 135.1, 133.9, 133.3, 126.3, 125.7, 116.4, 82.4, 79.7, 65.7, 53.7, 47.1, 45.9, 42.6, 38.7, 38.2, 36.4, 36.2, 36.0, 35.9, 33.9, 28.6, 28.3, 27.9, 27.3, 22.8, 21.1, 20.7, 17.9, 11.9. HRMS (ESI+) *m*/*z* found 638.3783; $C_{36}H_{52}N_3O_7$ (M^+ + H) requires 638.3806.

5.3.6. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-7-yl]but-2-en-1-yl (2S)-2-[(tert-butoxycarbonyl) amino]-3-(thiazol-4-yl)propanoate (**8f**)

Compound **8f** was synthesized from Boc-L-4-thiazolylalanine. Flash chromatography (hexanes to hexanes/ethyl acetate (50:50)). Yield, 67%; White solid; mp: 70–75 °C. IR *ν*: 2937 (Ar), 1714 (C=O), 1670 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.75 (s, 1H, thiazole), 7.06 (s, 1H, thiazole), 5.73 (d, 1H, *J* = 8.4 Hz, NH), 5.65–5.39 (m, 3H, 3× =CH), 4.63–4.48 (m, 4H, CH, CHOAc and CH_2), 3.2 (d, 2H, *J* = 4.9 Hz, CH_2), 2.38–1.07 (m, 35H, 4× CH, 8× CH_2, and 5×

CH_3), 0.80 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.9, 171.5, 171.1, 168.8, 155.4, 153.0, 152.6, 134.2, 126.2, 125.8, 115.6, 82.3, 79.7, 65.6, 53.3, 47.0, 46.0, 42.5, 38.6, 38.3, 36.4, 36.2, 36.0, 35.9, 34.0, 33.3, 28.6, 28.3, 27.3, 22.9, 21.1, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 655.3388; $C_{36}H_{51}N_2O_7S$ (M^+ + H) requires 655.3418.

5.4. General procedure for the synthesis of amino acid conjugates at position 7α of 17β-acetyl-testosterone (compounds **9a**–**f**)

Trifluoroacetic acid (7.8 mmol, 60 eq.) was added dropwise to a solution of the appropriate Boc amino acid (compounds **8a**–**f**, 0.13 mmol, 1 eq.) in dichloromethane (8 mL). The resulting mixture was stirred at room temperature for 5 h. Then, mixture was alkalinized (pH 14) with solution of NaOH (1 N, 15 mL) and the aqueous layer was extracted thrice with methylene chloride (10 mL), washed with brine (15 mL), dried with sodium sulfate, and the solvent was evaporated under reduced pressure to afford the amino acid conjugate at position 7α of 17β-acetyl-testosterone (compounds **9a**–**f**).

5.4.1. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl]but-2-en-1-yl (2S)-2-amino-3-(pyridin-2-yl) propanoate (**9a**)

Yield, 100%; White solid; mp: 95–100 °C. IR *ν*: 2932 (Ar), 1729 (C═O), 1670 (C═O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 8.49–8.47 (m, 1H, pyr), 7.61–7.52 (m, 1H, pyr), 7.18–7.06 (m, 2H, pyr), 5.64–5.41 (m, 3H, 3× ═CH), 4.61–4.50 and 4.05–3.93 (m, 4H, CH, CHOAc and CH_2), 3.29–2.95 (m, 2H, CH_2), 2.39–1.05 (m, 28H, 4× CH, 8× CH_2, 2× CH_3 and NH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 198.9, 174.9, 171.1, 168.9, 158.0, 149.3, 136.4, 134.2, 126.2, 126.0, 123.9, 121.6, 82.3, 65.3, 54.4, 47.0, 45.9, 42.5, 38.6, 38.2, 36.3, 36.1, 36.0, 35.9, 33.9, 29.6, 28.6, 27.3, 22.9, 21.1, 20.7, 17.9, 11.9. HRMS (ESI+) *m/z* found 549.3309; $C_{33}H_{45}N_2O_5$ (M^+ + H) requires 549.3329.

5.4.2. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl]but-2-en-1-yl (2R)-2-amino-3-(pyridin-2-yl) propanoate (**9b**)

Yield, 95%; Yellowish solid; mp: 76–80 °C. IR *ν*: 2927 (Ar), 1730 (C═O), 1670 (C═O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.49–8.48 (m, 1H, Pyr), 7.61–7.56 (m, 1H, pyr), 7.18–7.09 (m, 2H, Pyr), 5.68–5.43 (m, 3H, 3× ═CH), 4.60–4.47 and 4.06–3.99 (m, 4H, CH, CHOAc and CH_2), 3.32–3.02 (m, 2H, CH_2), 2.69 (brs, 2H, NH_2) 2.37–1.05 (m, 26H, 4× CH, 8× CH_2, and 2× CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.9, 174.4, 171.1, 168.9, 157.9, 149.2, 136.5, 134.3, 126.2, 126.0, 124.0, 121.7, 82.4, 65.4, 54.3, 47.0, 46.0, 42.5, 38.6, 38.3, 36.4, 36.2, 36.0, 35.9, 34.0, 29.7, 28.6, 27.3, 22.9, 21.1, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 549.3311; $C_{33}H_{45}N_2O_5$ (M^+ + H) requires 549.3329.

5.4.3. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl]but-2-en-1-yl L-methioninate (**9c**)

Yield, 95%; Yellowish solid; mp: 78–82 °C. IR *ν*: 2924 (Ar), 1730 (C═O), 1672 (C═O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 5.64–5.42 (m, 3H, 3× ═CH), 4.60–4.51 (m, 3H, CHOAc and CH_2), 3.59–3.53 (m, 1H, CH), 2.59 (t, 2H, *J* = 7.1 Hz, CH_2), 2.40–1.07 (m, 33H, 4× CH, 9× CH_2, 3× CH_3 and NH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 198.9, 175.5, 171.1, 168.8, 134.6, 126.2, 125.9, 82.3, 65.4, 53.3, 47.0, 45.9, 42.5, 38.6, 38.2, 36.4, 36.2, 35.9, 33.9, 33.9, 30.4, 28.7, 27.3, 22.9, 21.1, 20.7, 18.0, 15.4, 11.9. HRMS (ESI+) *m/z* found 532.3075; $C_{30}H_{46}NO_5S$ (M^+ + H) requires 532.3097.

5.4.4. (E)-4-((7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl)but-2-en-1-yl D-methioninate (**9d**)

Yield, 100%; Yellowish solid; mp: 85–88 °C. IR *ν*: 2932 (Ar), 1729 (C═O), 1670 (C═O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 5.63–5.49 (m, 3H, 3× ═CH), 4.59–4.51 (m, 3H, CHOAc and CH_2), 3.56–3.53 (m, 1H, CH), 2.59 (t, 2H, *J* = 7.0 Hz, CH_2), 2.39–1.03 (m, 33H, 4× CH, 9× CH_2, 3× CH_3 and NH_2), 0.80 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.0, 175.4, 171.1, 168.9, 134.5, 126.1, 125.9, 82.3, 65.4, 53.3, 47.0, 45.9, 42.5, 38.6, 38.2, 36.4, 36.2, 35.9, 35.9, 33.9, 33.8, 30.4, 28.7, 27.3, 22.8, 21.1, 20.7, 17.9, 15.3, 11.9. HRMS (ESI+) *m/z* found 532.3075; $C_{30}H_{46}NO_5S$ (M^+ + H) requires 532. 3097.

5.4.5. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl]but-2-en-1-yl L-histidinate (**9e**)

Yield, 100%; Orange solid; mp: 94–96 °C. IR *ν*: 2935 (Ar), 1729 (C═O), 1670 (C═O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 7.52 (s, 1H, imidazole), 6.81 (s, 1H, imidazole), 5.64–5.39 (m, 3H, 3× ═CH), 4.62–4.40 (m, 3H, CHOAc and CH_2), 3.79–3.72 (m, 1H, CH), 3.11–2.82 (m, 2H, CH_2), 2.45–1.04 (m, 29H, 4× CH, 8× CH_2, 2× CH_3, NH and NH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.6, 174.8, 171.2, 169.9, 169.9, 135.1, 134.1, 133.4, 126.3, 125.9, 82.3, 65.5, 54.6, 47.0, 45.9, 42.5, 38.7, 38.2, 36.4, 36.2, 35.9, 35.8, 33.9, 28.7, 27.3, 22.9, 21.1, 20.7, 17.9, 11.9. HRMS (ESI+) *m/z* found 538.3285; $C_{31}H_{44}N_3O_5$ (M^+ + H) requires 538.3282.

5.4.6. (E)-4-[(7R,10R,13S,17S)-17-Acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-7-yl]but-2-en-1-yl (2S)-2-amino-3-(thiazol-4-yl) propanoate (**9f**)

Yield, 87%; Yellowish solid; mp: 85–88 °C. IR *ν*: 2923 (Ar), 1730 (C═O), 1670 (C═O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.73 (s, 1H, thiazole), 7.10 (s, 1H, thiazole), 5.64–5.42 (m, 3H, 3× ═CH), 4.60–4.47 (m, 3H, CHOAc and CH_2), 3.95–3.91 (m, 1H, CH), 3.32–3.08 (m, 2H, CH_2), 2.39–1.02 (m, 28H, 4× CH, 8× CH_2, 2× CH_3 and NH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 198.9, 174.3, 171.1, 168.9, 153.5, 152.8, 134.4, 126.2, 126.0, 115.4, 82.3, 65.5, 54.3, 47.0, 46.0, 42.5, 38.6, 38.3, 36.4, 36.2, 35.9, 35.8, 33.9, 29.7, 28.7, 27.3, 22.9, 21.1, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 555.2867; $C_{31}H_{43}N_2O_5S$ (M^+ + H) requires 555.2893.

5.5. General procedure for the synthesis of platinum(II) complex conjugates at position 7α of 17β-acetyl-testosterone (17β-acetyl-testosterone-7α-platinum(II) complexes **10a**–**f**)

To a solution of the amino acid (compounds **9a**–**f**, 1.0 eq., 0.10 mmol) in DMF (3 mL) at room temperature was added potassium tetrachloroplatinate(II) (1.5 eq., 0.15 mmol) dissolved in water (1.5 mL). After 5 days of stirring in darkness at room temperature, a saturated KCl solution (4 mL) was added followed by solid KCl (0.5 g). The mixture was vigorously stirred for 30 min and the suspension was then filtered, washed with water (15 mL) and dried under vacuum. The crude compound was purified by flash chromatography on silica gel to afford cis-dichloroplatinum(II) complexes (compounds **10a**–**f**).

5.5.1. 17β-Acetyl-testosterone-7α-platinum(II) complex **10a**

Flash chromatography (methylene chloride to methylene chloride/ethyl acetate (50:50)). Yield, 33%; Yellowish solid; mp: 215–218 °C. IR *ν*: 2936 (Ar), 1729 (C═O), 1657 (C═O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 9.24–9.21 (m, 1H, pyr), 7.88–7.80 (m, 1H, pyr), 7.31–7.27 (m, 2H, pyr), 6.14–6.05 and 5.71–5.36 (m, 3H, 3× ═CH), 4.78–4.31 (m, 4H, CH, CHOAc and CH_2), 4.15–4.06 and 3.59–3.49 (m, 2H, CH_2), 2.43–1.08 (m, 28H, 4× CH, 8× CH_2, 2× CH_3 and NH_2),

0.84 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.2, 171.2, 169.4, 169.0, 155.5, 154.0, 138.9, 136.2, 126.1, 125.8, 124.6, 124.4, 82.3, 67.2, 50.2, 47.1, 46.0, 42.6, 38.6, 38.3, 36.4, 36.2, 35.9, 35.8, 34.0, 28.7, 27.3, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 814.2357; $C_{33}H_{45}Cl_2N_2O_5Pt$ ($M^+ + H$) requires 814.2354.

5.5.2. 17β-Acetyl-testosterone-7α-platinum(II) complex ***10b***

Flash chromatography (methylene chloride to methylene chloride/ethyl acetate (50:50)). Yield, 21%; Yellowish solid; mp: 202–205 °C. IR ν: 2941 (Ar), 1729 (C=O), 1659 (C=O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 9.23–9.20 (m, 1H, pyr), 7.87–7.79 (m, 1H, pyr), 7.36–7.22 (m, 2H, pyr), 6.13–6.02 and 5.77–5.33 (m, 3H, 3× =CH), 4.82–4.24 (m, 4H, CH, CHOAc and CH_2), 4.11–4.05 and 3.62–3.51 (m, 2H, CH_2), 2.43–1.12 (m, 28H, 4× CH, 8× CH_2, 2× CH_3 and NH_2), 0.84 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.2, 171.2, 169.4, 169.1, 155.6, 154.0, 138.9, 136.2, 126.2, 125.9, 124.7, 124.3, 82.3, 67.3, 50.2, 47.1, 46.0, 42.6, 38.6, 38.3, 36.4, 36.3, 36.0, 35.7, 34.0, 28.8, 27.3, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 814.2354; $C_{33}H_{45}Cl_2N_2O_5Pt$ ($M^+ + H$) requires 814.2354.

5.5.3. 17β-Acetyl-testosterone-7α-platinum(II) complex ***10c***

Flash chromatography (methylene chloride to methylene chloride/ethyl acetate (50:50) to methylene chloride/methanol (90:10)). Yield, 28%; White solid; mp: 192–195 °C. IR ν: 2933 (Ar), 1728 (C=O), 1660 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.67–5.41 (m, 4H, 3× =C and NH), 4.77–4.52 (m, 4H, CHOAc, CH_2 and NH), 4.15–4.03 (m, 1H, CH), 3.41–3.10 and 2.68–2.62 (m, 5H, CH_2 and CH_3), 2.41–1.14 (m, 28H, 4× CH, 9× CH_2 and 2× CH_3), 0.84 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.4, 171.1, 169.9, 169.5, 135.3, 126.0, 125.2, 82.4, 66.9, 53.1, 47.1, 45.9, 42.6, 38.6, 38.3, 36.4, 36.3, 36.0, 35.7, 35.6, 34.0, 28.9, 27.3, 23.1, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 797.2107; $C_{30}H_{46}Cl_2NO_5PtS$ ($M^+ + H$) requires 797.2122.

5.5.4. 17β-Acetyl-testosterone-7α-platinum(II) complex ***10d***

Flash chromatography (methylene chloride to methylene chloride/ethyl acetate (50:50) to methylene chloride/methanol (90:10)). Yield, 19%; White solid; mp: 192–195 °C. IR ν: 2942 (Ar), 1729 (C=O), 1658 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.69–5.44 (m, 4H, 3× =C and NH), 4.74–4.52 (m, 4H, CHOAc, CH_2 and NH), 4.12–4.03 (m, 1H, CH), 3.39–3.07 and 2.68–2.60 (m, 5H, CH_2 and CH_3), 2.41–1.13 (m, 28H, 4× CH, 9× CH_2 and 2× CH_3), 0.84 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.2, 171.1, 169.9, 169.4, 135.6, 126.1, 125.2, 82.4, 66.9, 53.0, 47.0, 45.9, 42.6, 38.6, 38.3, 36.4, 36.4, 35.9, 35.7, 35.6, 34.0, 28.9, 27.3, 23.1, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 797.2123; $C_{30}H_{46}Cl_2NO_5PtS$ ($M^+ + H$) requires 797.2122.

5.5.5. 17β-Acetyl-testosterone-7α-platinum(II) complex ***10e***

Flash chromatography (methylene chloride to methylene chloride/methanol (90:10)). Yield, 36%; White solid; mp: 208–213 °C. IR ν: 2936 (Ar), 1729 (C=O), 1655 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 11.6 (s, 1H, NH imidazole), 8.18 (s, 1H, imidazole), 6.95 (s, 1H, imidazole), 5.78–5.41 (m, 3H, 3× =CH), 4.79–4.55 (m, 3H, CHOAc and CH_2), 4.11–3.99 and 3.38–3.32 (m, 1H, CH), 3.06–3.00 and 2.42–1.06 (m, 30H, 4× CH, 9× CH_2, 2× CH_3, NH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 200.0, 171.2, 170.4, 169.5, 136.6, 135.1, 126.2, 125.9, 125.4, 125.1, 82.5, 66.7, 51.9, 47.1, 46.0, 42.6, 38.7, 38.3, 36.4, 36.3, 35.9, 35.8, 34.0, 28.8, 27.4, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 803.2301; $C_{31}H_{44}Cl_2N_3O_5Pt$ ($M^+ + H$) requires 803.2307.

5.5.6. 17β-Acetyl-testosterone-7α-platinum(II) complex ***10f***

Flash chromatography (methylene chloride to methylene chloride/ethyl acetate (50:50)). Yield, 34%; Yellowish solid; mp: 210–213 °C. IR ν: 2944 (Ar), 1730 (C=O), 1660 (C=O) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 9.63 (d, 1H, J = 2.6 Hz, thiazole), 7.39 (d, 1H, J = 2.6 Hz, thiazole), 5.64–5.46 (m, 4H, 3× =CH and NH), 4.98–4.87 and 4.64–4.57 (m, 4H, CHOAc, CH_2 and NH), 4.00–3.96 (m, 1H, CH), 3.76–3.69 and 3.42–3.31 (m, 2H, CH_2), 2.44–1.07 (m, 26H, 4× CH, 8× CH_2 and 2× CH_3), 0.84 (s, 3H, CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 199.3, 171.2, 169.2, 168.9, 157.3, 149.3, 135.7, 126.2, 125.0, 117.3, 82.4, 67.1, 52.0, 47.1, 46.0, 42.6, 38.6, 38.3, 36.4, 36.3, 35.9, 35.7, 34.0, 33.5, 28.9, 27.3, 22.9, 21.2, 20.7, 18.0, 11.9. HRMS (ESI+) *m/z* found 820.1898; $C_{31}H_{43}Cl_2N_2O_5PtS$ ($M^+ + H$) requires 820.1918.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.011.

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