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In vitro and *in vivo* evaluation of first-generation carbosilane arene Ru(II)-metallodendrimers in advanced prostate cancer

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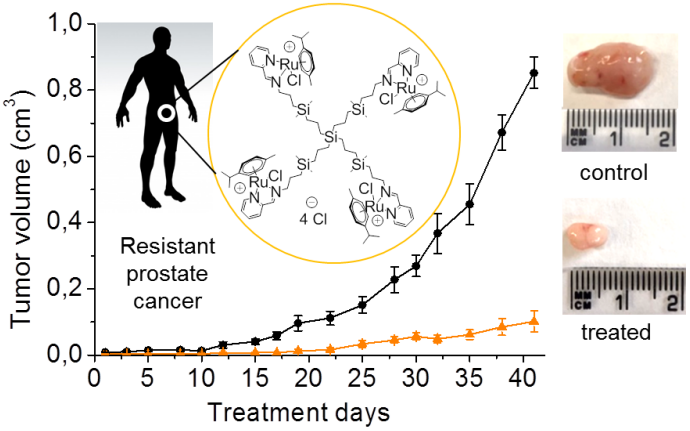

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ABSTRACT

Background: Prostate cancer is the fifth cause of death among men worldwide. Patients suffering resistant prostate tumor, unresponsive to common treatments, can only be treated with palliative therapy. *Materials & Methods*: Ruthenium(II) carbosilane metallodendrimers containing arene moieties were evaluated as a novel antitumor nanotherapy against resistant prostate cancer. The preclinical evaluation included relevant *in vitro*, *ex vivo* and *in vivo* assays, in an experimental mice model of human prostate cancer. *Results*: Promising cytotoxic, antiproliferative and antimetastatic behaviors were observed. After treatment with these nanocompounds, mice underwent a significant reduction of tumor volume, in comparison to non-treated animals. *Conclusions*: The selective antitumor behavior and the lack of toxicity, potentially make ruthenium(II) metallodendrimers promising agents for cancer therapy.

KEYWORDS. Dendrimer; metallodendrimer; ruthenium; cancer therapy; prostate cancer.

ABBREVIATIONS USED

BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; FITC, Fluorescein isothiocyanate; ICP, Inductively Coupled Plasma; IR, Infrared spectroscopy; MTT, 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PC3, human prostate cancer cell line; SEM, Standard Error of the Mean.

INTRODUCTION

Prostate cancer is an extremely frequent disease which appears in 50% male population over 70 years and around 100% male population over 90 years, being the fifth cause of death among men worldwide [1]. Prostate cancer is classified in four different stages [2], from a slow-progressing localized tumor in phase I to a metastatic aggressive tumor in phase IV. At the first stages, its asymptomatic nature in occasions leads to a late diagnosis when the cancer already is in an advanced phase [3].

The growth of prostate tumor cells is androgen-dependent thus making hormone-based therapy the most common treatment. However, a special type of tumor – non-responsive to this treatment- usually appears in late phases, after local treatment such as radiotherapy, radical prostatectomy or hormone-therapy, or in patients suffering a disseminated disease from the beginning of diagnosis, towards bone (80% cases) or other organs such as lung, brain or liver (20% cases) [4]. In these cases, the only existing therapy is a systemic palliative treatment such as docetaxel o cabazitaxel [5], or immunotherapy like denosumab, to avoid dissemination towards bone [6]. Consequently, new therapeutic strategies that can overcome this non-responsive behavior of prostate tumor are highly desired.

The field of cancer therapeutics is currently facing a major challenge related to tumor cell chemoresistance. Despite the success of platinum-based metallodrugs, such as cisplatin and its analogues, the appearance of toxic side-effects and chemoresistance forced the development of new strategies [7]. In the search of new metallodrugs with different modes of action, improved efficacy and minimum side-effects, several approaches have been explored [8]. A potential strategy, already successful for many types of cancers, is the use of organometallic and inorganic Ru(II) and Ru(III) complexes [9]. For example, the ruthenium(III) complex NAMI-A and its analogue UNICAM-1 can overcome drug chemoresistance through the activation of tumor infiltrating lymphocites present in the tumor microenvironment [10]. Other examples to overcome chemoresistance include gold nanoparticles which inhibit cisplatin-induced Epithelial to Mesenchymal Transition, thus reducing tumor cells enhanced

migratory capacity and invasiveness [11]; and Cu(I) oxide nanoparticles, which shift tumor mitochondrial metabolism and induce apoptosis of cancer cells [12].

The emergence of nanotechnology has provided new tools for the detection, imaging and treatment of prostate cancer [13]. Nanosized particles display unique physical and biochemical properties, including an exceptionally high surface area, and are known to improve the efficacy of cancer treatment through the Enhanced Permeation and Retention (EPR) effect, which favors a specific delivery of nanoparticles to tumor sites not accomplished with common drugs. Liposomes, micelles, dendrimers, carbon nanotubes as well as metallic and polymeric NPs have been evaluated as nanocarriers for cancer treatment. Nevertheless, few nanoparticle-based formulations have progressed into clinical trials [14]. The successful transition of nanoparticles from the lab bench to a real clinical setting requires addressing important challenges related to their safety, reproducibility and batch-to-batch manufacturing.

Among the new approaches targeting tumors, dendrimers appear to be particularly promising due to their precise synthesis and controllable multivalence [15, 16]. These unique properties deliver monodisperse entities, thus overcoming the batch-to-batch inconsistencies and enabling an exact structure-to-property relationship. Furthermore, dendrimers are excellent nanocarriers for different cargos such as nucleic acids, drugs and metallodrugs, increasing the drug selectivity, specificity, stability and bioactivity [17].

Our previous results in the use of carbosilane metallodendrimers based on arene ruthenium(II) complexes showed relevant *in vitro* cytotoxic activity towards a range of cisplatin-resistant cancer cell lines in the low micromolar range as well as low haemotoxicity [18]. In general, the dendritic macromolecules displayed a stronger cytotoxicity than their mononuclear counterparts, highlighting the impact of the dendritic structure on the antitumor activity. However, similar cytotoxic behavior was observed between first and second generation dendrimers, containing 4 and 8 metallic centers respectively, ruling out a clear relationship between the size of the dendrimer and its cytotoxic properties, and including other factors such as flexibility, accessibility and solubility. Interestingly, the first

generation metallodendrimers exhibited significant inhibitory properties on Cathepsin-B, protease overexpressed in invasive and metastatic processes.

Herein, we describe the use of first generation carbosilane metallodendrimers based on arene ruthenium(II) complexes G_1-[$NH_2Ru(\eta^6$-*p*-cymene)$Cl_2]_4$ (**1**), G_1-[NCPh(*p*-N)$Ru(\eta^6$-*p*-cymene)$Cl_2]_4$ (**2**) and G_1-[[NCPh(*o*-N)$Ru(\eta^6$-*p*-cymene)Cl]$Cl]_4$ (**3**) (Figure 1) as potential anticancer drugs against PC3 tumor cell line, derived from hormone-resistant prostate adenocarcinoma with bone metastasis (phase IV) [19]. Further *in vivo* evaluation in a human prostate tumor mice model revealed the potential of these metallodendrimers as antitumor agents in resistant prostate cancer.

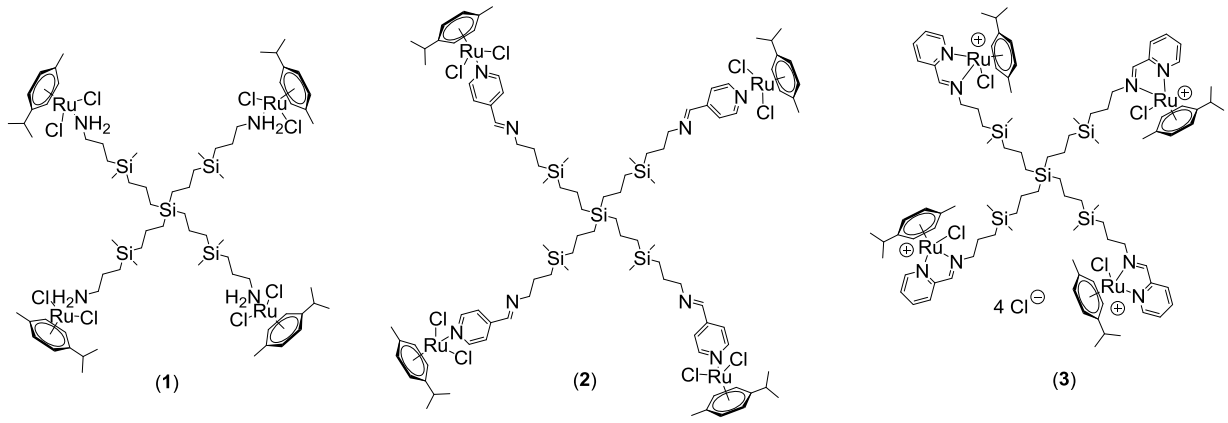

Figure 1. Chemical structure of selected metallodendrimers.

MATERIALS & METHODS

Metallodendrimers. First generation metallodendrimers G_1-[$NH_2Ru(\eta^6$-*p*-cymene)$Cl_2]_4$ (**1**), G_1-[NCPh(*p*-N)$Ru(\eta^6$-*p*-cymene)$Cl_2]_4$ (**2**), G_1-[[NCPh(*o*-N)$Ru(\eta^6$-*p*-cymene)Cl]$Cl]_4$ (**3**) and G_1-{[[NCPh(*o*-N)$Ru(\eta^6$-*p*-cymene)Cl]$Cl]_3$[FITC]} were synthesized according to previously described protocols [18, 20].

PC3 cell culture. The androgen-unresponsive cell line PC3 was obtained from the American Type Culture Collection (Manassas, VA) and may be related to recurrent prostate cancers that have achieved androgen independence. PC3 cells were grown routinely in RPMI-1690, containing 10% fetal bovine

serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Life Technologies, Barcelona, Spain) at 37 °C and 5% CO_2. After the cells reached 70–80% confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin/0.2% ethylenediaminetetraacetic acid (EDTA) and seeded at 3-4 x 10^4 cells/cm^2. The culture medium was changed every 3 days.

Cytotoxicity assays. Cells were seeded in 96-well plates (Nunclon Delta Surface, Thermo Fischer Scientific) as monolayers (approximately 5x10^4 cells/cm^2) and grown for 24 h in complete medium (90 ml). Solutions of compounds were prepared by diluting a freshly prepared stock solution in dimethylsulfoxide (DMSO) of the corresponding compound in aqueous medium (DMEM or RPMI). Afterward, the intermediate dilutions of the compounds were serially diluted to the appropriate concentration (ranging from 0 to 100 µM) and the cells were incubated for another 24 h (0.6% (v/v) is the maximum final content of DMSO). DMSO at comparable concentrations did not show any effects on cell cytotoxicity. Cytotoxicity was determined using a MTT assay (MTT, 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). MTT (5.0 mg/ml solution) was added to the cells and the plates were further incubated for 3.5 h. Then the culture medium was removed and the purple formazan crystals formed by the mitochondrial dehydrogenase and reductase activity of vital cells were dissolved in DMSO. The optical density, directly proportional to the number of surviving cells, was quantified at 570 nm (background correction at 690 nm) using a multiwell plate reader and the fraction of surviving cells was calculated from the absorbance of untreated control cells. The Inhibitory Concentration 50 (IC_{50}) value indicates the concentration needed to inhibit the biological function of the cells by half and is presented as the mean (± standard error) of three independent experiments, each comprising three microcultures per concentration level.

Cell proliferation assays. PC3 cells (2x10^5 cells/cm^2) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% penicillin/streptomycin/amphotericin B for 16 h. Then, cells were subjected to different treatments for 24 h. During the last 30 min of incubation, cells were labeled with 10 µM bromodeoxyuridine (BrdU).

Thereafter, cells were washed with PBS, fixed with ice-cold absolute ethanol, and stored at 20°C for 30 min. The fixative was removed by centrifugation and pellets were washed with PBS. DNA was partially denatured by incubation with 1 M HCl for 30 min at room temperature and then cells were washed three times with PBS containing 0.05% Tween-20 (pH 7.4) and 0.1% Bovine Serum Albumin (BSA). Cells were incubated with 20 ml of anti-BrdU monoclonal antibody linked to fluorescein isothiocyanate (FITC, BD Bioscience, San Agustin de Guadalix, Spain) for 30 min in the dark. For flow cytometry analysis, cells were stained with propidium iodide (PI) staining solution (50 mg/ml PI and 10 mg/ml RNase). The number of BrdU-positive cells was counted using a FACSCalibur cytometer (BD Bioscience). The results were analyzed using Cyflogic v1.2.1 program.

Cell cycle assays. PC3 cells ($2x10^5$ cells/cm^2) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% penicillin/streptomycin/amphotericin B for 16 h. After that, cells were subjected to various treatments for 8 h and subsequently washed with PBS and detached with 0.25% trypsin/0.2% EDTA. The cells were centrifuged at 500 g for 5 min at 4°C and the pellets were mixed with ice-cold 70% ethanol and then kept at 20°C for 30 min. After removing the ethanol by centrifugation, the pellets were washed with PBS and centrifuged again. The supernatants were discarded and the pellets were suspended in PBS, 0.2 mg/ml RNase A and 20 mg/ml PI before flow cytometry analysis was performed using FACSCalibur cytometer. The results obtained were analyzed using Cyflogic v1.2.1 program.

Cell adhesion assays. Concentrated type-I collagen solution was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 h at 37 °C. Plates were washed twice with PBS (pH 7.4). Cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were resuspended in RPMI medium/0.1% (w/v) containing BSA (pH 7.4). Then, cells were cultured at a density of $2.5x10^4$ cells/100 ml. The assay was terminated at indicated time intervals by aspiration of the wells. Cell adhesion was quantified by adding 1 mg/ml MTT followed by 1 h incubation. Isopropanol (50 µl) was

added to each well to dissolve the formazan precipitate and absorbance at 540 nm with a reference wavelength at 630 nm was measured.

Internalization assays. PC3 cells ($5x10^4$ cells/ml) were cultured in 60 m-dishes as monolayers and grown for 48 h. Afterward, the media was removed and cells were treated with the different compounds for 24 h, i.e. FITC (positive control), metallodendrimer **3** (negative control) and metallodendrimer **4** (fluorescent sample). Solutions were prepared by diluting a freshly prepared stock solution of the compound (DMSO for FITC, H_2O for **3** and **4**) in aqueous medium (RPMI-1640). After treatment, cells were washed with PBS and nuclei were labelled by incubation with PBS-HOESCH (Gibco Life Technologies, 1.5 ml) for 15 min. Cells were observed using a confocal laser microscope (Leica SP5 confocal microscopy). Images were acquired using double excitation at 488 nm (FITC) and 405 nm (HOESCH) and were recorded through separate channels. Serial plane images were collected at 0.1, 0.5, 1, or 2 μm intervals.

Animals, xenografts, *ex vivo* and *in vivo* assays. The Ethics Committee of Research and Animal Experimentation as Animal Welfare Body of the University of Alcalá (Madrid/Spain) in compliance with the article 33.1 of the Royal Decree 53/2013 of Spain and the European Directive 2010/63/EU on animal experimentation, has evaluated the present animal research project (code PROEX 194/16). The project has been performed at the Animal Research Center of the University of Alcalá (Legal registration code ES280050001165). After examining the application for evaluation, the Ethics Committee has considered that the research project and their procedures on animals are technically, ethically and methodologically suitable and therefore give this adequacy report, having regard to its possible effect on the animals used and having determined and evaluated the elements that best contribute to the replacement, reduction and refinement. In the same way, the Committee had been in charge of monitoring the project, in accordance to the article 38 of the mentioned Royal Decree.

Athymic male nude mice (*nu/nu*) 5–6 weeks old were obtained from Harlan (Oxon, UK) and maintained in microisolator units on a standard sterilisable diet. Mice were housed under humidity- and

temperature-controlled conditions and the light/dark cycle was set at 12 h intervals. For preparation of xenografts, PC3 cells were washed with PBS, detached with 0.25% trypsin/0.2% EDTA, centrifuged at 400 xg and resuspended in fresh medium at $5x10^7$ cells/ml. The cell suspension was mixed with Matrigel synthetic basement membrane (1:1, v/v, Becton Dickinson, Madrid, Spain) and then injected subcutaneously (s.c.) into the right flank of nude mice ($5x10^6$ cells/mouse). Three types of trials were performed in order to determine the antitumor behavior of compound **3**.

Experiment #1 – ex vivo antitumor behavior assay. Xenografts were developed according to two different treatment groups: group I (ten mice), injection of non-treated PC3 cells; group II (ten mice), injection of PC3 cells treated with metallodendrimer **3** (7.8 μM, IC_{50}) for 24 h. None of the animals died during the experiment. The experiment was finished after 41 days. Animals blood was collected under anaesthesia and the animals were sacrificed through cervical dislocation. During the assay, the mice health was visually examined, their weight was controlled twice a week as well as any behavioral anomaly. The animals´ condition was evaluated according to established tests on severity for the efficiency of new antitumor drugs, which were set as end point criteria. Tumor volume was measured with a microcaliper and calculated with formula I:[21]

$$\text{Volume} = \text{high} \times \text{wide} \times \text{deep} \times 0.5236 \quad \text{(I)}$$

Experiment #2 – in vivo lethal dose assay. Xenografts were developed in the animals and then the mice were randomly divided into two treatment groups: group 1 (one mouse), control and vehicle solution; group 2, seven mice treated s.c. with compound **3** at a dose of 5, 7.5, 10, 15, 20, 35 or 50 mg/kg body weight, once a day. The behavior and weight of the animals were examined daily. The experiment was ended on day 12 when the body weight of several mice was reduced by approximately 20%. There were no changes in the behavior of any of the animals studied.

Experiment #3 –in vivo antitumor behavior assay. Xenografts were developed in the animals and the experiment started when the tumors had grown to ≈ 85 mm^3. Animals were randomly divided into three

treatment groups and received the following treatment as daily s.c. injections: group 1 (ten mice), control and vehicle solution; group 2 (eight mice), compound **3** at a dose of 5 mg/kg body weight; and group 3 (eight mice), compound **3** at a dose of 15 mg/kg body weight. Tumor volume (Formula I) and body weight were measured twice a week and daily, respectively. The experiment was ended on day 36. The mice were sacrificed under isoflurane anesthesia. The tumors were dissected and weighed. Spleen, liver, kidneys, lungs, heart, and axillary nodes were removed carefully, cleaned, and weighed. Organ and tumor specimens were divided into two approximately equal portions: one portion was processed for immunohistochemistry (10% formalin fixed and paraffin embedded) and the other portion was frozen and maintained at -80 ºC for further experiments.

Inductively Coupled Plasma (ICP). ICP was used to evaluate de presence of Ru(II) in different mice tissue samples after the *in vivo* assay. The plasma conditions, sample preparation and calibration protocol are described in the Supporting Information.

Statistical analysis. Results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical significance was assessed through Bonferroni´s test for multiple comparisons after one or two-way analysis of variance (ANOVA). Differences were considered statistically significant $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

RESULTS AND DISCUSSION

The high incidence, aggressiveness and resistance to common treatments of advanced prostate cancer demand the development of new approaches that overcome these problems. A potential strategy is the attachment of organometallic or inorganic Ru(II) complexes into nanocarriers, improving the penetration of the drug, as well as certain pharmacological barriers such as solubility, degradation, targeting ability and bioavailability [9]. According to our previous results, carbosilane dendrimers functionalized with *N*-, *O*-, and *N*,*N*-chelating imine ligands coordinate Ru(II) ions and the corresponding metallodendrimers display significant cytotoxic activities in the low micromolar range in several human cancer cell lines, through a mode of action different from that of cisplatin [18]. We demonstrated that the attachment of the metal compound into the dendritic scaffold produced a substantial increase of the cytotoxicity; indeed, first generation dendrimers accounted for IC_{50} values in the range 2.5-10.7 μM for the different cell lines evaluated, while the analogue mononuclear counterparts displayed IC_{50} values up to 90 μM. Due to the simple-synthesis, water-solubility and promising cytotoxic and antimetastatic properties of first generation metallodendrimers, they were chosen as potential candidates towards resistant prostate cancer.

In vitro antitumor evaluation in PC3 cells

A set of experiments was designed to evaluate the antitumor behavior of first generation metallodendrimers **1**, **2** and **3**, displaying 4 ruthenium units, in PC3 cell line. The analysis included *in vitro* cytotoxicity, internalization and influence on proliferation, adhesion and cell cycle (Table 1).

Table 1. Comparison of *in vitro* antitumor activity of metallodendrimers **1**-**3** in PC3 cells.

Metallo dendrimer	*Cytotoxicity* [IC_{50} (μM)]	*Cathepsin B inhibition*[a] [IC_{50} (μM)]	*Proliferation inhibition* [% cel. BrdU]	*Adhesion to collagen-I* [% cel.]
ct	-	-	34.3±2.4	100±2.4
1	2.7±0.4	14.4	16.8±0.5***	63±2.8**
2	1.9±0.1	47.1	-	-
3	7.8±1.4	4.4	6.3±0.6***	45±1.7***

[a]Previously published results.[18] **p<0.01; ***p<0.001.

Aiming for an antitumor candidate highly cytotoxic towards tumor cells even at low doses, we evaluated metallodendrimers cytotoxicity on PC3 cells through MTT assay. The three metallodendrimers were cytotoxic, displaying IC_{50} values below 10 μM after 24 h treatment (Table 1), in agreement with other organometallic ruthenium (II) chelate complexes with *N*-, *N*,*N*- or *N*,*O*- ligands previously described, which inhibit the growth of PC3 cells even at nanomolar concentrations [22, 23]. Despite the high cytotoxic activity of metallodendrimer **2** (1.9 ± 0.1 μM), it was discarded for subsequent experiments due to its low inhibition of Cathepsin B activity, protease involved in metastatic processes [18]. Nevertheless, compounds **1** and **3** present low IC_{50} values (2.7 ± 0.4 and 7.8 ± 1.4 μM, respectively) in PC3 cells while keeping promising antimetastatic behavior (14.4 and 4.4 μM, respectively, for Cathepsin B inhibition).

Tumors uncontrolled invasion is related to a deregulation of cell proliferation and a suppression of cell apoptosis. The effect of metallodendrimers **1** and **3** on these critical events in PC3 was evaluated through flow cytometry, according to previous studies using Ru(II) complexes [24-27]. The effect was evaluated after 24 h treatment using concentrations 20% below IC_{50}, i.e. 2.1 μM (**1**) and 6.3 μM (**3**). As depicted in Figure 2, a change in all cycle phases was observed for PC3 cells treated with either **1** or **3**. The most important changes were found in subG_0 and G_1 phases, where treatment with metallodendrimers **1** or **3** increased the amount of apoptotic cells in subG_0 phase (10.8 and 15.6 fold higher, $p < 0.001$) and decreased those in G_1 phase (1.3 and 1.4 fold lower, $p < 0.01$ and $p < 0.001$, respectively), in comparison

to non-treated cells. These results indicated that selected metallodendrimers increased cell death through apoptosis and produced a cycle detention mainly in G_1 phase, when many signals are involved that determine cell development and cell decisions such as renew, differentiate or die.[11,16] Furthermore, treatment with compounds **1** and **3** led to a significant decrease of *in vitro* proliferative activity of PC3 cell line, already after 24 h treatment. Using concentrations 20% below IC_{50} values (2.1 μM (**1**), 6.3 μM (**3**)), we found a 51% decrease for compound **1** and 82% reduction for compound **3**, in comparison to non-treated cells ($p < 0.001$). Our metallodendrimers surpass the antiproliferative behavior of similar mononuclear Ru(II) complexes containing *p*-cymene and *N,N*-chelate ligands, which inhibited proliferation of lung adenocarcinoma A549 cells to 50%, using drug concentrations similar to the IC_{50} values reported here [28].

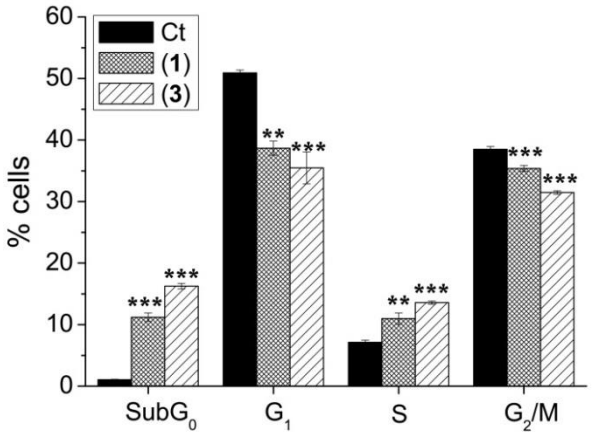

Figure 2. Distribution (% PC3 cells) in each phase of cell cycle after 24 h treatment with metallodendrimers **1** (2.1 μM) and **3** (6.3 μM). Results are the mean ± SEM of 3 independent assays.

Prostate cancer metastasis to bone is partially regulated by the ability of prostate cancer cells to adhere to the extracellular matrix and the bone marrow endothelial cells [29]. In order to evaluate the effect of treatment with metallodendrimers **1** and **3** on cell adhesion, different time-response assays of PC3 adhesion to collagen type-I (component of the extracellular matrix) in the presence of the different compounds were performed. Again, we used concentrations 20% below IC_{50} values (2.1 μM (**1**), 6.3 μM

(**3**)). A decrease on the adhesion of PC3 cells to type-I collagen sheet was observed after 80 min treatment with compound **1** (37% reduction, $p < 0.01$) and **3** (55% reduction, $p < 0.001$), Table 1. These results suggest that treatment with compounds **1** and **3** does not promote cellular localization on secondary metastatic tissues, such as bone, whose matrix is mainly formed by type-I collagen.

The efficacy and selectivity of cancer therapy are related to the drug uptake by cancer cells. Ru(II) complexes are known to penetrate cells through multiple mechanisms, including passive diffusion, active transport and endocytosis, while most nanostructured Ru(II) complexes enter cells by endocytosis.[9] In order gain further insight into the potential mechanism of action of our ruthenium metallodendrimers, we performed an attempt to evaluate the internalization of metallodendrimer **3** into PC3 cells using confocal microscopy. The absence of fluorescence emission from compound **3** at these wavelengths prevented the acquisition of relevant information. Consequently, we evaluated the analogous metallodendrimer **4**, displaying 3 ruthenium units and 1 fluorescein traceable moiety. The synthesis and structural characterization of **4** has been recently reported [20].

Unlike non-treated cells and those treated with compound **3** (both used as negative controls), cells treated with traceable metallodendrimer **4** showed green fluorescence in the cytoplasm (Figure 3), confirming its ability to cross the cell membrane and remain in the cytoplasm. The absence of green fluorescence in the cell nucleus indicates that compound **4** was not internalized in this compartment, ruling out a similar mechanism of action to that of cisplatin, which exerts its cytotoxic action through strong covalent bonds with DNA.[7] This fact confirms our previous results, where we demonstrated that compound **3** displays a weak electrostatic interaction with DNA and produces cell death through both apoptosis and necrosis [18]. It is worth mentioning that, in contrast to other cationic molecules [30, 31], compound **4** was internalized without any apparent disturbance of the cell membrane and toxicity to the cells. The balance between the charge density and the hydrophobicity of the dendritic scaffold might play a role in this action. Finally, the longer time required to accomplish the internalization of **4** (24 h), in

comparison to free FITC (2 h), is related to the bigger size of the dendritic molecule and a possible endocytic mechanism of internalization.

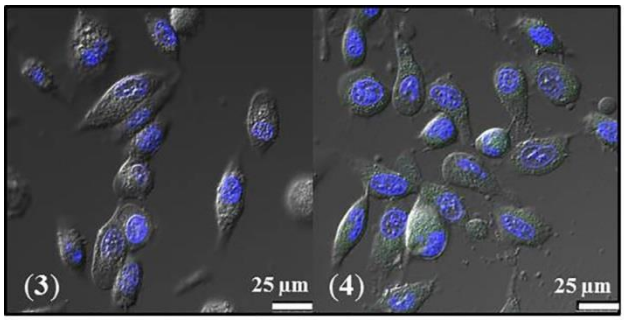

Figure 3. Confocal microscopy imaging of the cellular uptake of non-traceable metallodendrimer **3** (negative control) and its traceable analogue **4**, after incubation for 24 h with PC3 cells. Blue: cell nuclei stained with DAPI (excitation: 405 nm laser line), green: FITC-labeled metallodendrimer (excitation: 488 nm laser line) (Bar: 25 µm).

The overall results from the *in vitro* assays revealed important differences between metallodendrimers **1**, **2** and **3**, ascribed to their structural differences. Under the assay conditions, our former studies showed that all Ru(II) complexes were stable, both the monodentate complexes in compounds **1** and **2** and the chelate complexes in compound **3**, pointing out that the biological activity was due to the complete metallodendrimer and ruling out any Ru(II) release [18]. The structure flexibility, the lipophilic-lipophobic balance as well as the solubility in water are key factors in order to explain the different biological behavior. As example, the presence of cationic charges in compound **3** are responsible for its high solubility in water, while compounds **1** and **2** can only be dissolved in 1:99 DMSO:H_2O mixtures. To sum up, metallodendrimer **3** was selected for subsequent evaluation as a potential antitumor drug in resistant prostate cancer due to the following reasons: i) it is water-soluble enabling an easy administration; ii) it is cytotoxic at low micromolar doses and alters PC3 cell cycle, increasing cell death through apoptosis; iii) at concentrations lower than its IC_{50} inhibits proliferation as well as metastatic processes.

Ex vivo antitumor evaluation in a mice model

An experimental *ex vivo* mice model of human prostate cancer was established to evaluate the antitumor behavior of metallodendrimer **3**. Compound **3** was selected due to the promising cytotoxicity in *in vitro* assays, its low haemotoxicity and inhibition of proliferation as well as Cathepsin B activity (see Table 1). Untreated PC3 cells or cells treated with compound **3** at 7.8 µM (IC_{50}) for 24 h were inoculated subcutaneously into two different groups of immunodepressed mice. Tumor growth was measured twice a week for 41 days (Figure 4). The mice weight after treatment did not significantly change, in comparison to the non-treated group, and no mice died during the treatment. The results indicated that treatment with metallodendrimer **3** inhibited tumor growth, up to 82% smaller tumor size in comparison to non-treated group. The size of the tumor was doubled in 9.6 days, while the non-treated tumor required only 6.2 days, highlighting the delay in tumor progression after treatment with metallodendrimer **3**.

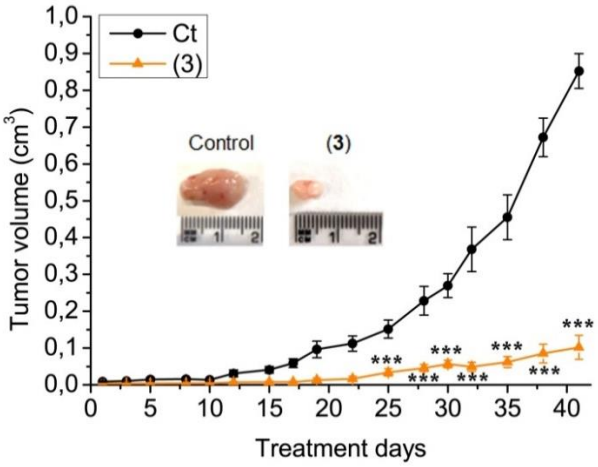

Figure 4. Tumor growth on athymic mice generated by PC3 cells pretreated with metallodendrimer **3** (7.8 µM, orange), in comparison to non-treated cells (control, black). Insert picture shows final tumor size for mice injected with pretreated and non-treated PC3 cells. Data represents mean ± SEM. ***p<0.001 vs control (10 mice).

In vivo antitumor evaluation in a mice model

An *in vivo* study was designed to establish a scenario as closer to clinical practice as possible. Initially, a lethal dose assay was performed in order to select a satisfactory working concentration of compound **3**,

ranging from 0 to 50 mg/kg/day. Mice received their treatments once a day through subcutaneous injection. The mice behavior and weight were examined daily, ending the assay on day 12 when the body weight of several mice was reduced by approximately 20%. A body weight decrease was perceived for mice treated with compound **3** at the highest doses already at day 5 (35 and 50 mg/kg) or day 7 (20 mg/kg), while concentrations below 15 mg/kg appeared to be safe during the span of the experiment. There were no changes in the behavior of any of the mice studied.

Two safe doses were selected for the subsequent *in vivo* assay, *i.e.* 5 and 15 mg/kg/day. In this assay, statistically significant tumor volume reduction was observed from day 29 for both doses. Metallodendrimer **3** administered subcutaneously resulted in up to 36% decreased tumor growth in subcutaneous tumors, using a concentration of 5 mg/kg/day (Figure 5.A). Triplicating the dose of compound **3** did not produce a significant improvement in antitumor activity. The mice weight after treatment did not significantly change, in comparison to the non-treated group (Figure 5.B). Biodistribution of metallodendrimer **3** was analyzed through ICP (Table S.1). The results confirmed a predominant presence of ruthenium(II) in the tumor mass, the liver and the kidneys, with ICP (ppb) values below the limit of quantification. In the rest of organs tested, the presence of ruthenium was below the limit of detection. Overall, this evaluation confirmed the selective action of compound **3** in the tumor as well as a fast elimination via urine route.

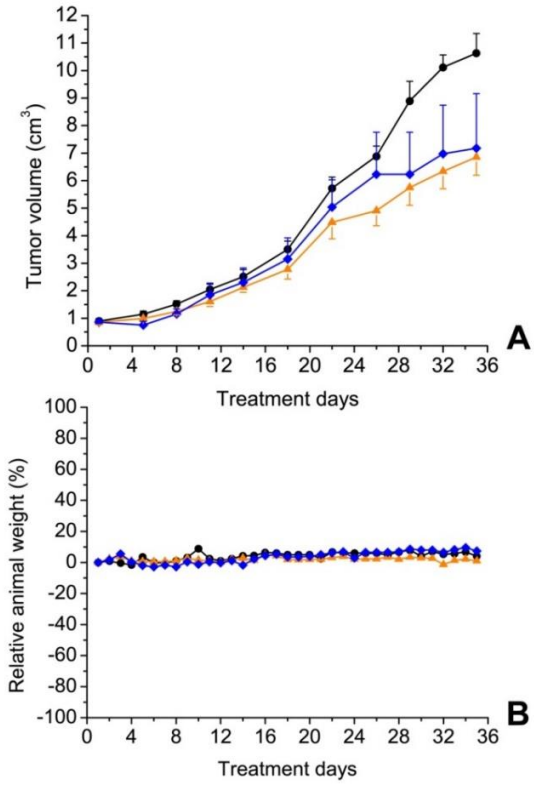

Figure 5. A. Tumor growth on athymic mice treated with metallodendrimer **3** at 5 (orange) and 15 (blue) mg/kg, in comparison to non-treated mice (black). B. Changes on the animals body weight during treatment (same color code as in A). Data represent mean ± SEM of the control group (10 mice) or treatment group (8 mice/group). *$p < 0.05$; ***$p<0.001$ vs control.

Arene Ru(II) monometallic compounds have been previously reported as successful *in vivo* antitumor agents. For example, the arene Ru(II) compound with a *N,N*-chelating ligand UNICAM-1 presented a high IC_{50} value (231 μM) in A17 triple negative breast cancer cells, but showed effective inhibition of tumor growth of A17 cells transplanted into mice, after four intraperitoneal administrations of 52.5 mg/kg/every three days [10]. Another arene Ru(II) complex bearing an iminophosphorane *N,O*-chelating ligand was subcutaneously administered (14 doses of 5 mg/kg/every other day) to mice with xenografted breast carcinoma [32]. The tumor size decreased 56% after 28 days of treatment. RAPTA-C is an arene Ru(II) complex bearing a 1,3,5-triaza-7-phosphaadamantane ligand which can reduce the growth of

colorectal tumors in a mice model up to 50%, using a intraperitoneal dose of 100 mg/kg/day for 11 days [33]. In comparison to these studies, our dendritic arene Ru(II) complexes bearing *N*,*N*-chelating iminopyridine ligands were used at much lower daily dose, minimizing the toxicity to healthy tissues. Despite the milder growth inhibition observed in our mice model, the obtained results are quite satisfactory due to the resistant nature of PC3 cells and the absence of alternative treatments for these patients. Furthermore, our assays confirm that the attachment of Ru(II) metal complexes into nanocarriers improves the selective *in vivo* delivery into tumor cells, in agreement with diverse studies found in the literature relying on single-walled carbon nanotubes, gold nanoparticles or nanocapsules [9].

CONCLUSIONS

In the field of prostate cancer, precision nanomedicine can overcome the limitations of current therapies. In particular, dendrimers appear as the flagship among precision nanoparticles, providing an unprecedented structure-to-property relationship and avoiding batch-to-batch inconsistencies. In this work, we have demonstrated that novel subcutaneously administered ruthenium(II) carbosilane metallodendrimers resulted in a significant decrease in resistant prostate tumor growth, with long-term survival of the animals and no signs of toxicity. By attaching the cytotoxic Ru(II) complexes into these precision nanoparticles, which are not substrates for the Multi-Drug Resistance transporters, we found a significant anticancer efficacy in resistant cancer cells. This antitumor behavior, together with the lack of toxicity, potentially make ruthenium(II) metallodendrimers promising agents for cancer therapy and should be further investigated. New administration routes and patterns, as well as a thorough study on the theranostic properties of the candidates are currently being explored.

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SUPPLEMENTARY MATERIAL

Supporting Information. ICP analysis.

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Notes

The authors declare no competing financial interest.

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