Journal of Organometallic Chemistry 695 (2010) 1175–1181

Contents lists available at ScienceDirect

Journal of Organometallic Chemistry

journal homepage: www.elsevier.com/locate/jorganchem

Antitumor activity of vanadocene Y and its selenocyanate derivative in xenografted caki-1 tumors in mice

Iduna Fichtner [a], James Claffey [c], Anthony Deally [c], Brendan Gleeson [c], Megan Hogan [c], Maria Rivera Markelova [b], Helge Müller-Bunz [c], Holger Weber [d], Matthias Tacke [c,*]

[a] Max Delbrück Center (MDC) for Molecular Medicine, Robert-Rössle-Str. 10, D-13125, Berlin, Germany
[b] Experimental Pharmacology and Oncology (EPO) GmbH, Robert-Rössle-Str. 10, D-13125, Berlin, Germany
[c] UCD School of Chemistry and Chemical Biology, Centre for Synthesis and Chemical Biology (CSCB), Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland
[d] ProQinase GmbH Freiburg, Breisacher Str. 117, D-79106 Freiburg, Germany

ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 15 January 2010
Accepted 21 January 2010
Available online 29 January 2010

Keywords:
Anticancer drug
Vanadocene dichloride
Renal-cell cancer
Cytotoxicity
Xenograft
Anti-angiogenesis

ABSTRACT

The *para*-methoxybenzyl-substituted vanadocene dichloride (Vanadocene Y) (**1**) and its diselenocyanate (Selenocyanato-Vanadocene Y) (**2**) were tested in vitro in an anti-angiogenesis assay against human umbilical vein endothelial cells (HUVEC) delivering IC50 values of 0.92 ± 0.03 μM (**1**) and 37 ± 11 μM (**2**). In a cytotoxicity assay against the human renal cancer cells, CAKI-1, the compounds demonstrated IC50 values of 0.55 ± 0.09 μM (**1**) and 0.25 ± 0.03 μM (**2**). Then both compounds were given at their maximum tolerable dose, MTD, of 20 mg/kg/d (**1**) or 40 mg/kg/d (**2**) on four consecutive days or at 50% of the MTD on five consecutive days per week for three weeks to overall four cohorts of eight CAKI-1 tumor-bearing female NMRI:nu/nu mice each, while a further cohort was treated with solvent only. Both MTD-treated mouse cohorts showed a statistically significant tumor growth reduction with respect to the solvent-treated control group with an optimal T/C value of 47% on day 39 of the experiment. Immunohistological analysis revealed that the expression of the proliferation marker Ki-67 was reduced due to long-term treatment with **2**.

1. Introduction

Renal-cell carcinoma (RCC) is the most common malignant disease of the adult kidney, which accounts for approximately 3% of adult malignancies [1]. If not detected early, these cancers develop to an invasive adenocarcinoma, which have very limited treatment options and poor outcomes. New targeted compounds like Bevacizumab, Sunitinib, Sorafenib, Temsirolimus and others give a certain amount of hope to patients with advanced renal-cell cancer, since these compounds can block the VEGF or mTOR pathway and are therefore anti-angiogenic [2]. Nevertheless these new drugs cannot cure advanced or metastatic renal-cell cancer and give the patient only a few extra months of survival. These clinical facts suggest new therapeutic regimens must be explored in the quest to develop an effective therapy for these metastatic or advanced forms of renal-cell cancer.

There is significant unexplored space for titanium-based drugs targeting cancer. Titanocene dichloride reached clinical trials, but the efficacy of this compound in Phase II clinical trials in patients with metastatic renal-cell carcinoma [3] or metastatic breast cancer [4] was too low to be pursued. The field got renewed interest with P. McGowan's elegant synthesis of ring-substituted cationic titanocene dichloride derivatives, which are water-soluble and show significant activity against ovarian cancer [5]. More recently, novel methods starting from fulvenes [6] allow direct access to anti-proliferative titanocenes via reductive dimerisation with titanium dichloride, hydridolithiation or carbolithiation of the fulvene followed by transmetallation with titanium tetrachloride in the latter two cases.

Hydridolithiation of 6-anisyl fulvene and subsequent reaction with titanium tetrachloride led to bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium(IV) dichloride (Titanocene Y) [7], which has an IC50 value of 21 μM when tested on the LLC-PK cell line. In addition, the anti-proliferative activity of Titanocene Y and other titanocenes has been studied in 36 human tumor cell lines [8] and against explanted human tumors [9,10]. Previous work has demonstrated that Titanocene Y induces apoptosis in a caspase-independent manner in a range of prostate cancer cells and it maintained its cytotoxic effects in Bcl-2 over expressing PC-3 cells [11]. Besides the direct apoptosis-inducing effects these titanocene derivatives give a positive immune response by up-regulating the number of natural killer (NK) cells in mice [12] and Titanocene Y proved itself as a strong anti-angiogenic compound with an IC50

* Corresponding author.
E-mail address: matthias.tacke@ucd.ie (M. Tacke).

0022-328X/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jorganchem.2010.01.026

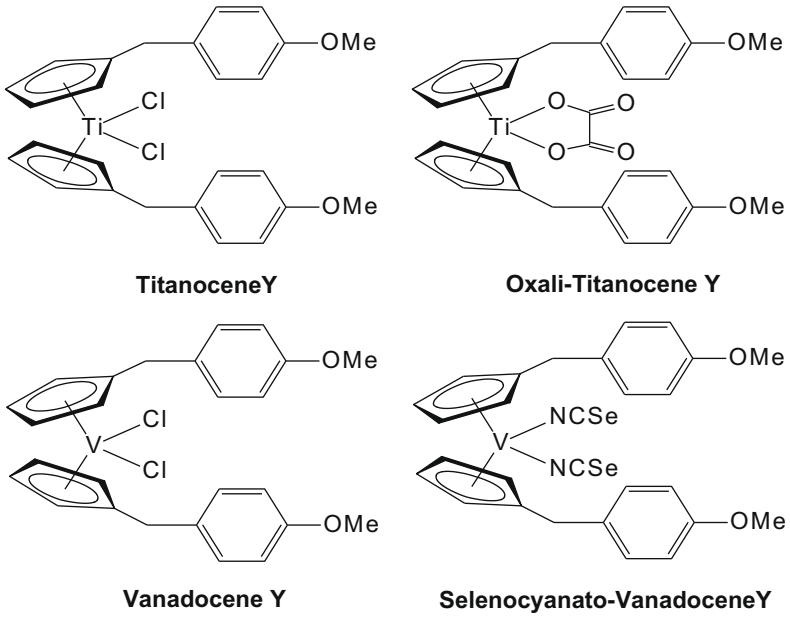


Fig. 1. Molecular structures of Titanocene Y, Oxali-Titanocene Y and Vanadocene Y.

value of 4.9 μM shown in a spheroid-based cellular angiogenesis assay [13]. Recently, animal studies reported the successful treatment of mice bearing xenografted A431 [14], PC-3 [15], Caki-1 [16] and MCF-7 [17] tumors with Titanocene Y. In these xenograft experiments a good tumor volume growth reduction (CAKI-1) or even a reduction of the tumor volume upon long-term treatment (MCF-7) and no severe side effects were observed. The body-weight loss due to diarrhoea was moderate; no influence on haematological (white blood cells, platelets) and renal toxicity - associated (creatinine, blood urea nitrogen) parameters was noticed. Recently, Oxali-Titanocene Y was synthesised [18] and evaluated in vitro and in vivo [19] and the chemistry was extended to vanadium [20,21], which led to the synthesis of Vanadocene Y. Vanadocene Y showed a high cytotoxic activity against LLC-PK cells with an IC50 value of 3.0 μM and shares therefore the activity level of clinically used platinum-based anticancer drugs. The structures of Titanocene Y, Oxali-Titanocene Y, Vanadocene Y (**1**) and its diselenocyanate derivative bis-[(*p*-methoxybenzyl)cyclopentadienyl] vanadium (IV) diselenocyanate (**2**) are shown in Fig. 1.

This paper is now investigating the synthesis of *para*-methoxybenzyl-substituted vanadocene diselenocyanate as seen in Scheme 1 and the anti-proliferative effect of **1** and **2** against CAKI-1 and HUVE cells in vitro and in CAKI-1 xenograft mouse models in vivo.

2. Materials and methods

2.1. Synthesis of bis-[(p-methoxybenzyl)cyclopentadienyl] vanadium (IV) diselenocyanate $[\eta^5\text{-}C_5H_4\text{–}CH_2\text{–}C_6H_4\text{–}O\text{–}CH_3]_2V(SeCN)_2$

0.24 g (0.49 mol) of **1** was dissolved in 40 ml of dry THF. To this solution 0.14 g (0.98 mmol) of KSeCN was added and allowed to stir at room temperature for five days, after which the solution had changed colour from dark green to red. The solution was then filtered and the solvent removed under reduced pressure to yield the red solid (0.25 g, 0.40 mmol, 80.9%).

Anal. Calc. for $VSe_2O_2N_2C_{28}H_{26}$ (631.39): C, 53.26; H, 4.15; N, 4.44, Found: C, 52.84; H, 4.12; N, 4.57%.

ESR (CH_2Cl_2 solution, RT): 8-line hyperfine coupling, g_{iso} = 2.024, A_{iso} = 7.25 mT.

MS (*m*/*z*, QMS-MS/MS): 421.43 $[M\text{-}SeCN_2]^-$

IR absorptions (KBr, cm^{-1}): 3075 (m), 2953 (w), 2928 (w), 2832 (w), 2070 (s), 1608 (m), 1511 (s), 1485 (w), 1437 (w), 1246 (m), 1175 (m), 1031 (w), 931 (w).

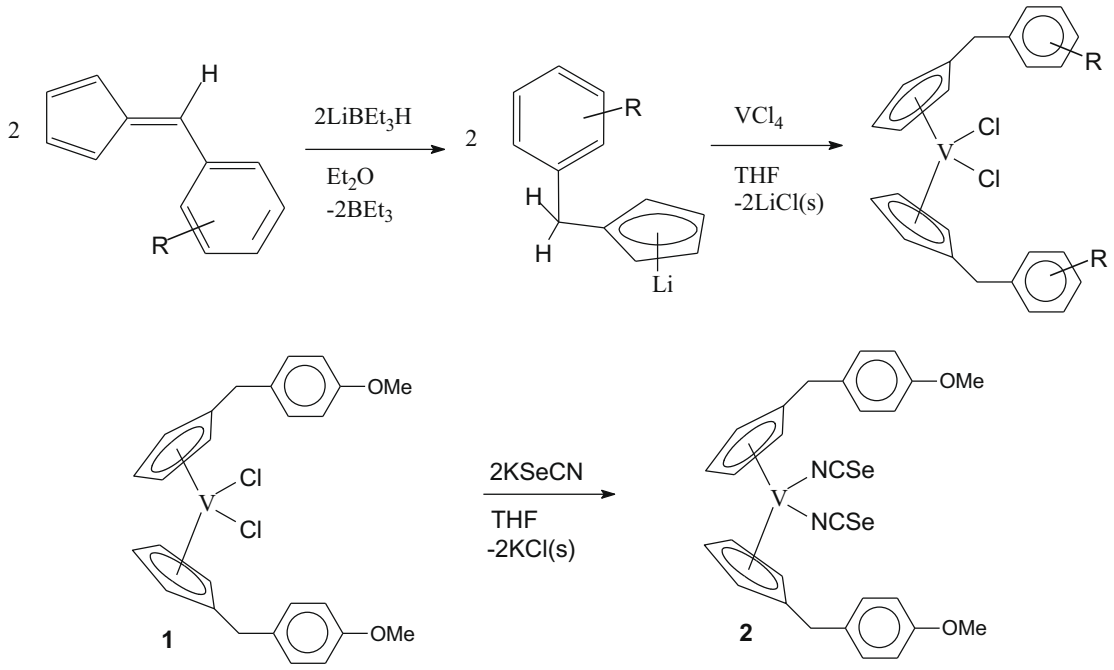
UV–Vis (CH_2Cl_2, nm): λ 235 (ε 31,919), λ 286 (ε 16,555), λ 384 (ε 3,179), λ 650 (ε 761).

2.2. CAKI-1 cell tests

CAKI-1 cells (ATCC HTB-46) are derived from a human clear cell carcinoma of the kidney, which represents the vast majority of human kidney cancer cases. The cells were seeded in 96 well plates (5000 cells per well) and cultivated with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 1% (v/v) penicillin streptomycin and 1% (v/v) L-glutamine. 24 h after seeding, **1** or **2** were dissolved in 0.7% DMSO, added in a concentration range of 10^{-4}–10^{-9} M and incubated for 48 h at 37 °C. Then, the solutions were removed from the wells and the cells were washed with PBS (phosphate buffer solution) and fresh medium was added to the wells. Following a recovery period of 24 h incubation, a routine MTT-assay [22] was performed, and IC50 values were determined. Each in vitro assay was performed three times in duplicates.

2.3. Spheroid-based cellular angiogenesis assay

HUVE cells were purchased from Promocell (Heidelberg, Germany) and used at passage 3–4. The experiments were pursued in modification of the originally published protocol [23,24]. HUVEC monolayers were trypsinised and suspended in ECGM (PromoCell) and methocel (4:1). The methocel was generated by dissolving 6 g of carboxymethylcellulose (Sigma–Aldrich, Steinheim, Germany) in 500 ml ECBM (PromoCell). Only the clear supernatant was used after centrifugation. Endothelial cell (EC) spheroids were prepared by pipetting 500 EC in a hanging drop (25 μl) on plastic dishes to allow overnight spheroid aggregation under culture conditions of 37 °C, 5% CO_2 and 100% humidity. The generated spheroids were harvested and suspended in the methocel solution containing 20% FCS (Cambrex, Verviers, Belgium). Subsequently, the ice-cold collagen solution (rat tail type I in 0.1% acidic acid) was neutralised



Scheme 1. General reaction scheme for the synthesis of benzyl-substituted vanadocene dichloride derivatives along with the synthesis of **2**.

by adding 10% Medium 199 10x (Sigma-Aldrich, Steinheim, Germany) and approx. 10% 0.2 N NaOH to adjust the pH to 7.4 EC spheroid/methocel solution was mixed 1:1 with the neutralised collagen solution and 0.9 ml of the mixed solution containing approximately 50 EC spheroids were pipetted into individual wells of a 24 well plate to allow polymerisation in the incubator at 37 °C, 5% CO_2 and 100% humidity. The test compounds were added after 30 min by pipetting 100 µl of a 10-fold concentrated working dilution on top of the polymerised gel in combination with VEGF-A [25 ng/ml,ProQinase,Freiburg,Germany]. VEGF-A activates ECs and induces the formation of tube-like EC structures ("sprouting EC"). Plates were incubated at 37 °C, 5% CO_2 and 100% humidity for 24 h and fixed by adding 4% paraformaldehyde.

Sprouting intensity of EC spheroids was quantitatively determined by the cumulative sprout length per spheroid using an inverted microscope and the digital imaging software Analysis 3.2 from Soft Imaging System (Münster, Germany). The mean of the cumulative sprout length of 10 randomly selected spheroids was analysed as an individual datum point.

2.4. CAKI-1 xenografts

In the first mouse experiment the maximum tolerable dose (MTD) of **1** and **2** were determined. Both compounds were solubilised in 10% Tween 80 in saline, which resulted in turbid but homogeneous microsuspensions for the two compounds. Then male NMRI:nu/nu mice (2 mice per group) were treated with 20, 40 and 60 mg/kg/d intraperitoneally (i.p.) in single injections over five days in order to determine the MTD, which resulted in 20 mg/kg/d for **1** and 40 mg/kg/d for **2**.

In the second in vivo experiment 10^7 CAKI-1 cells were injected subcutaneously (s.c.) to male NMRI:nu/nu mice (8 mice per group) on day 0. When tumors were grown to a palpable size (5–6 mm diameter) treatment was initiated on day 32. Compound **1** was injected into mice i.p. using a dose of 20 mg/kg/d (group C) and compound **2** using a dose of 40 mg/kg/d (group E) once daily at four consecutive days, while a further group (A) of mice was treated with the solvent only as negative control. In groups B and D compounds **1** and **2** were injected into mice i.p. using a reduced dose of 10 mg/kg/d or 20 mg/kg/d once daily at five consecutive days per week for three weeks (days 32–35, 39–43, 46–49). Tumor size was measured with a caliper-like instrument. Tumor volumes, relative tumor volumes (relation to the first treatment day) and treated/control (T/C) values were calculated. Body-weight and lethality of the mice were determined continuously during the experiments for an estimation of tolerability. Statistical evaluation was performed with the *U*-test of Mann and Whitney with a cut-off level of $p < 0.05$. On day 50 tumors of five mice per group were taken, snap frozen and used for immunohistological investigations. The animal experiments were performed according to the German Animal Protection Law and with approval from the responsible authorities. The in vivo procedures were consistent and in compliance with the UKCCCR guidelines.

2.5. Immunohistology

5 µm thick sections from snap frozen CAKI-1 tumors were fixed with 3.7% paraformaldehyde, blocked with H_2O_2 and goat serum. After the primary antibody (anti-mouse CD31, clone: MEC13.3, BD Pharmingen, Heidelberg, Germany), slides were incubated with an HRP-labelled antibody (Jackson ImmunoResearch, Hamburg, Germany), DAB substrate (Dako, Hamburg, Germany) and counterstained with hematoxylin. Evaluation of micro vessel density was performed with AxioVision 4.5 from ZEISS (Jena, Germany). Vessels were labelled in six representative pictures of each tumor and quantified for following parameters: Micro vessel size, number and ratio (micro vessel area versus total tumor area). Staining of the Ki-67 protein was performed according to CD31 and the antibody was purchased from Dako (clone: MIB-1). Instead of a secondary antibody the LSAB2 system from Dako was used. For evaluation of Ki-67 expression five pictures of each tumor were taken and the number of positive/negative cells was counted in three fields of view.

2.6. Crystal structure determination

Crystal data were collected using a Bruker SMART APEX CCD area detector diffractometer. A full sphere of reciprocal space

was scanned by phi-omega scans. Pseudo-empirical absorption correction based on redundant reflections was performed by the program SADABS [25]. The structures were solved by direct methods using SHELXS-97 [26] and refined by full matrix least-squares on F^2 for all data using SHELXL-97 [26]. Hydrogen atoms were added at calculated positions and refined using a riding model. Their isotropic temperature factors were fixed to 1.2 times (1.5 times for methyl groups) the equivalent isotropic displacement parameters of the carbon atom the H-atom is attached to. Anisotropic thermal displacement parameters were used for all non-hydrogen atoms. C–C bond lengths of the solvent molecule were restrained to be 1.5 Å. DELU (rigid bond) restraints were applied to all thermal displacement parameters of the solvent. CCDC 756029 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via <www.ccdc.cam.ac.uk/data_request/cif>.

3. Results and discussion

3.1. Synthesis

Vanadocene Y (**1**) was synthesised according to a previously published method [20].

The vanadocene diselenocyanate derivative was synthesised by dissolving **1** in dry THF and stirring with 2 equivalents of KSeCN for 5 d, resulting in the desired air stable red solid with a yield of 81% and KCl as a solid by-product. The compound is readily soluble in organic chlorinated solvents and DMSO, which allows for its separation from the reaction mixture and the formulation in the in vitro and in vivo experiments.

3.2. Structural discussion

A suitable crystal structure of compound **2** was isolated (Fig. 2). The vanadocene complex crystallised in the orthorhombic space group Pbcn (#60). The structure of **2** contained four molecules in the unit cell, with the respective molecule placed on a twofold axis. The structure also contained two solvent molecules, which were found to be C_5H_{12} and C_6H_{14}. Selected bond lengths and angles are displayed in Table 1, while the crystal data and refinement details for all three compounds are found in Table 2.

The bond length between the vanadium centre and the centroid of the cyclopentadienyl rings for compound **2** was found to be 1.954 Å. This bond length is slightly shorter than the V–Cp(Centroid) bond in Vanadocene Y, which was in the range of 1.974–1.979 Å. These lengths are comparable to those found in the literature for Cp–V bonds. These bond lengths are also shorter than the corresponding Ti–Cp(Centroid) bond, which has a bond length of 2.060 Å for Titanocene Y. This is due to the unpaired electron at the vanadium d^1 centre, which leads to back bonding at the Cp ligands, resulting in shorter bond lengths.

Table 1
Selected bond lengths and angles from the crystal structure of **2**.

Complex **2**	
Bond lengths [Å]	
V–C(1)	2.355(6)
V–C(2)	2.286(6)
V–C(3)	2.238(7)
V–C(4)	2.254(8)
V–C(5)	2.294(7)
V–Cent	1.954(1)
C(1)–C(5)	1.386(9)
C(1)–C(2)	1.403(9)
C(2)–C(3)	1.375(11)
C(3)–C(4)	1.375(14)
C(4)–C(5)	1.492(13)
C(1)–C(6)	1.482(9)
C(6)–C(7)	1.527(8)
V–N	2.034(6)
Bond angles [°]	
Cent(1)–V–Cent(2)	134.95(3)
Cent(1)–V–N(1)	106.3(1)
Cent(1)–V–N(2)	106.7(1)
N(1)–V–N(2)	84.5(3)

The V–Cl bond lengths in compound **1** were 2.410 Å and 2.419 Å. The V–N bond length in compound **2**, which corresponds to the selenocyanate ligands, yielded a bond length of 2.034 Å. This leads to a stronger bond between the vanadium centre and selenocyanate ligand, which could result in slower hydrolysis of the compound in aqueous solution compared to compound **1**.

The Cp–V–Cp angle in compound **1** was previously found to be 132.79°, whereas the same angle was widened in the case of compound **2** to 134.95°.This is due to the smaller Van de Waals radius of the nitrogen element of the pseudo-halide ligand. As a result the anion–V–anion bond angle in compound to is narrowed to 84.5° for the N–V–N angle. The corresponding Cl–Ti–Cl bond angle was 88.5°.

The structure of compound **2** displays a distorted tetrahedral conformation.

There is also a variance in the ESR spectra between compounds **1** and **2**. **1** gave g_{iso} = 2.012 and A_{iso} = 7.47 mT, whereas **2** yielded results of g_{iso} = 2.024, A_{iso} = 7.25 mT. This highlights the difference in the environment surrounding the vanadium centre in both compounds due to the different anions.

The vanadocene complex features the expected band at 2070 cm^{-1} in the IR spectrum, which is typical for a nitrogen atom bound iso-selenocyanate group [27]. This band appears broadened, which is an indication of an overlap of the two expected stretching vibrations of the iso-selenocyanate groups. These groups donate

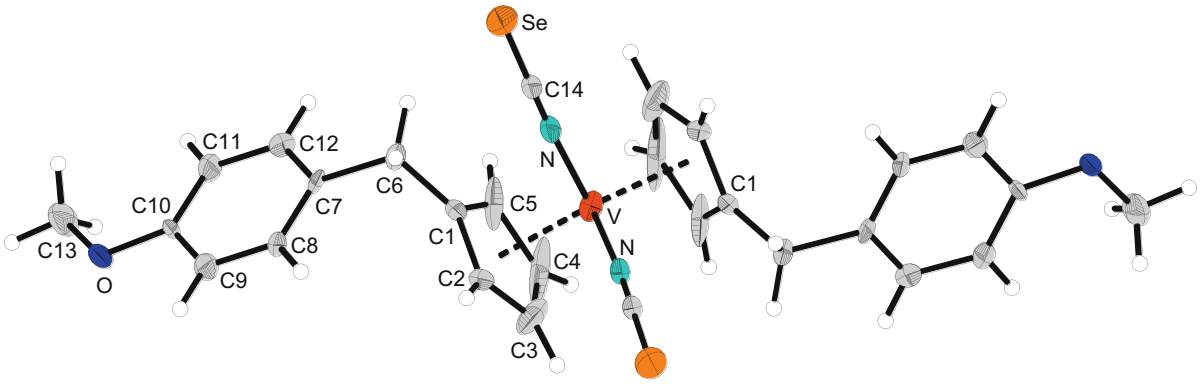


Fig. 2. X-Ray diffraction structure of 2; thermal ellipsoids are drawn on the 50% probability level.

Table 2
Crystal data and structure refinement for **2**.

Identification code	**2**
Empirical formula	$C_{67}H_{78}N_4O_4V_2Se_4$
Molecular formula	$(C_{28}H_{26}N_2O_2VSe_2)_2 \times C_5H_{12} \times C_6H_{14}$
Formula weight	1421.05
T (K)	100(2)
λ (Å)	0.71073
Crystal system	Orthorhombic
Space group	Pbcn (#60)
Unit cell dimensions	
a (Å)	16.019(3)
b (Å)	7.9387(14)
c (Å)	24.482(4)
α (°)	90
β (°)	90
γ (°)	90
V (Å^3)	3113.4(9)
Z	2
D_{calc} (Mg m^{-3})	1.516
μ (mm^{-}1)	2.690
F (0 0 0)	1444
Crystal size (mm)	0.60 × 0.20 × 0.02
θ range (°)	1.66–21.65
Index ranges	$-16 \leqslant h \leqslant 16$, $-8 \leqslant k \leqslant 8$, $-25 \leqslant l \leqslant 25$
Reflections collected	16333
Independent reflections (R_{int})	1821(0.0516)
Completeness to θ = 21.65°	99.20%
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9482 and 0.6511
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	1821/10/205
Goodness-of-fit (GOF) on F^2	1.048
Final R indices [$I>2\sigma(I)$]	R_1 = 0.0543, wR_2 = 0.1379
R indices (all data)	R_1 = 0.0688, wR_2 = 0.1472
Largest difference in peak and hole (e Å^{-3})	0.670 and −0.811

additional electron density to the vanadium centre when compared to the chloride ligands, resulting in an enhanced resistance to hydrolysis.

3.3. In vitro efficacy

The effect of **1** and **2** on in vitro angiogenesis was tested in the spheroid-based angiogenesis assay using HUVE cells. The IC50 value was determined to be 0.92 ± 0.03 μM for **1** and 37 ± 11 μM for **2** as shown in Fig. 3.

Compared to the standard set by Titanocene Y, which exhibits an IC50 value of 0.94 ± 0.02 μM this time and 4.9 μM in a previous report [13], **1** is as strong as Titanocene Y and **2** is a considerably weaker anti-angiogenic compound in vitro.

The in vitro cytotoxicity of **1** and **2** were determined in a MTT-based CAKI-1 assay, which show IC50 values of 0.55 ± 0.09 μM (**1**) and 0.25 ± 0.03 μM (**2**) respectively as shown in Fig. 4.

Since the IC50 value of Titanocene Y against CAKI-1 is 36 μM, it is obvious that both compounds are significantly more cytotoxic than Titanocene Y. Therefore it was decided to precede with the CAKI-1 xenograft experiments and to evaluate both vanadocene derivatives in vivo.

3.4. In vivo efficacy

In the MTD investigating mouse experiment three groups of two mice each were treated with 20, 40 and 60 mg/kg/d of **1** or **2**. The mice showed a dose dependent body-weight loss (BWC) of 11 or 16%, respectively. The highest dose of both compounds led to two out of two toxic deaths after 3–5 d, whereas 40 mg/kg/d of **1** induced complete mortality. The other mice recovered and

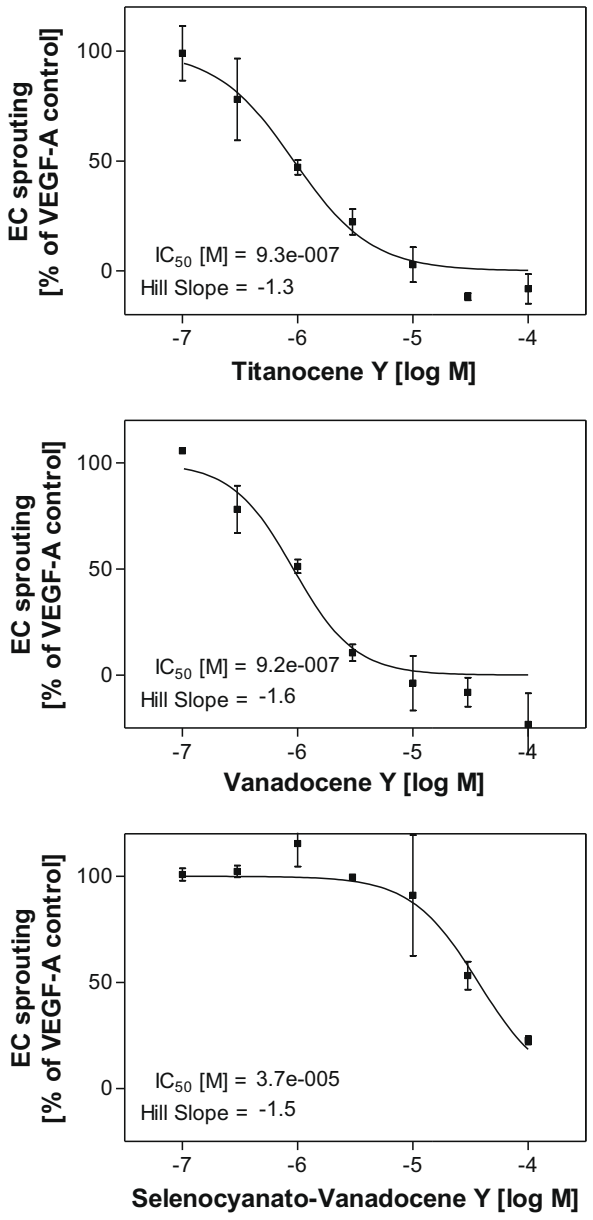


Fig. 3. Dose response curve showing the relative cumulative sprout length inhibition of HUVEC spheroids treated with **1** or **2**.

the body-weight loss was found to be reversible. From these investigations a dose of 20 mg/kg/d of **1** and 40 mg/kg/d of **2** were chosen for further experiments.

In the CAKI-1 xenograft experiment tumors revealed a long latency period before growing and treatment did not start before day 32, when the tumors reached a palpable size. Then five groups of 8 mice each were treated from day 32 onwards intraperitoneally with solvent or **1** or **2** at 100% or 50% at four/five consecutive days per week for one or three weeks. All observed parameters resulting from the in vivo experiment using CAKI-1 xenografts are presented in Table 3.

As shown in Fig. 5, in both MTD-treated groups C and E tumors grew slowly at almost the same rate and reached tumor volumes of 90% (both) on day 39 and 150% (C) and 190% (E) on day 50, while in the control cohort of group A the tumors grew to 190% on day 39 and 280% on day 50. Because of the significant body-weight loss of 24 or 22% in groups C and E treatment was cancelled on day 36, but three mice in group C and one mouse of group E died be-

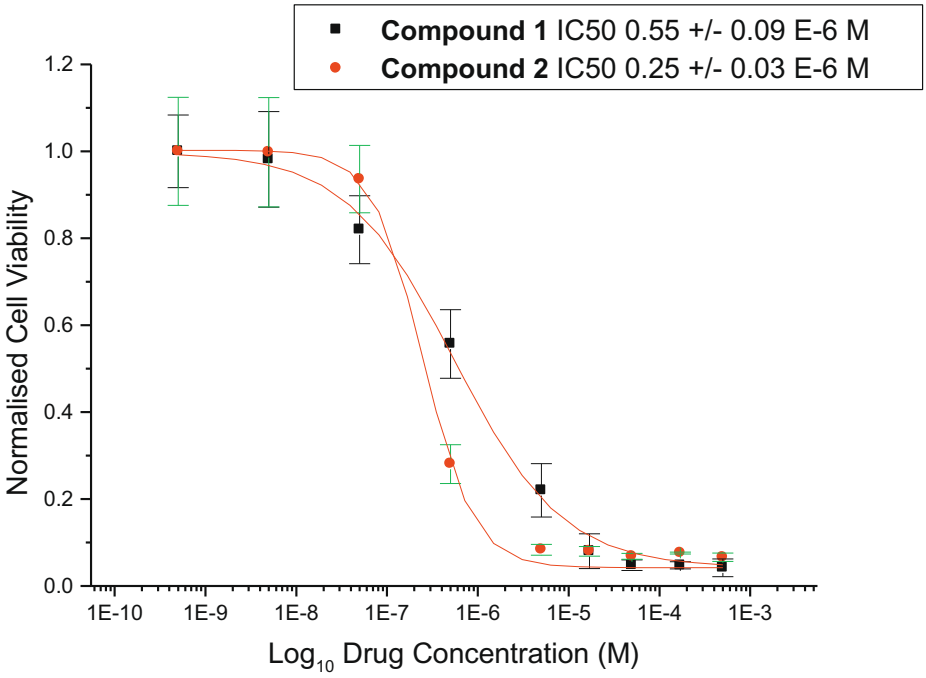


Fig. 4. Dose response curve showing the viability of CAKI-1 cells treated with **1** or **2**.

tween day 37 and 39. Nevertheless, treatment in groups C and E displayed statistically relevant tumor volume reductions with respect to group A and had both optimal T/C values of 47% on day 39 shortly after the end of the treatment. In groups B and D, which were treated at 50% MTD of **1** or **2**, tumor volumes reached 160% (B) and 155% (D) on day 39 and 255% (C) and 225% (E) on day 50. The body-weight loss of 4 or 5% in groups C or E was tolerable and none of the mice died. In groups B and D relatively small tumor volume reductions with respect to group A with optimal T/C values of 77% on day 42 (B) and 73% on day 46 (D) were observed, but only the effect in group D was statistically significant.

3.5. Immunohistology

When the experiment was finished at day 50, tumors were snap frozen. Cryoslices of five tumors each of the control and the two long-term treatment groups were analysed for CD31 and Ki57 expression.

The ratio of Ki-67 positive cells in the solvent-treated tumors was found to be 17.4%. The fraction of Ki-67 expressing cells remained practically unchanged upon treatment with **1**, while it was significantly decreased to 12.8% upon treatment with **2** implying a reduction of approximately 25%.

Micro vessels within a tumor area were detected by specific CD31 staining; the blood vessels did not change in number, but after treatment with compound **2** enlargement and dilatation was observed.

4. Conclusion and outlook

Through a facile anion exchange reaction **2** became available from **1**; **2** is more lipophilic and therefore an interesting alternative for further biological evaluation.

The in vitro experiments of **1** and **2** on the CAKI-1 cell line showed impressive cytotoxic effects with an IC50 values reaching into the nanomolar range for the first time for metallocene anticancer drug candidates. Furthermore, the HUVEC assay proved, that **2** is a medium and **1** a strong anti-angiogenic compound, which could possibly be used in a long-term treatment of human kidney tumors.

The in vivo experiments showed that **1** induces a statistically significant inhibition of tumor growth with a T/C value of 47% on day 39 in the CAKI-1 xenograft experiment when using the MTD and 77% when the dose is halved. Unfortunately, the toxicity of Vanadocene Y (**1**) is too high and results in unacceptable body-weight loss and three dead mice in the high dosage group. The Selenocyanato–Vanadocene Y (**2**) was better tolerated in the MTD group, led to a T/C value of 47% and only to one toxic death despite relatively high body-weight loss. At lower concentration of 50% MTD **2** showed a small but statistically relevant tumor volume reduction to 73% and the toxicity monitored by body-weight loss was tolerable.

In addition, the expression of the proliferation marker Ki-67 was reduced and the morphology of micro vessels in a defined tumor area was changed due to treatment with **2**.

Table 3
Overview on results obtained in the CAKI-1 xenograft experiment. Male nude mice received subcutaneous tumor cell injections on day 0. At palpable tumor size on day 32 the mice were randomised and treated with the compounds **1** or **2** as indicated. Tumor size in the treated group in relation to the control group (T/C) was measured as a therapeutic marker, while body-weight change (BWC) was used as a toxicity parameter. * Significant to control ($p < 0.05$). Solvent was 10% Tween 80 in saline.

Group	Mice	Substance	Treatment (*d*)	Dose (mg/kg/inj)	Toxic deaths (*d*)	BWC (%) d 32–39	Optimum T/C (%) [day]
A	8	Solvent[0]	32–35,39–43, 46–49		0	1	
B	8	1	32–35,39–43, 46–49	10	0	−4	77 [42]*
C	8	1	32–35	20	3 (37,37,38)	−24	47 [39]*
D	8	2	32–35,39–43, 46–49	20	0	−5	73 [46]*
E	8	2	32–35	40	1 (39)	−22	47 [39]*

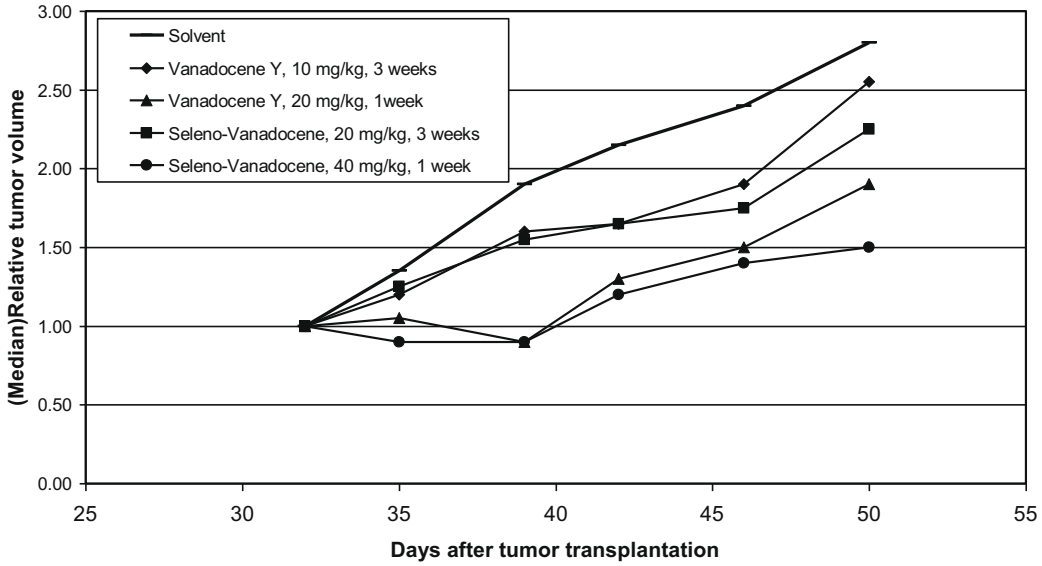


Fig. 5. Tumor growth curves of CAKI-1 xenografts in nude mice comparing 1 or 2 treated cohorts at MTD or 50% MTD against a control cohort.

This promising CAKI-1 mouse model should be a catalyst to promote more research into metallocene derivatives allowing them to re-enter clinical studies against renal-cell cancer in the future.

Acknowledgements

The authors thank the Higher Education Authority (HEA), the Centre for Synthesis and Chemical Biology (CSCB), University College Dublin (UCD), COST D39 and the Central European Society for Anticancer Drug Research (CESAR) for funding. Furthermore, the authors gratefully acknowledge the excellent technical support of M. Lemm, B. Büttner, and S. Gromova.

References

[1] Surveillance Epidemiology and End Results. National Cancer Institute, 2007. www.seer.cancer.gov.
[2] A.J. Schrader, R. Hofmann, Anti-Cancer Drugs 19 (2008) 235–245.
[3] G. Lummen, H. Sperling, H. Luboldt, T. Otto, H. Rubben, Cancer Chemotherapy Pharmacol. 42 (1998) 415–417.
[4] N. Kröger, U.R. Kleeberg, K.B. Mross, L. Edler, G. Saß, D.K. Hossfeld, Onkologie 23 (2000) 60–62.
[5] O.R. Allen, L. Croll, A.L. Gott, R.J. Knox, P.C. McGowan, Organometallics 23 (2004) 288–292.
[6] K. Strohfeldt, M. Tacke, Chem. Soc. Rev. 37 (2008) 1174–1187.
[7] N.J. Sweeney, O. Mendoza, H. Müller-Bunz, C. Pampillón, F.J.K. Rehmann, K. Strohfeldt, M. Tacke, J. Organomet. Chem. 690 (2005) 4537–4544.
[8] G. Kelter, N. Sweeney, K. Strohfeldt, H.H. Fiebig, M. Tacke, Anti-Cancer Drugs 16 (2005) 1091–1098.
[9] O. Oberschmidt, A.R. Hanauske, F.J.K. Rehmann, K. Strohfeldt, N. Sweeney, M. Tacke, Anti-Cancer Drugs 16 (2005) 1071–1073.
[10] O. Oberschmidt, A.R. Hanauske, C. Pampillón, N.J. Sweeney, K. Strohfeldt, M. Tacke, Anti-Cancer Drugs 18 (2007) 317–321.
[11] K. O'Connor, C. Gill, M. Tacke, F.J.K. Rehmann, K. Strohfeldt, N. Sweeney, J.M. Fitzpatrick, R.W.G. Watson, Apoptosis 11 (2006) 1205–1214.
[12] M.C. Valadares, A.L. Ramos, F.J.K. Rehmann, N.J. Sweeney, K. Strohfeldt, M. Tacke, M.L.S. Queiroz, Eur. J. Pharmacol. 534 (2006) 264–270.
[13] H. Weber, J. Claffey, M. Hogan, C. Pampillón, M. Tacke, Toxicol. in Vitro 22 (2008) 531–534.
[14] I. Fichtner, J. Bannon, A. O'Neill, C. Pampillón, N.J. Sweeney, K. Strohfeldt, R.W.G. Watson, M. Tacke, M.M. Mc Gee, Br. J. Cancer 97 (2007) 1234–1241.
[15] C.M. Dowling, J. Claffey, S. Cuffe, I. Fichtner, C. Pampillón, N.J. Sweeney, K. Strohfeldt, R.W.G. Watson, M. Tacke, Lett. Drug Des. Discovery 5 (2008) 141–144.
[16] I. Fichtner, C. Pampillón, N.J. Sweeney, K. Strohfeldt, M. Tacke, Anti-Cancer Drugs 17 (2006) 333–336.
[17] P. Beckhove, O. Oberschmidt, A.R. Hanauske, C. Pampillón, V. Schirrmacher, N.J. Sweeney, K. Strohfeldt, M. Tacke, Anti-Cancer Drugs 18 (2007) 311–315.
[18] J. Claffey, M. Hogan, H. Müller-Bunz, C. Pampillón, M. Tacke, ChemMedChem 3 (2008) 729–731.
[19] I. Fichtner, J. Claffey, B. Gleeson, M. Hogan, D. Wallis, H. Weber, M. Tacke, Lett. Drug Des. Discovery 5 (2008) 489–493.
[20] B. Gleeson, J. Claffey, M. Hogan, H. Müller-Bunz, D. Wallis, M. Tacke, J. Organomet. Chem. 694 (2009) 1369–1374.
[21] B. Gleeson, J. Claffey, A. Deally, M. Hogan, L.M. Menéndez Méndez, H. Müller-Bunz, S. Patil, D. Wallis, M. Tacke, Eur. J. Inorg. Chem. (2009) 2804–2810.
[22] T.J. Mosmann, Immunol. Methods 65 (1983) 55–63.
[23] T. Korff, H.G. Augustin, J. Cell. Biol. 143 (1998) 1341–1352.
[24] T. Korff, H.G. Augustin, J. Cell. Sci. 112 (1999) 3249–3258.
[25] SADABS, Bruker AXS Inc., Madison, Wisconsin, USA, 2001
[26] G.M. Sheldrick, Acta Crystallogr., Sect. A64 (2008) 112–122.
[27] A. Turco, C. Pecile, M. Nicolini, J. Chem. Soc. (1962) 3008–3015.