The Addition of a Pregnenolone Pendant Group Enhances the Anticancer Properties of Titanocene Dichloride in a MCF-7 Xenograft Model

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Abstract. *Background/Aim: Titanocene dichloride held great promise as a chemotherapeutic compound in pre-clinical studies. However, subsequent clinical trials revealed hepatoxicity and nephrotoxicity, which limited its use in clinical applications. Therefore, we used steroid pendant groups to improve the targeting of titanocene in MCF-7 breast cancer cells, and demonstrated a 10-fold lower effective dose compared to titanocene in in vitro assays. The aim of the present study was to test the efficacy of a titanocene functionalized with pregnenolone (Ti-Preg) in an in vivo breast cancer model. Materials and Methods: Xenografts from the MCF7 breast cancer cell line were implanted into athymic nu/nu mice to evaluate the potential of Ti-Preg as an anti-breast cancer agent. Results: Ti-Preg demonstrated significant inhibition of MCF-7 tumor growth when compared to vehicle and to titanocene controls. Conclusion: Our findings demonstrate the potential of steroid pendent groups for targeting chemotherapeutics to steroid hormone-dependent cancer.*

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Key Words: Titanocene, steroid pendant groups, TLT-1, breast cancer, titanocenyl-pregnenolone.

Titanocene dichloride (Cp_2TiCl_2) is a transition metal complex containing a bis-cylopentandienyl titanium unit that has been proven to be effective against a wide range of cancer types, and therefore holds great promise as an anti-cancer drug (1, 2). Although its mechanism of action has not been confirmed, Cp_2TiCl_2 has been shown to induce DNA damage in human ovarian carcinoma cell lines (3), leading to blockage in the late S and early-G_2 phase of the cell cycle. Cp_2TiCl_2 has also been shown to induce apoptosis; Christodoulou *et al*. demonstrated that Cp_2TiCl_2 complexes induce apoptosis in cisplatin-resistant prostate cancer cells, as well as in B-cell lymphoma-2 (BCL-2)-overexpressing cells (3), further validating its promise as an anticancer agent. In clinical trials, however, hepotoxicity and nephrotoxicity were found to be the dose-limiting factors (4-7). Therefore, it is reasonable to hypothesize that if Cp_2TiCl_2 could be specifically-targeted to cancer cells; it may overcome the dose-limiting toxicities seen in clinical trials.

In light of titanocene's promise as an anticancer compound, our group has investigated the functionalization of titanocene as a means of improving its efficacy (8-12). Our recent attempts have led to the modification of Cp_2TiCl_2 with biologically-active steroid pendent groups. We have characterized seven of the most promising functionalized Cp_2TiCl_2 and have demonstrated that several of these functionalized titanocenes have better efficacy in controlling the growth of breast cancer cells than their parent compound, Cp_2TiCl_2, *in vitro* (Figure 1A) (11). We used the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay to assess the effect of different concentrations of functionalized titanocene on MCF-7 breast cancer cell viability. Cp_2TiCl_2 bound to the steroid

Table I. *The effect of titanocenyl-pregnenolone (Ti-Preg) dose on blood cell counts in nu/nu mice. Data are cell counts ±S.D, (n=3).*

		Vehicle	Titanocene mg/kg	Ti-Preg mg/kg		
	Initial	10% DMSO	0.1	0.01	0.02	0.04
Red blood cells, $\times10^6$/ml	9.2±1	9.1±0.2	8.4±0.41	8.9±0.8	9.3±0.5	9.5±1
Platelets $\times10^3$/ml	1,232±260	339±19	957±45***	659±202	622±159	227±113
Neutrophils $\times10^3$/ml	2.12±0.7	2.0±0.6	4.0±1.9	2.9±1	3.2±0.5	2.7±0.5
Lymphocytes $\times10^3$/ml	4.2±1.6	5.2±1.1	4.1±2.3	7.8±1.7	6.9±1.1	7.7±0.85
Monocytes $\times10^3$/ml	0.52±0.2	0.51±0.02	0.8±0.3	0.9±0.15	0.9±0.13*	0.6±0.3

*$p<0.05$, **$p<0.005$ compared to vehicle-treated mice.

Table II. *The effect of titanocenyl-pregnenolone (Ti-Preg) on blood cell counts of tumor bearing nu/nu mice after indicated treatments, Data are cell counts ±S.D, (n=3).*

	Initial	Vehicle	Titanocene	Ti-Preg
Red Blood cells $\times10^6$/ml	9.2±1	10.42±1.1	9.615±0.9	10.11±0.5
Platelets $\times10^3$/ml	1,232±260	562±177	417±252	892±312
Neutrophils $\times10^3$/ml	2.12±0.7	0.52±54	0.145±0.06	0.96±0.6
Lymphocytes $\times10^3$/ml	4.2±1.6	4.85±1.2	2.4±1.7*	2.15±1.2**
Monocytes $\times10^3$/ml	0.52±0.2	0.34±0.2	0.04±0.03*	0.12±0.18

*$p<0.05$, **$p<0.005$ compared to vehicle-treated mice.

pregnenolone, titanocenyl-pregnenolone (Ti-Preg), was more efficient than Cp_2TiCl_2 at inhibiting breast cancer cell viability (Figure 1B) (11). Since Cp_2TiCl_2 is known to cause DNA adducts and affect cell replication, it is possible that these compounds have adverse effects on all host cells, as evidenced by the observed liver and kidney toxicity during clinical trials. Therefore, *in vivo* studies are required to demonstrate the anticancer potential of functionalized Cp_2TiCl_2.

In the present study we evaluated the potential of Ti-Preg as an anti-breast cancer therapeutic agent using an athymic *nu/nu* mouse model of breast cancer. We demonstrate that Ti-Preg significantly inhibits tumor growth when compared to the parent compound, Cp_2TiCl_2.

Materials and Methods

Drugs and reagents. Titanocene was purchased from Sigma Aldrich (St. Luis, MO, USA). Ti-Preg was synthesized as described in reference (11).

Cell culture. MCF7 cells were obtained from American Type Culture Collection (ATCC). The human breast cancer cell line MCF7 was cultured as described in (13).

Cell-cycle analysis. Cell-cycle analysis of MCF7 cells treated with DMSO, Cp_2TiCl_2, or Ti-preg was performed as described elsewhere, (13) using an Acurri C6 (Acurri Cytometers, Durham, NC, USA).

Animals. All protocols were approved by the Institutional Animal Care and Use Committees (IACUC) at Universidad Central del Caribe (approval number 10-XI-01). Female athymic *nu/nu* (nude) mice (Charles River Laboratories, Inc., Wilmington, MA, USA), were maintained in high efficiency particulate air–filtered cages in a pathogen-free facility. Mice were fed autoclaved diet (Tek Global; Harlan Teklad, Madison, WI, USA) containing 14% protein and 3.5% fat and minimal alfalfa or soybean meal. In the preliminary study, blood was collected from untreated nude mice to establish their baseline blood profiles. Eight-week-old mice (3/group) were treated with Ti-Preg (0.01 mg/kg, 0.02 mg/kg or 0.04 mg/kg), Cp_2TiCl_2 (0.1 mg/kg) or vehicle (10% DMSO) in saline by intraperitoneal injections (*i.p.*) every other day for five days (three doses). Mice were sacrificed after 21 days after the initial dose and evaluated for changes in their hematological parameters and gross malformalities using hematoxylin and eosin (H&E) staining.

Tumor establishment. MCF-7 (2×10^6 cells in Matrigel) breast cancer cells were implanted into the right mammary fat pad of 15 female athymic nude mice. Mice were also implanted subcutaneously with slow-release estrogen pellets (0.18 mg/pellet: Innovative Research, Sarasota, FL, USA) to maintain MCF-7 tumor growth.

Treatment administration. After three weeks of breast cancer cell xenograft implantation (tumors were ~20 mm^3), mice were randomly divided into three groups (Five mice/group) and administered vehicle (10% DMSO), Cp_2TiCl_2 (0.1 mg/kg), or Ti-Preg (0.04 mg/kg) by *i.p.* injections every other day for five days (three doses).

Tumor growth. Mice were monitored for 21 days after the last treatment. Tumor volume was determined using caliper measurements. Mouse weights and tumor volume (mm^3= h × l × w × 0.523) were recorded once a week. Following sacrifice, blood and tissues (liver, spleen, kidneys, and lungs) were harvested for evaluation of abnormalities.

Blood counts. Whole blood was collected by cardiac puncture, using a syringe containing 3.8% sodium citrate, from mice anesthetized with CO_2. Platelet and leukocyte counts were determined using a Sysmex KX-21 hematology cell analyzer (Sysmex, Kobe, Japan).

Enzyme-linked immunosorbant Assay (ELISA) and colorimetric assays. Plasma was obtained by centrifuging whole blood at 100 ×*g* for 10 min to remove red cells followed by two spins at 16,000 ×*g* to remove platelets. Plasma samples were frozen and later analyzed for mouse soluble triggering receptor expressed in myeloid cells (TREM) like transcript-1 (sTLT-1) (R&D Systems, Minneapolis, MN, USA) or plasma alanine transaminase (ALT) levels (Cayman Chemical, Ann Harbor, MI, USA), according to the manufacturers' instructions.

Histology. Tissues were fixed in 10% buffered formalin, and paraffin sections were stained with H&E. The sections were examined by light microscopy.

Results

We have previously demonstrated that Ti-Preg inhibits cell proliferation of MCF-7 cells with a >10-fold lower inhibitory concentration (IC_{50}) than Cp_2TiCl_2 (20 μM *vs*. 570 μM) (11). To determine whether Ti-Preg causes a similar arrest in cell cycle progression as Cp_2TiCl_2, cells were incubated with vehicle, Cp_2TiCl_2, or Ti-Preg for 12 h, and stained with propidium iodide (PI) for cell-cycle analysis. Treatment times of longer than 12 h at selected doses of Cp_2TiCl_2 and Ti-Preg led to significant cell death with a sub-G_1 peak believed to represent apoptotic cells (data not shown). Flow cytometry showed that 20 μM Ti-Preg caused a highly significant increase in the percentage of cells at the S phase (Figure 2) with a concomitant decrease in cells at G_1 phase of the cell cycle, when compared to vehicle or 200 μM Cp_2TiCl_2 (Figure 2B). These results suggest that Ti-Preg leads to a G_2/M block, which is consistent with previous studies on titanocene (3).

To evaluate the effect of Ti-Preg *in vivo*, we compared three doses of Ti-Preg to Cp_2TiCl_2 and vehicle for 21 days, in nude mice implanted with MCF7 breast cancer cell xenografts. Mice were treated with vehicle, Cp_2TiCl_2, (0.1 mg/kg) or (0.01 mg/kg, 0.02 mg/kg or 0.04 mg/kg) Ti-Preg by *i.p*. injection and were evaluated for changes in their hematological profile and gross malformations. All treatments were tolerated by the mice, with only one death from the titanocene treatment group. No gross malformations of internal organs were observed on histological evaluation, suggesting that the selected doses were acceptable for experimentation (Figure 3). Evaluation of the blood components showed a platelet count reduction at all Ti-Preg doses; the most severe reduction was observed at the highest dose of Ti-Preg (Table I). It should be noted that a drop in platelet count was also observed in the vehicle-treated group, but not in the Cp_2TiCl_2-treated group. Both neutrophil and lymphocyte levels showed a trend for increase. Since platelet count was the only parameter that decreased on treatment, even though this decrease was in the same range as that of the controls, the highest dose of Ti-Preg (0.04 mg/kg) was selected for the subsequent evaluation of the effects of Ti-Preg on tumor growth.

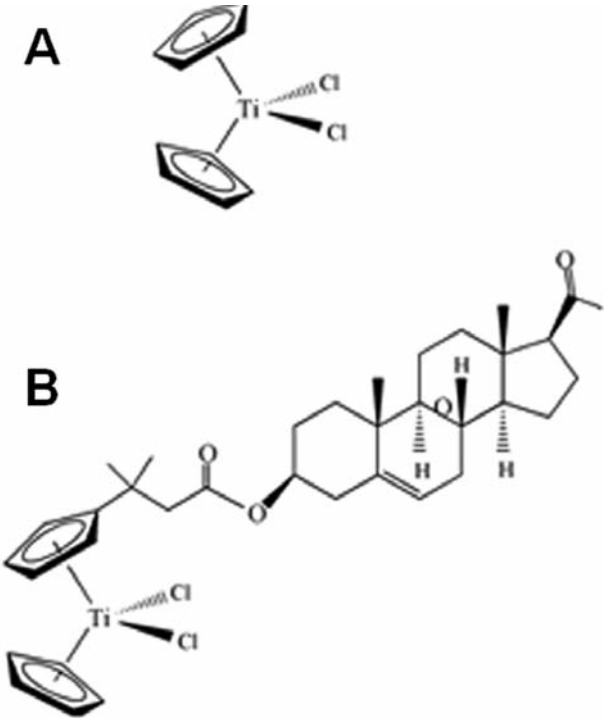

Figure 1. *Titanocene functionalized with pregnenolone: titanocene (A) and titanocenyl-pregnenolone (B).*

Ti-Preg demonstrated a significant inhibition on tumor growth compared to both vehicle and Cp_2TiCl_2 (Figure 4A). After day seven, tumor size increased exponentially in vehicle-treated or Cp_2TiCl_2-treated mice. However, mice treated with Ti-Preg demonstrated minimal growth over the 21-day period when compared to vehicle-treated or Cp_2TiCl_2 -treated groups, suggesting that Ti-Preg inhibited tumor growth ($p<0.05$ by two-way ANOVA). Consistent with these findings, mice treated with vehicle or Cp_2TiCl_2 presented a weight loss of 0.96±0.16 g and 4.2±2.5 g, respectively (Figure 4B), while Ti-Preg-treated mice showed an overall increase in weight of 0.52±0.6 g ($p=0.04$ *vs*. vehicle and 0.08 *vs*. Cp_2TiCl_2), which is consistent with a lower tumor burden.

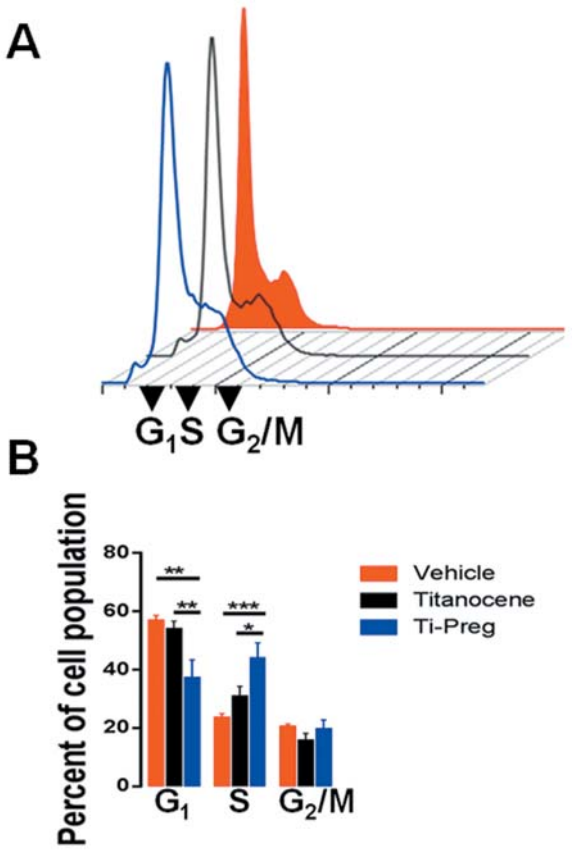

Figure 2. *Effect of titanocenyl-pregnenolone (Ti-Preg) on cell cycle distribution. A: Representative cell cycle curves from titanocene (Cp_2TiCl_2) (black) Ti-Preg (blue) or vehicle (red) treated cells, representative of at least five experiments. B: Quantification of cell cycle progression from five separate experiments. Two-way ANOVA with Tukey multiple comparisons test: n=5, *p≤0.01, **p=≤0.001, and ***p=0.0001.*

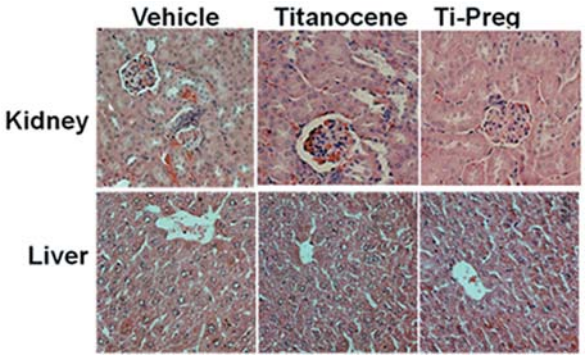

Figure 3. *Examination of the in vivo effects of titanocenyl-pregnenolone (Ti-Preg). Representative hematoxylin and eosin-staining of kidneys and liver from mice following a one-week regimen of vehicle, titanocene, or 0.04 mg/kg Ti-Preg and harvested after 21 days (N=3).*

Tumor interaction with its microenvironment causes heightened platelet activation and often leads to disseminated intravascular coagulation (DIC). The presence of platelet protein, sTLT-1, in plasma has been shown to correlate with DIC (14). To evaluate the extent of DIC in our mice, we measured levels of sTLT-1. Consistent with inhibited tumor growth, we found significantly reduced levels of sTLT-1 in the plasma of the mice treated with Ti-Preg compared to either Cp_2TiCl_2-treated or vehicle-treated mice (p=0.046: Figure 5). These results suggest that there is less platelet involvement, as well as lower levels of DIC, in the Ti-Preg-treated mice compared to their control counterparts.

Mason trichrome staining of histological sections (Figure 6A) demonstrated no gross histological differences in the kidneys; evaluation of lungs, heart, and spleen were also normal (data not shown). Nevertheless, we detected slight vacuolization of hepatocytes in the liver of the Cp_2TiCl_2-treated mice. This vacuolization effect was not seen in vehicle-treated or Ti-Preg-treated mice. Because of the hepatic toxicity reported in clinical trials and the potential vacuolization seen in Cp_2TiCl_2-treated mice, we further evaluated serum levels of ALT. Both the Cp_2TiCl_2- and Ti-Preg treated mice demonstrated increased ALT levels. However, neither of these differences reached statistical significance (vehicle *vs.* Cp_2TiCl_2 p=0.08; vehicle *vs.* Ti-Preg p=0.1).

Evaluation of blood samples revealed significant differences in various populations of blood cells (Table II). Lymphocyte counts in the vehicle-treated mice remained stable, while mice treated with Cp_2TiCl_2 or Ti-Preg demonstrated a 43% and 49%, reduction respectively (p≤0.05). Monocytes were reduced in average cell count in both Cp_2TiCl_2-treated and Ti-Preg-treated mice when compared to vehicle treatment. However, such reduction was significant only in the Cp_2TiCl_2-treated group (p≤0.05). The red blood cell (RBC) count increased slightly in each of the treatment groups compared to initial values but these increases were not significant. Surprisingly, Ti-Preg had a protective effect on platelet count, preserving total platelet counts (892±312) compared with both control (562±177) and Cp_2TiCl_2 (417±252). Finally, Ti-Preg also had a protective effect on neutrophils, preserving average neutrophil counts compared to both controls.

Discussion

These data extend our previous *in vitro* findings and give strong support for further development and testing of chemotherapeutic agents functionalized with steroid pendent groups. In our early studies, we demonstrated that functionalized Cp_2TiCl_2 has a lower IC_{50} than titanocene against the breast cancer cell line MCF-7 (11). These intriguing results prompted us to pursue the compounds with

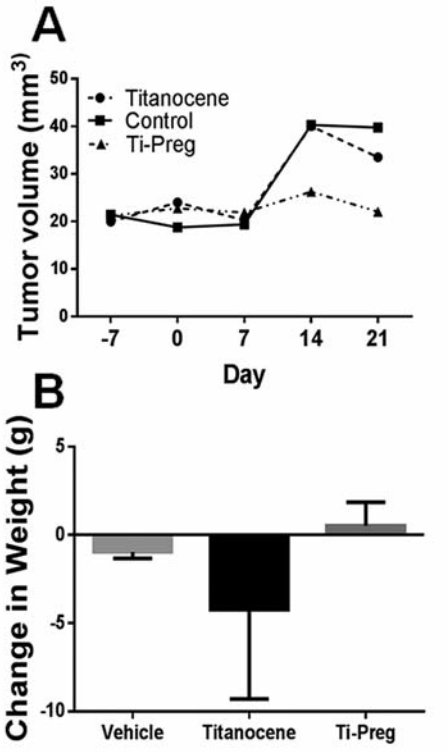

Figure 4. *Effect of titanocenyl-pregnenolone (Ti-Preg) in comparison to titanocene on the growth of MCF-7 cell tumors in nude mice. Athymic nu/nu mice bearing MCF-7 xenografts were treated with either vehicle, titanocene (0.9 mg/kg), or Ti-Preg (0.04 mg/kg). A: Mean tumor volumes for each treatment group as a function of time. Results represent mean for N=5, p=0.025; p-values were evaluated by ANOVA. B: Changes in animal weights over the 21-day observation period. Treatments were given between day -7 and 0.*

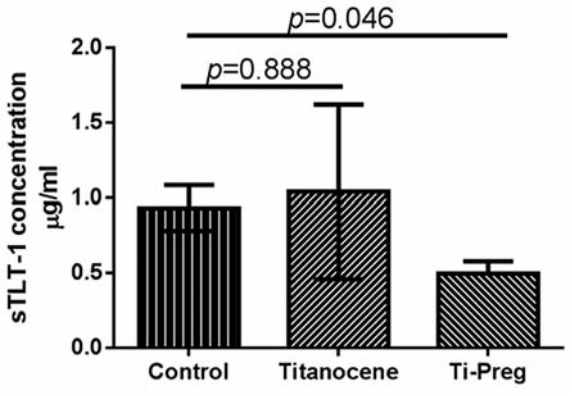

Figure 5. *Assessment of disseminated intravascular coagulation in cancer treated mice. Levels of soluble TLT-1 (sTLT-1) were measured in the plasma from mice bearing MCF-7 xenografts and subsequently treated with vehicle, titanocene, or titanocenyl-pregnenolone (Ti-Preg). Student's t-test was used for statistical analysis.*

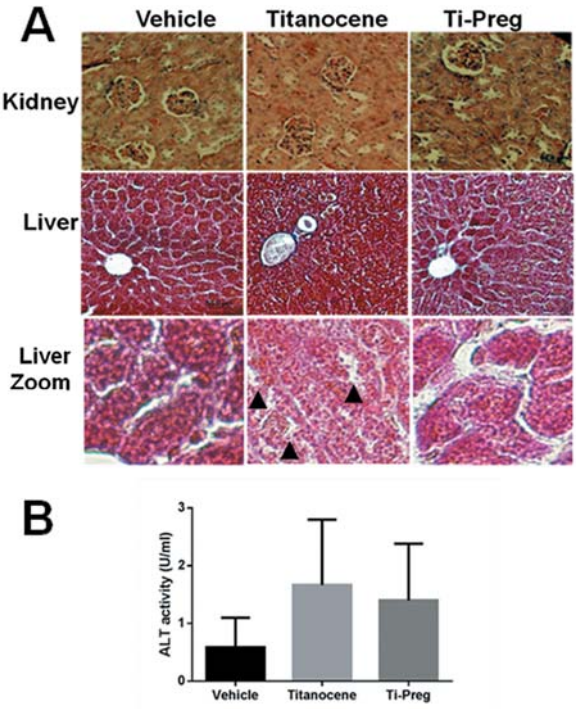

Figure 6. *In vivo effects of titanocenyl-pregnenolone (Ti-Preg) on kidney and liver toxicity. A: Mason Trichrome staining of kidneys and livers from mice with MCF-7 Xenografts treated for one week (every other day) with vehicle, titanocene, or Ti-Preg, and evaluated after 21 days of xenograft implantation. Black arrow heads show areas of vacuolization. B: Alanine transaminase (ALT) levels in mouse plasma, n=5. Student's t-test was used for statistical analysis.*

the lowest IC_{50}s as potentials anticancer agents. Out of the seven tested compounds, the Cp_2TiCl_2 functionalized with pregnenolone consistently yielded the best combination of efficacy, solubility, and ease of fabrication; therefore we selected Ti-Preg for further characterization.

Consistent with our growth-inhibition studies, we found that Ti-Preg inhibited cell-cycle progression with a concentration that was an order of magnitude lower than that for Cp_2TiCl_2. Our early studies used longer incubation times or higher concentrations of Cp_2TiCl_2 or Ti-Preg, all of which led to a significant fraction of the cells (>90%) with a sub-G_1 peak, considered to be apoptotic. Ti-Preg at lower concentrations caused a significant increase in the percentage of cells in the S phase of the cell cycle (Figure 2). These results are consistent with previous findings on the effects of Cp_2TiCl_2 on cell-cycle progression *via* a G_2/M phase block (3). Changes in platelet counts are commonly observed with chemotherapeutic drugs such as cisplatin and Cp_2TiCl_2 (15). Our dosing studies show that Ti-Preg causes only mild myelotoxicity with the exception of the platelet/megakaryocyte axis (15). As such, it is not surprising to see a drop in platelet count in the dosing studies. It was surprising, however, to see this effect reversed in the

presence of a tumor, where Ti-Preg played a platelet protective role in the tumor environment. Platelet counts dropped to approximately half in vehicle-treated and Cp_2TiCl_2-treated mice, while Ti-Preg-treated mice had only a ~10% drop in counts. This change may reflect an additional specificity of the attached steroid functional group, thereby targeting it to steroid receptor-positive tumor cells, such as the MCF-7 model. The preservation of platelet numbers in the Ti-Preg-treated group is consistent with the lower presence of DIC in this group.

Our histological evaluation revealed no gross defects or deviations in Ti-Preg-treated mice compared to the vehicle-treated mice. Considering that Cp_2TiCl_2 has been used at concentrations as high as 40 mg/kg and Ti-Preg is predicted to have an order of magnitude lower IC_{50}, we used the highest dose of Ti-Preg for the treatment of MCF7-derived mammary tumor-bearing mice. We selected a dose of Cp_2TiCl_2 that was lower than the published values, yet higher than the dose of Ti-Preg in order to emphasize the effects of the steroid pendent group.

Our data show that at the time when the tumors reached the exponential growth phase in the vehicle and Cp_2TiCl_2 treatment groups, tumor growth in mice treated with Ti-Preg only demonstrated a very modest increase in size. Notably, at 21 days, Ti-Preg-treated tumors remained significantly smaller than the tumors treated with either vehicle or Cp_2TiCl_2. Published studies with Cp_2TiCl_2 use concentrations between 30 and 40 mg/kg. The concentration used in our study, which was considerably lower, is probably the reason for the insensitivity of the mouse tumors to Cp_2TiCl_2. Regardless of the effect of Cp_2TiCl_2, Ti-Preg-treated mice demonstrated retarded growth and the tumors never achieved the exponential growth phase.

DIC is a hallmark of diseases such as sepsis, cardiovascular disease and cancer (16). Soluble TLT-1 is released from platelets upon activation. Our investigations demonstrated that in mice and humans, sTLT-1 levels are undetectable in the plasma of healthy individuals (17). However, in individuals suffering from various diseases that cause platelet activation in the periphery and diffuse coagulation, increased sTLT-1 levels correspond to the severity of DIC (14). Because cancer is associated with DIC, we investigated whether sTLT-1 levels are elevated as a measure of DIC, but also to give an indication of whether TLT-1 may play a role in cancer progression. Our results suggest that implantation of these tumors leads to DIC, and that TLT-1 may indeed play a role in cancer progression. These possibilities are being further explored in our laboratory.

Evaluation of the changes in blood cell counts between Ti-Preg- and vehicle-treated tumor-bearing mice revealed a significant reduction only in lymphocyte numbers. Cp_2TiCl_2-treated mice, however, exhibited significant declines in monocytes, in addition to lymphocytes. As with platelets, a protective effect was seen on neutrophils with Ti-Preg. Our results strongly suggest that Ti-Preg would be less toxic to the hemotological compartment than Cp_2TiCl_2.

Although there were no gross differences in kidney histology, there were subtle differences in the cellular topography of the Cp_2TiCl_2-treated tumor-bearing mice (Figure 6). However there were no signs of dilation, congestion of central vein, sinusoidal spaces, or portal vein. Early signs of vacuolization, indicative of changes in storage potential were seen throughout the liver tissue from Cp_2TiCl_2-treated mice. These signs were not seen in either the vehicle-treated or the Ti-Preg-treated groups. Surprisingly, both groups treated with titanium-containing compounds demonstrated less collagen deposition than the vehicle-treated tumor group. Collagen deposition is an early sign of liver fibrosis. The differences in serum ALT levels between the mice were not significant suggesting liver damage by the chemotherapeutic compounds was minimal, if any.

Herein, we demonstrate that using a steroid pendent group increases the efficacy of the Cp_2TiCl_2 without increasing its toxicity to the host tissue. We were able to demonstrate tumor growth inhibition with Ti-Preg using a lower dose than Cp_2TiCl_2, which showed no appreciable benefits. Although significantly higher doses were used in previously-published studies to achieve efficacy with Cp_2TiCl_2, our data demonstrate that lower doses of functionalized Cp_2TiCl_2 are effective. We have established the potential of steroid pendent groups on chemotherapeutic agents, and validated the screening process that was used to identify compounds with therapeutic potential. Moreover, these data demonstrate the need to validate these novel compounds on other types of steroid receptor-positive cancer such as prostate cancer, as well as their effects on metastasis.

Acknowledgements

Support for these projects was obtained through grants G12RR-03035 and 8U54MD007587-03 from the National Institute of Minority Health and Health Disparities, a component of the National Institute of Health and (1R01HL090933-01A2) from the National Heart Lung and Blood Institute.

References

1 Kopf H and Kopf-Maier P: Titanocene dichloride--the first metallocene with cancerostatic activity. Angew Chem Int Ed Engl *18*: 477-478, 1979.

2 Kopf-Maier P and Kopf H: Tumor inhibition by titanocene dichloride: First clues to the mechanism of action. Naturwissenschaften *67*: 415-416, 1980.

3 Christodoulou CV, Eliopoulos AG, Young LS, Hodgkins L, Ferry DR and Kerr DJ: Anti-proliferative activity and mechanism of action of titanocene dichloride. Br J Cancer *77*: 2088-2097, 1998.

4 Lummen G, Sperling H, Luboldt H, Otto T and Rubben H: Phase II trial of titanocene dichloride in advanced renal cell carcinoma. Cancer Chemother Pharmacol *42*: 415-417, 1998.
5 Mross K, Robben-Bathe P, Edler L, Baumgart J, Berdel WE, Fiebig H and Unger C: Phase I Clinical Trial of a Day-1, -3, -5 Every 3 Weeks Schedule with Titanocene Dichloride (MKT 5) in Patients with Advanced Cancer. (Phase I Study Group of the AIO of the German Cancer Society). Onkologie *23*: 576-579, 2000.
6 Korfel A, Scheulen ME, Schmoll HJ, Grundel O, Harstrick A, Knoche M, Fels LM, Skorzec M, Bach F, Baumgart J, Sass G, Seeber S, Thiel E and Berdel WE: Phase I clinical and pharmacokinetic study of titanocene dichloride in adults with advanced solid tumors. Clin Cancer Res *4*: 2701-2708, 1998.
7 Christodoulou CV, Ferry DR, Fyfe DW, Young A, Doran J, Sheehan TM, Eliopoulos A, Hale K, Baumgart J, Sass G and Kerr DJ: Phase I trial of weekly scheduling and pharmacokinetics of titanocene dichloride in patients with advanced cancer. J Clin Oncol *16*: 2761-2769, 1998.
8 Melendez E: Titanium complexes in cancer treatment. Crit Rev Oncol Hematol *42*: 309-315, 2002.
9 Gao LM, Hernandez R, Matta J and Melendez E: Synthesis, Ti(IV) intake by apotransferrin and cytotoxic properties of functionalized titanocene dichlorides. J Biol Inorg Chem *12*: 959-967, 2007.
10 Gao LM and Melendez E: Cytotoxic properties of titanocenyl amides on breast cancer cell line MCF-7. Met Based Drugs *2010*: 1-6, 2010.
11 Gao LM, Vera JL, Matta J and Melendez E: Synthesis and cytotoxicity studies of steroid-functionalized titanocenes as potential anticancer drug: Sex steroids as potential vectors for titanocenes. J Biol Inorg Chem *15*: 851-859, 2010.
12 Gao LM, Matta J, Rheingold AL and Melendez E: Synthesis, Structure and Biological Activity of Amide-Functionalized Titanocenyls: Improving their Cytotoxic Properties. J Organomet Chem *694*: 4134-4139, 2009.
13 Schlachterman A, Valle F, Wall KM, Azios NG, Castillo L, Morell L, Washington AV, Cubano LA and Dharmawardhane SF: Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model. Transl Oncol *1*: 19-27, 2008.
14 Washington AV, Gibot S, Acevedo I, Gattis J, Quigley L, Feltz R, De La Mota A, Schubert RL, Gomez-Rodriguez J, Cheng J, Dutra A, Pak E, Chertov O, Rivera L, Morales J, Lubkowski J, Hunter R, Schwartzberg PL and McVicar DW: TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. J Clin Invest *119*: 1489-1501, 2009.
15 Kopf-Maier P and Gerlach S: Pattern of toxicity by titanocene dichloride in mice. Blood and urine chemical parameters. J Cancer Res Clin Oncol *111*: 243-247, 1986.
16 Levi M and ten CH: Disseminated intravascular coagulation. N Engl J Med *341*: 586-592, 1999.
17 Gattis JL, Washington AV, Chisholm MM, Quigley L, Szyk A, McVicar DW and Lubkowski J: The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1, and evidence for a naturally occurring soluble fragment. J Biol Chem *281*: 13396-403, 2006.

Received October 31, 2013
Revised December 19, 2013
Accepted December 21, 2013