Bioorganometallic fulvene-derived titanocene anti-cancer drugs

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6-Substituted fulvenes are interesting and easily accessible starting materials for the synthesis of novel substituted titanocenes *via* reductive dimerisation, carbolithiation or hydridolithiation reactions, which are followed by a transmetallation reaction with titanium tetrachloride in the latter two cases. Depending on the substitution pattern, these titanocenes prove to be bioorganometallic anti-cancer drugs, which have significant potential against advanced or metastatic renal-cell cancer. Patients bearing these stages of kidney cancer have a poor prognosis so far and therefore real progress in the area of metal-based anti-cancer drugs may come from this simple and effective synthetic approach. This *tutorial review* provides an insight into the synthesis of fulvene-derived titanocenes and their activity in preclinical experiments.

Introduction

Beyond the field of platinum anti-cancer drugs there is significant unexplored space for further metal-based drugs targeting cancer. Titanium-based reagents have significant potential against solid tumors. Budotitane ([*cis*-diethoxy-bis(1-phenylbutane-1,3-dionato)titanium(IV)]) looked very promising during its preclinical evaluation, but did not go beyond Phase I clinical trials, although a Cremophor EL® based formulation was found for this rapidly hydrolysing molecule.[1] Much more robust in this aspect of hydrolysis is titanocene dichloride (Cp_2TiCl_2), which shows medium anti-proliferative activity *in vitro* but promising results *in vivo*.[2,3] Titanocene dichloride reached clinical trials, but the efficacy of Cp_2TiCl_2 in Phase II clinical trials in patients with metastatic renal cell carcinoma[4] or metastatic breast cancer[5] was too low to be pursued (Fig. 1).

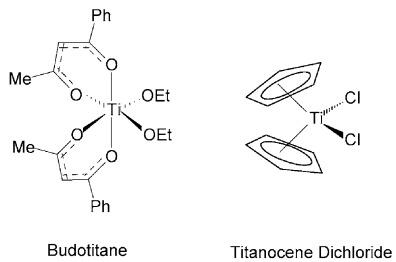

Fig. 1 Structures of budotitane and titanocene dichloride.

The field got renewed interest with McGowan's elegant synthesis of ring-substituted cationic titanocene dichloride derivatives, which are water-soluble and show significant activity against ovarian cancer.[6] More recently, novel methods starting from fulvenes and other precursors[7,8] allow direct access to substituted titanocenes *via* reductive dimerisation with titanium dichloride, carbolithiation or hydridolithiation of the fulvene followed by transmetallation with titanium tetrachloride in the last two cases. Within this article we will concentrate on the following four classes of substituted titanocenes, which can be synthesised using the above mentioned novel methods: *ansa*-

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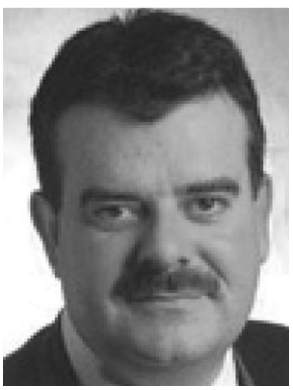

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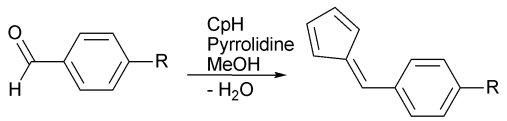


Scheme 1 Synthesis of 6-aryl fulvenes.

titanocenes, benzyl-substituted titanocenes, chiral 6-*N*,*N*-dimethylamino-functionalised titanocenes and achiral 6-bis-*N*,*N*-dimethylamino-functionalised titanocenes.

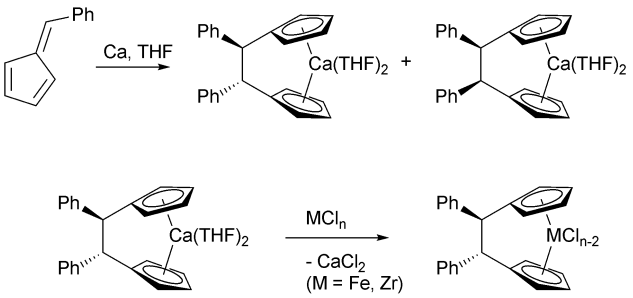
All four compound classes have the common feature that they can be synthesized using fulvenes as starting material. Fulvenes are easily accessible through the condensation reaction of the accordingly substituted benzaldehyde and freshly cracked cyclopentadiene in methanol in the presence of catalytic amounts of pyrrolidine. 6-Arylfulvenes can be obtained as orange solids, in rare cases as oils in yields up to 95%[9] and their use is an elegant approach to introduce biologically active groups to the titanocene dichloride moiety (Scheme 1).

Substituted titanocene dichlorides have potential application as metal-based anti-cancer drugs and therefore the cytotoxic activity of members of all four titanocene classes has been determined and compared. The cytotoxicity was consistently determined on the pig kidney cell line LLC-PK using the so-called MTT-based assay, in order to establish structure–activity relationships. The LLC-PK cell line was chosen in accordance to the Phase I and II studies of Cp_2TiCl_2, where renal cell cancer was identified as one of the main targets. For the *in vitro* cytotoxicity experiments LLC-PK cells were seeded into a 96-well plate and treated with various concentrations of the *ansa*-titanocene. After 48 h the drug was removed and the viability of the cells was determined by treatment with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Only living cells are able to transform MTT into formazan, which forms purple crystals, which can be measured by light absorption.

Using this method IC_{50} values (50% inhibitory concentration) between 930×10^{-6} mol l^{-1}(micromolar) and 5.4×10^{-6} mol l^{-1} have been determined for substituted titanocene dichlorides, with 6-*N*,*N*-dimethylamino-functionalised titanocene dichlorides showing the lowest IC_{50} values. For comparison we want to mention that the unsubstituted Cp_2TiCl_2 has an IC_{50} value of 2000×10^{-6} mol l^{-1} and *cis*-platinum, the most widely used metal-based anti-cancer drug, has an IC_{50} value of 3.3×10^{-6} mol l^{-1} when tested on the same cell line.

ansa-Titanocenes as metal-based anti-cancer drugs

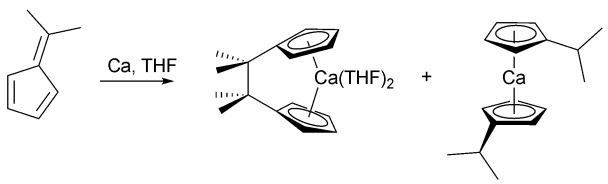
ansa-Titanocenes are characterised by an interannular bridge between the cyclopentadienyl (Cp) rings of the metallocene. As a consequence of the bridging moiety, the electronic behaviour of the metal centre is changed, because of the restricted geometry of the Cp rings.[10] The main application of *ansa*-metallocenes is their use as catalysts for stereoselective polymerisation of α-olefins.[11] Nevertheless, due to the cytotoxic activity of Cp_2TiCl_2, a range of substituted *ansa*-titanocenes have been tested for their potential application as anti-cancer drugs.



Scheme 2 Synthesis of bridged metallocenes by reductive dimerisation.

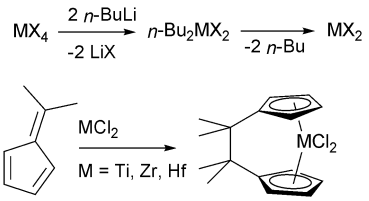
Reductive dimerisation of substituted fulvenes with activated alkali or alkaline earth metals and subsequent interaction of the resulting dianion with group 4 or other transition metal halides is one way of synthesising bridged metallocenes.[10] The reductive dimerisation can be achieved through the use of metal vapour synthesis or more conventionally by solution methods such as activated powders and the subsequent interaction of the resulting dianion with group 4 or other transition metal halides if necessary (see Scheme 2).[12]

Unfortunately, the reductive dimerisation is not the only process which takes place. For certain fulvenes, or at increased temperatures, hydrogen abstraction is a well known side reaction (see Scheme 3).[10]



Scheme 3 Hydrogen abstraction as side reaction.

Furthermore, *ansa*-titanocenes can be synthesized by using low valent transition metal complexes, *e.g.* group 4 halides, in a reductive dimerisation process involving two units of substituted fulvenes. Group 4 dihalides can be prepared by treating group 4 tetrahalides with two equivalents of *n*-BuLi in THF, followed by brief warming to rt in order to complete the reductive elimination of the intermediate di-*n*-butyl metal dihalide (see Scheme 4).[11]

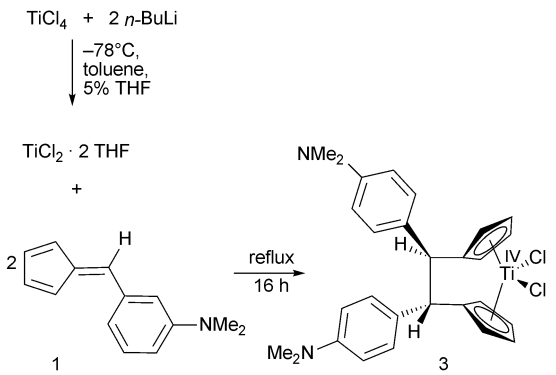


Scheme 4 Synthesis of *ansa*-metallocenes of Ti, Zr and Hf using reductive dimerisation of fulvenes.

C_2-Symmetrical *ansa*-metallocenes become available, if fulvenes—which carry two different substituents at the C6 position—are used. This compound class is known for their leading role as novel catalysts for the stereoselective

polymerisation of propene.[11] Unfortunately, the stereocentre is a disadvantage for a medical application as long as the isomers cannot be separated.

A variety of *ansa*-titanocene dichlorides have been synthesized by reacting two units of aryl-substituted fulvene with titanocene dichloride and their cytotoxic activity have been studied. A first series included *N*,*N*-dimethyl-phenyl and pentamethyl-phenyl substituted *ansa*-titanocene dichlorides (**3** and **4**).[13] These were synthesised by reacting 6-(*p*-*N*,*N*-dimethylanilinyl)fulvene (**1**) or 6-(pentamethylphenyl)fulvene (**2**), respectively with titanocene dichloride, which was obtained by reacting titanium tetrachloride with two equivalents *n*-BuLi in THF. The substituted *ansa*-titanocene dichlorides **3** and **4** were obtained in yields of 47 and 10% as dark solids, with *cis* : *trans* ratios of 60 : 40 and 93 : 7 (Scheme 5).



Scheme 5 Synthesis of *ansa*-titanocene dichloride **3**.

The evaluation of the cytotoxic potential of titanocene **3** using the above-mentioned standard assay revealed an IC_{50} of 270×10^{-6} mol l^{-1}, which represents a 10-fold increase in cytotoxicity compared to Cp_2TiCl_2 itself. Unfortunately, the 6-(pentamethylphenyl)-substituted *ansa*-titanocene (titanocene **4**) is even in DMSO fairly insoluble and therefore, it was not possible to determine any IC_{50} value (Fig. 2).

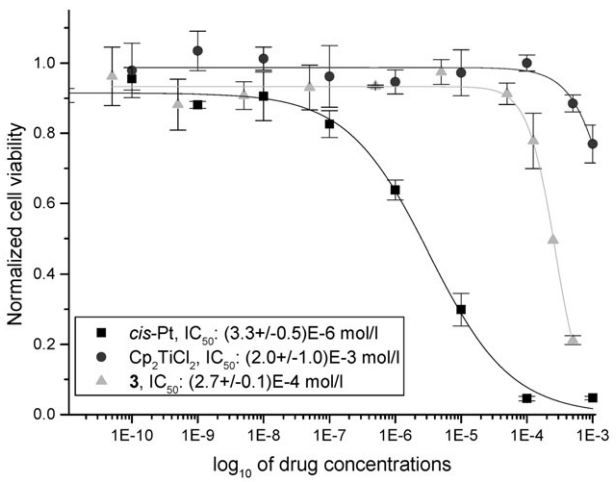


Fig. 2 Cytotoxicity curves from typical MTT assays comparing the effect of *cis*-platinum, Cp_2TiCl_2, and *ansa*-titanocene **3** on the viability of LLC-PK cells. (Reprinted from ref. 13 Fig. 5 with permission of Elsevier. © 2004 Elsevier.)

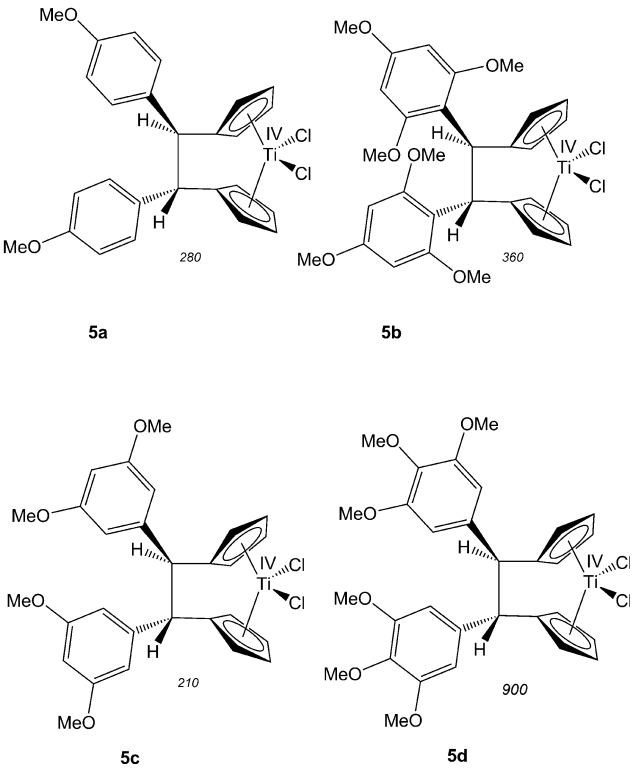


Fig. 3 IC_{50} values (italics; in 10^{-6} mol l^{-1}) showing the effect of titanocenes **5a–d** on the viability of LLC-PK cells.

Following the encouraging result from *ansa*-titanocene **3** a series of methoxyphenyl substituted *ansa*-titanocene dichlorides were synthesized and their cytotoxic potential evaluated.[14] 6-(4′-Methoxyphenyl)fulvene, 6-(2′,4′,6′-trimethoxyphenyl)-fulvene, 6-(3′,5′-dimethoxyphenyl)fulvene and 6-(3′,4′,5′-trimethoxyphenyl)fulvene, respectively, were used as starting material and the according substituted *ansa*-titanocenes were obtained as dark solids in yields of 44–66%. Cytotoxicity studies showed similar results compared to *ansa*-titanocene **3** with most IC_{50} values ranging between 210×10^{-6} mol l^{-1} and 360×10^{-6} mol l^{-1}. However, *ansa*-titanocene **5d** showed an exceptionally high IC_{50} value with 900×10^{-6} mol l^{-1} (Fig. 3).

It is essential for a successful reductive dimerisation process without the formation of by-products that the fulvene can be easily reduced to form a stable radical anion intermediate, which subsequently dimerises. For example, the use of dimethyl-substituted fulvene results in the formation of unbridged titanocene dichlorides as by-product. Therefore, it was very interesting to study a series of heteroaryl (furan, thiophene and *N*-methyl pyrrole) substituted fulvenes and the according substituted titanocenes.[15] Titanocene dichlorides **6a–c** were obtained as dark solids in yields of 40–63% and they showed a medium cytotoxic activity with IC_{50} values ranging from 450×10^{-6} mol l^{-1} to 200×10^{-6} mol l^{-1} (Fig. 4).

One major problem with the application of *ansa*-titanocenes as anti-cancer drugs is their low water solubility. Therefore, the benzo[1,3]dioxole,[16] the glycol methyl ether or the bis-(2-methoxyethyl)amino group[17] was introduced in order to improve the water solubility and the bioavailability of the titanocene compound. Benzo[1,3]dioxole substituted

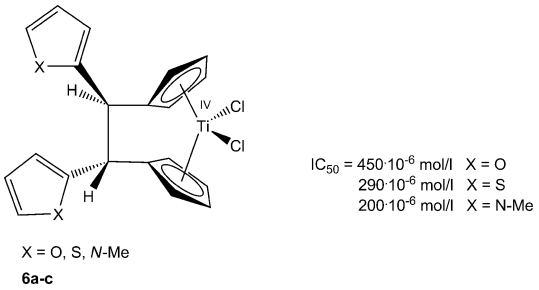


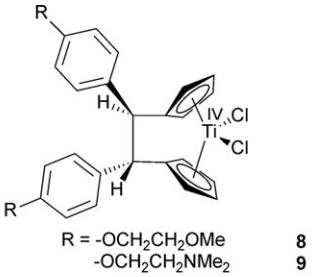
Fig. 4 IC_{50} values showing the effect of titanocenes **6a–c** on the viability of LLC-PK cells.

ansa-titanocene (**7**) showed an IC_{50} value of 160×10^{-6} mol l^{-1} when tested on the LLC-PK cell line and was obtained as a brown solid in a yield of 51%. This value represents a more than 10-fold improved cytotoxicity compared to Cp_2TiCl_2 and a slight improvement compared to the previously mentioned *ansa*-titanocene dichlorides.

4-(2-Methoxyethoxy)phenyl and bis-(2-methoxyethyl)amino substituted *ansa*-titanocenes **8** and **9** were isolated as black solids in yields of 22% and 42%. Cytotoxicity tests delivered very surprising results: for the first time a proliferative effect, in contrast to the anti-proliferative effects observed beforehand, was seen. This means that the cell growth was significantly increased and not reduced, when tested on the LLC-PK cell line. Only at very low concentrations (in the range of 10^{-5} to 10^{-8} mol l^{-1}) was a small cytotoxic effect observed in the treatment with titanocene **8** (Fig. 5). A comparable unexpected proliferative effect was observed by Jaouen and co-workers when they tested a titanocene derivative of the anti-cancer drug Tamoxifen on the human hormone-dependent breast cancer cell line MCF-7.[18] His compound contains a similar glycol amine group and hydrolysis processes were used as explanation for this surprising effect. Further explanations concerning the hydrolysis process of titanocenes can be found within the literature[18] and especially in studies by Sadler.[19]

As already mentioned, *ansa*-titanocenes contain stereocentres, which is a disadvantage for medical applications and for crystallisation experiments. Therefore, just a few *ansa*-titanocenes have been crystallised and their solid-state structure determined up to now. One of these rare examples is the crystal structure of titanocene **8**, where only the *trans* isomer is found in the solid state. In accordance with the centrosymmetric space group $P\bar{1}$, the (*R*,*R*) and (*S*,*S*) isomers are present in equal amounts. The molecule itself has a pseudo-C_2 symmetry in the solid state and all the bond lengths are in the normal range. The crystal structure and the structural elements are in accordance with results from quantum chemical calculations (Fig. 6).

Substituted *ansa*-titanocene dichlorides are available through reductive dimerisation of substituted fulvenes with $TiCl_2$. The obtained *ansa*-titanocenes show a medium high cytotoxicity in the range of 930–210 $\times$ 10^{-6} mol l^{-1}, highly depending on the substitution pattern. This does not appear to be a very high cytotoxic activity, but compared to Cp_2TiCl_2, which entered Phase II clinical trials, it still represents an up to 10-fold increase in cytotoxicity. Nevertheless, the target area of the lower micromolar range (*cis*-platinum has an IC_{50} value of 3.3×10^{-6} mol l^{-1} when tested on the same cell line) is far



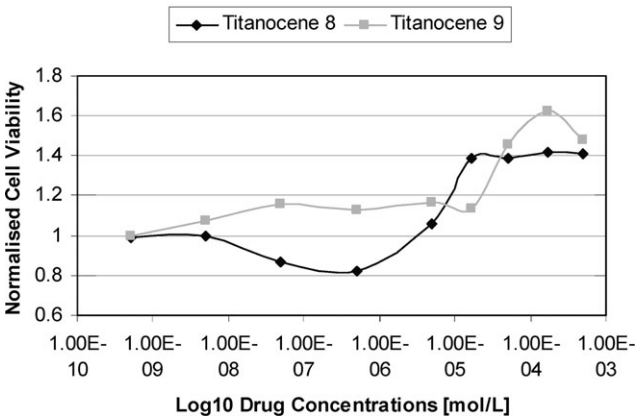


Fig. 5 Cytotoxicity curves from typical MTT assays showing the effect of *ansa*-titanocenes **8** and **9** on the viability of LLC-PK cells. (Adapted from Fig. 3 from ref. 17*a* and from Fig. 3 from ref. 17*b* with permission of Wiley-VCH Verlag GmbH & Co and Springer. © 2006 Wiley-VCH Verlag GmbH & Co; © 2007 Springer).

away and additionally, the presence of stereocentres does not make this compound class a very promising area as anti-cancer drugs. Therefore, new synthetic routes and new classes of substituted titanocene dichlorides, ideally without stereocentres, had to be developed (Fig. 7).

Novel benzyl-substituted titanocene dichlorides as potential anti-cancer drugs

In order to avoid stereocentres and to increase the cytotoxicity a novel class of substituted titanocene dichlorides, the

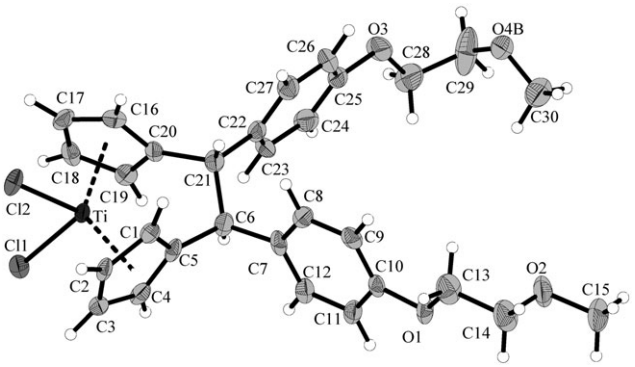


Fig. 6 Molecular structure of **8** in the crystal; thermal ellipsoids are drawn on the 50% probability level. (After Fig. 2 from ref. 17*a* with permission of Wiley-VCH Verlag GmbH & Co. © 2006 Wiley-VCH.)

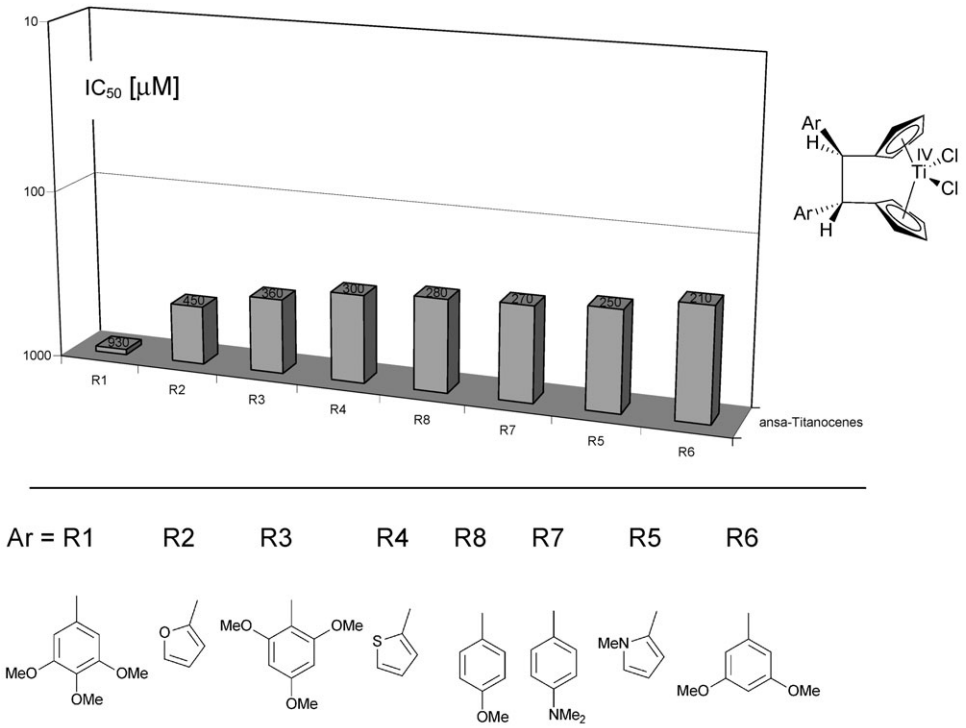


Fig. 7 IC_{50} values (LLC-PK) of titanocene derivatives obtained by reductive dimerisation.

so-called ''benzyl-substituted titanocenes'', were developed and tested for their potential application as anti-cancer drugs.

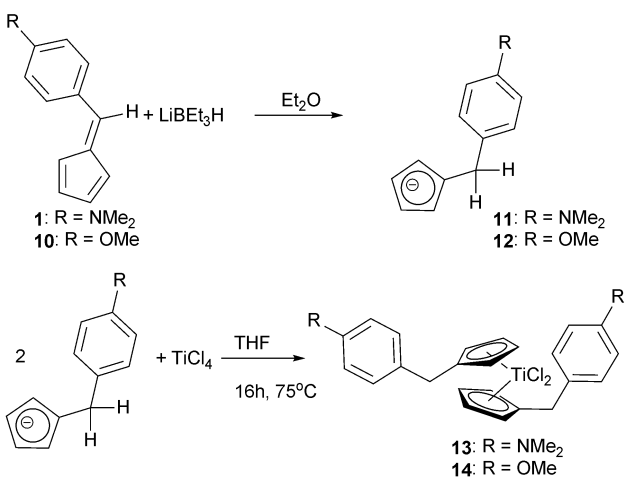
Benzyl-substituted titanocenes do not have stereocentres and therefore stereoisomers do not exist, unlike their *ansa* analogues. In terms of *in vivo* and *in vitro* cell testing this is advantageous. Previously, the presence of unseparated stereoisomers means that the issue of whether the compounds' cytotoxicities are related to specific isomers was not addressed. This is not of concern in the achiral benzyl-substituted titanocenes presented here.

The novel reaction of Super Hydride ($LiBEt_3H$) and phenyl-substituted fulvenes, so-called hydridolithiation, results in the formation of lithium cyclopentadienide intermediates. This is in general an interesting and applicable method towards the synthesis of a wide range of new benzyl-substituted metallocenes. By employing titanium tetrachloride a wide variety of benzyl-substituted titanocene dichlorides have been synthesised,[16,17,20,21] starting with the *p*-methoxy and the *p*-dimethylamino benzyl-substituted titanocene dichloride (Scheme 6).[20]

The nucleophilic addition of a hydride to the exocyclic double bond of fulvenes **1** or **10**, using $LiBEt_3H$ as the hydride transfer reagent, resulted in the formation of the appropriate substituted lithium cyclopentadienide intermediates **11** or **12**. Two equivalents of either **11** or **12** underwent a transmetallation reaction when reacted with one mol equivalent of titanium tetrachloride in THF under reflux, to give the appropriate non-bridged substituted titanocene dichloride **13** or **14** in yields of 50 and 54%.

Super Hydride is one of the most powerful nucleophilic reducing agents available, capable of reducing many functional groups. It is also highly selective: the exocyclic double bond in aryl-substituted fulvenes has an increased polarity, due to the inductive effects of their respective aryl groups. This increased polarity allows for selective nucleophilic attack at this double bond and not at the diene component of the fulvenes. Other examples of the nucleophilic addition of hydrides to substituted fulvenes (albeit with alkyl or unsubstituted phenyl group functionality) include the use of lithium aluminium hydride and the use of alkyl lithium species as highly reactive-hydride transfer reagents.[22]

The *in vitro* cytotoxicity of the benzyl-substituted titanocenes was determined using the above-mentioned standard assay. These activity studies were undertaken to the same protocol used for the studies with *ansa*-titanocene dichlorides, and therefore, the results are directly comparable. Titanocenes



Scheme 6 Synthesis of ''unbridged'' benzyl-substituted titanocene dichlorides.

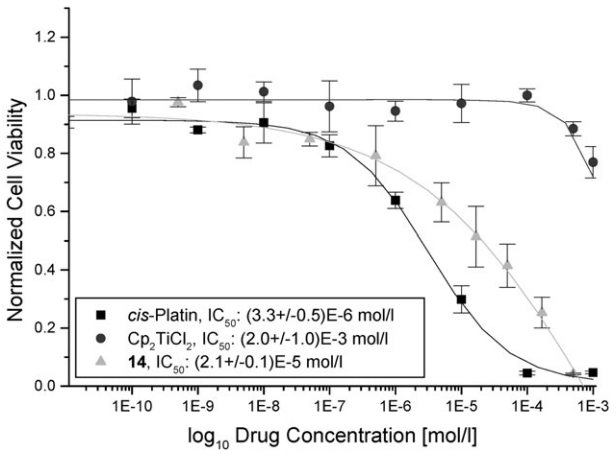
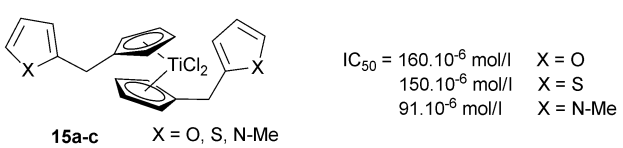


Fig. 8 Cytotoxicity curves from typical MTT assays showing the effect of *cis*-platinum, Cp_2TiCl_2, and benzyl-substituted titanocene **14** on the viability of LLC-PK cells. (After Fig. 6 from ref. 20 with permission of Elsevier. © 2005 Elsevier.)

13 and **14** showed an impressive cytotoxic activity with IC_{50} values of 120×10^{-6} mol l^{-1} for titanocene **13** and 21×10^{-6} mol l^{-1} for titanocene **14**.[20] The IC_{50} value of the *p*-methoxy benzyl substituted titanocene dichloride (**14**) represents a 10-fold increase in cytotoxicity compared to the *ansa*-analogue **5a** and an impressive 100-fold improvement compared to the unsubstituted Cp_2TiCl_2 (Fig. 8).

In analogy to the *ansa*-titanocenes, a series of unbridged heteroaryl-substituted titanocene dichlorides was synthesized.[21] Therefore, the furyl, thienyl and *N*-methyl pyrrolyl substituted fulvenes were reacted with $LiBEt_3H$ and the resulting lithium intermediates were successfully transmetallated with titanium tetrachloride. The resulting heteroaryl-substituted titanocenes dichlorides were obtained in yields of 24–63%. When tested for cytotoxicity on LLC-PK the IC_{50} values were in the lower 10^{-4} and upper 10^{-5} mol l^{-1} range. This represents an increase in cytotoxicity when compared to the unsubstituted Cp_2TiCl_2 and a significant increase when compared to the analogous *ansa*-bridged compounds.[15] In comparison to their *ansa*-analogues they show a similar series of increasing cytotoxicity, depending on the heteroaryl substituent. This would indicate that *N*-methyl pyrrole substitution increases cytotoxicity more than thienyl or furyl substitution on the cyclopentadienyl rings, the latter two being very similar for both the *ansa* and unbridged analogues (Fig. 9).

In order to improve the bioavailability of the benzyl-substituted titanocenes, different substitution patterns known for their biological activity, such as the benzo[1,3]dioxole,[16] the glycol methyl ether and the morpholino group,[17] were introduced into the molecules. The unbridged benzo[1,3]dioxole-substituted titanocene dichloride (**16**) was obtained in a yield of 59% as an orange solid.[16] When tested on the LLC-PK cell line an IC_{50} value of 280×10^{-6} mol l^{-1} was determined. Surprisingly, this represents even a slight decrease in cytotoxicity compared to the *ansa*-analogue compound (**16**, $IC_{50} = 160 \times 10^{-6}$ mol l^{-1}). In general, the benzyl-substituted titanocenes show a higher cytotoxicity than the analogous *ansa*-compound.



15a-c

Fig. 9 Structures and IC_{50} values of the heteroaryl-functionalised titanocenes **15a–c**.

Within this series it was very interesting to study the glycol methyl ether substituent, as previous experiments on *ansa*-titanocenes containing this substituent showed surprisingly a proliferate effect.[17a] Titanocene **17** was obtained as a brown solid in a yield of 67% after the reaction of 6-[4-(2-methoxyethoxy)phenyl]fulvene and $LiBEt_3H$, followed by transmetallation with titanium tetrachloride. Cytotoxicity tests revealed an IC_{50} value of 43×10^{-6} mol l^{-1}, which is in the lower micromolar range, and shows a clear anti-proliferative effect.

The unbridged morpholino-substituted titanocene **18** has an IC_{50} value of 25×10^{-6} mol l^{-1} and represents therefore, together with the *p*-methoxyphenyl substituted titanocene **14**, one of the most cytotoxic unbridged titanocene dichlorides within this series.[17b] Titanocene **18** was synthesised starting with the conversion of 4-(4-bromo-phenyl)morpholine into the corresponding benzaldehyde and thereafter into the corresponding fulvene. Following the above described method, titanocene **18** was obtained in 61% yield as a red solid (Fig. 10).

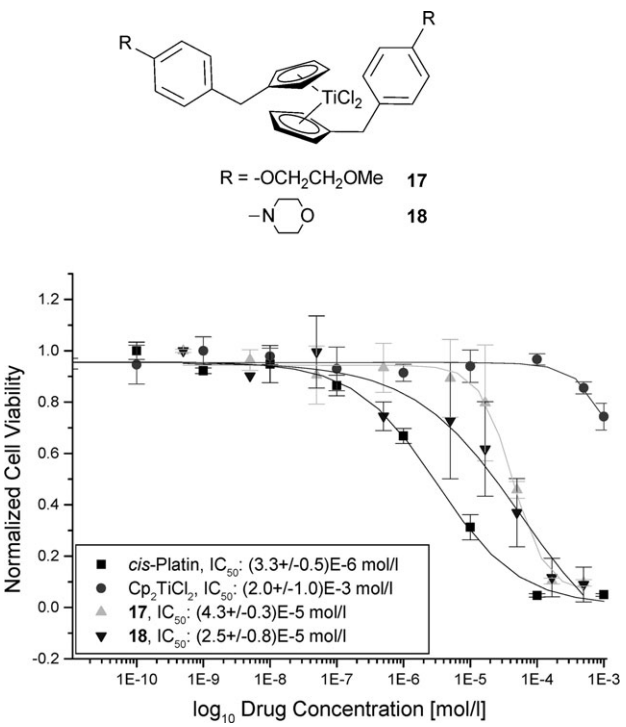


Fig. 10 Cytotoxicity curves from typical MTT assays showing the effect of *cis*-platinum, Cp_2TiCl_2, and titanocenes **17**, **18** on the viability of LLC-PK cells. (Adapted from Fig. 4 from ref. 17*a* and Fig. 2 from ref. 17*b* Wiley-VCH Verlag GmbH & Co and Springer. © 2006 Wiley-VCH Verlag GmbH & Co; © 2007 Springer.)

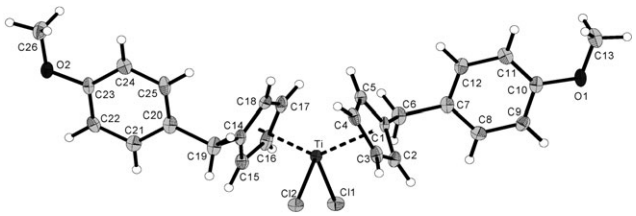


Fig. 11 Molecular structure of **14** in the crystal; thermal ellipsoids are drawn on the 50% probability level. (After Fig. 3 from ref. 20 with permission of Elsevier. © 2005 Elsevier.)

Due to the lack of stereoisomers, benzyl-substituted titanocenes are a promising class in terms of a medical application and of crystallisation experiments. Therefore, a number of solid state structures have been determined up to now[16,20,21] and the crystal structure presented within this review represents no exception. The molecule itself has C_2 symmetry in the solid state. The cyclopentadienyl bond lengths are of the expected values for titanocenes, ranging from 138 pm to 142 pm. The Ti–Cl bond lengths, both of them being in this example between 233 and 242 pm, and the Cl–Ti–Cl angle, being 95.94°, are also typical for titanocenes (Fig. 11).

Hydridolithiation as a new synthetic approach to novel benzyl-substituted titanocene dichlorides allows the synthesis of substituted titanocenes, which are up to 100-fold more cytotoxic than the unsubstituted Cp_2TiCl_2 itself. In general, these novel unbridged titanocene dichlorides were synthesised as solids in good yields of up to 67% and free of any stereocentres. They showed IC_{50} values in the range of 280×10^{-6} to 21×10^{-6} mol l^{-1} with titanocenes **14** and **17** being the most cytotoxic ones. Furthermore, this represents a significant improvement compared to the analogous *ansa* compounds, as seen in Fig. 12.

Carbolithiation leading to chiral mixtures of titanocenes

Carbolithiation proved to be a very effective approach to highly cytotoxic titanocenes. The reaction of aryl or heteroaryl lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation reaction led to dimethylamino-functionalised titanocenes with IC_{50} values of 6.8×10^{-6} mol l^{-1}, 5.5×10^{-6} mol l^{-1} and 5.4×10^{-6} mol l^{-1}. Furthermore, this reaction proved to be tolerant to many substituents and to be a versatile tool for the synthesis of a wide variety of substituted titanocene dichlorides (Scheme 7).

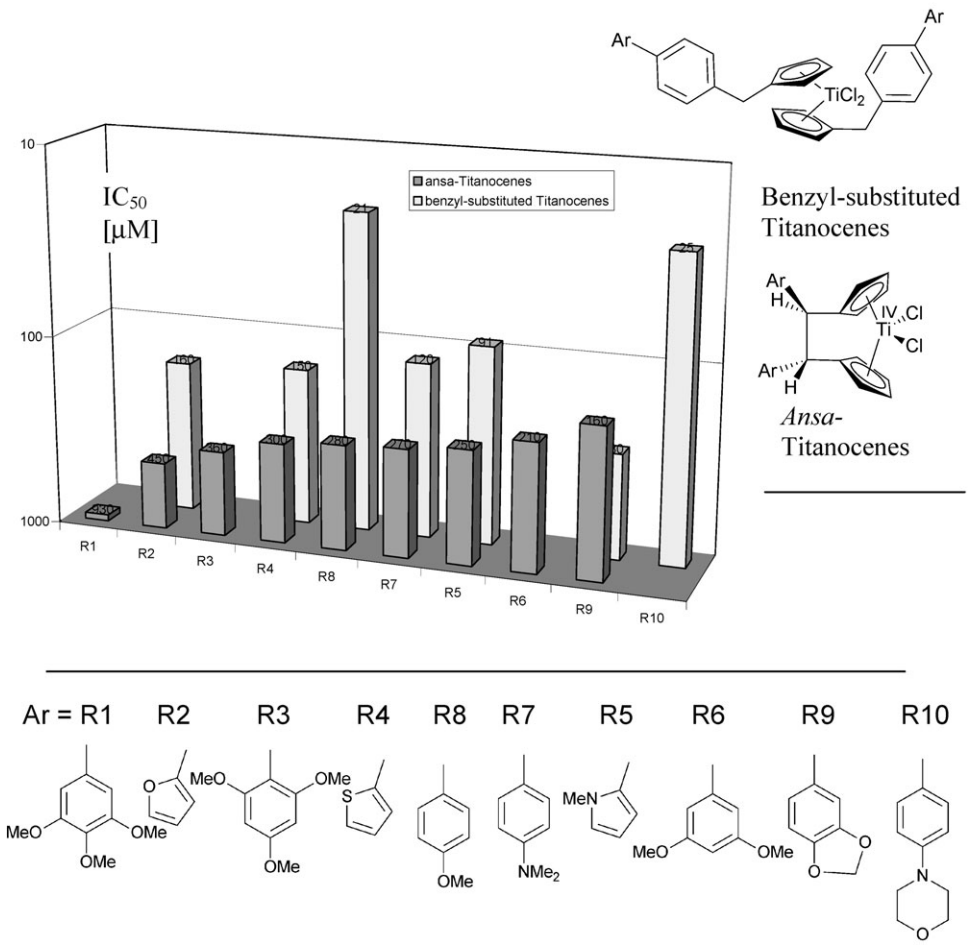
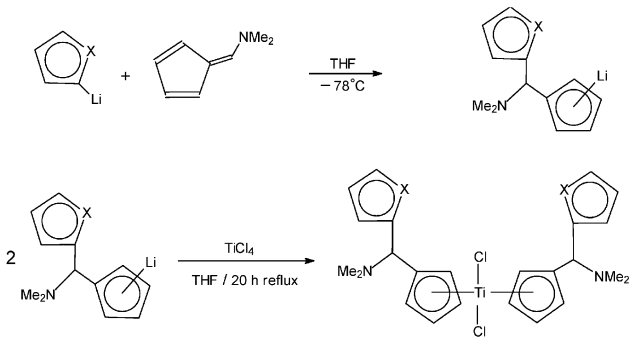


Fig. 12 IC_{50} values (LLC-PK) of titanocene derivatives obtained by reductive dimerisation (*ansa*-titanocenes, in dark grey) or hydridolithiation (benzyl-substituted titanocenes, in light grey) reactions.



Scheme 7 Synthesis of dimethylamino-functionalised titanocene dichloride derivatives.

A first series was reported on the reaction of simple aryl lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation. Thereby, it was possible to synthesize *p*-methoxy phenyl (**19**), *N*,*N*-dimethyl-phenyl (**20**) and benzo[1,3]dioxole phenyl (**21**) substituted dimethylamino functionalised titanocene dichlorides, which show IC_{50} values in the range of 54–26 $\times$ 10^{-6} mol l^{-1}.[23] This represents a significant improvement in cytotoxicity compared to the benzyl-substituted titanocenes by a factor of 3 for titanocene **20** (IC_{50} for the benzyl-substituted analogue: 120 $\times$ 10^{-6} mol l^{-1}) and even 10 for titanocene **21** (IC_{50} for the benzyl-substituted analogue: 280 $\times$ 10^{-6} mol l^{-1}). Solely a decrease in cytotoxic activity was observed for the *p*-methoxy phenyl substituted dimethylamino functionalised titanocene **19**, as the corresponding benzyl-substituted titanocene **14** has an IC_{50} of 21 $\times$ 10^{-6} mol l^{-1}.

In correspondence to previous experiments a series of heteroaryl substituted dimethylamino functionalised titanocene dichlorides have been synthesised and evaluated, as shown in Fig. 13.[24] Unfortunately, no uniform pattern compared to benzyl-substituted titanocenes was detected. A decrease in cytotoxicity was observed for the thienyl-substituted titanocene (**22b**) from 150 $\times$ 10^{-6} mol l^{-1} (for the unbridged analogous compound **15b**) to 240 $\times$ 10^{-6} mol l^{-1}. On the other hand a significant increase in cytotoxicity was observed for the furyl-substituted titanocene (**22a**) (160 $\times$ 10^{-6} mol l^{-1} to 28 $\times$ 10^{-6} mol l^{-1}) and especially for the *N*-methyl pyrrole derivative (**22c**), which exhibits an IC_{50} value of 5.5 $\times$ 10^{-6} mol l^{-1}. Therefore, titanocene **22c** is one of the two most cytotoxic substituted titanocene dichlorides reported up to now. This represents an impressive 400-fold increase in cytotoxic activity compared to the unsubstituted Cp_2TiCl_2, which reached Phase II clinical trials.

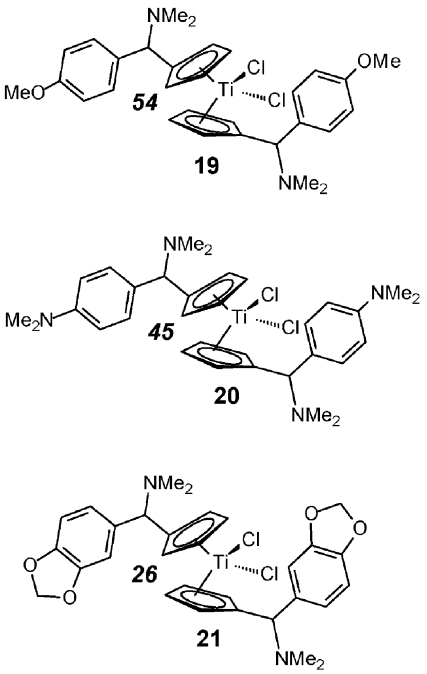


Fig. 13 Structures and IC_{50} values (italics; in 10^{-6} mol l^{-1}) of the chiral dimethylamino-functionalised titanocenes **19–21**.

In a second series the same heteroaryl lithium species were reacted with 6-morpholino substituted fulvene.[25] This led to three new titanocene dichlorides **23a–c** with again very mixed results in terms of the cytotoxic activity compared to the corresponding dimethylamino-functionalised titanocene. The thienyl-substituted titanocene **23b** showed an increase in cytotoxic activity, whereas titanocenes **23a** and **23c** showed a decrease (Fig. 14).

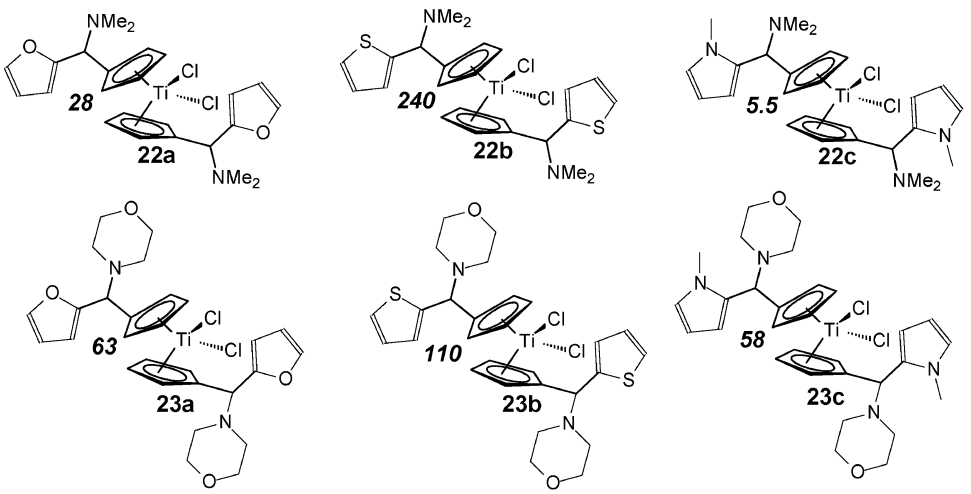


Fig. 14 Structures and IC_{50} values (italics; in 10^{-6} mol l^{-1}) of the chiral dimethylamino-functionalised titanocenes **22a–c** and **23a–c**.

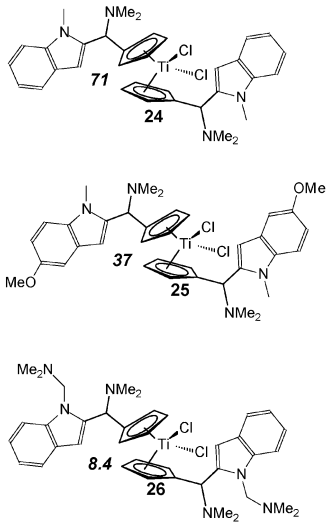


Fig. 15 Structures and IC_{50} values (italics; in 10^{-6} mol l^{-1}) of the chiral dimethylamino-functionalised titanocenes **24–26**.

The use of different *ortho*-lithiated indole derivatives (*N*-methylindole, 5-methoxy-*N*-methylindole and *N*,*N*-dimethylaminomethylindole) led to novel titanocene dichlorides **24–26** with IC_{50} values of 71×10^{-6} mol l^{-1}, 37×10^{-6} mol l^{-1} and the impressive value of 8.4×10^{-6} mol l^{-1} for **26** (see Fig. 15).[26] Again, this represents a nearly 250-fold increase in cytotoxic activity compared to Cp_2TiCl_2.

> Further lithiated small heterocyclic groups [2-thiazolyl, 5-*N*-methylpyrazolyl and 2-*N*(*N*,*N*-dimethylamino)methylimidazolyl] were the key reagents for the synthesis of novel *N*,*N*-dimethylamino-functionalised titanocene dichlorides **27–29**, which exhibit IC_{50} values of 61×10^{-6} mol l^{-1}, 53×10^{-6} mol l^{-1} and 5.4×10^{-6} mol l^{-1}, when tested against LLC-PK (Fig. 16).[27] This shows progress in respect to the best indole derivative and titanocene **29** with an IC_{50} value of 5.4×10^{-6} mol l^{-1} is, next to the *N*-methyl pyrrole derivative **22c**, the most cytotoxic titanocene derivative published up to now.

The use of lithiated *N*-heterocyclic compounds, such as 2,4-[bis(*N*′,*N*′-dimethylaminomethyl)]-*N*-methyl pyrrole, 1-methylimidazole and *N*,*N*-dimethylaminomethyl pyrrole, gave access to novel *N*,*N*-dimethyl amino functionalised titanocenes **30–32** with promising IC_{50} values of 63×10^{-6} mol l^{-1} and 13×10^{-6} mol l^{-1}, and the very impressive value of 6.8×10^{-6} mol l^{-1} in **32**, which is illustrated in Fig. 16.[28]

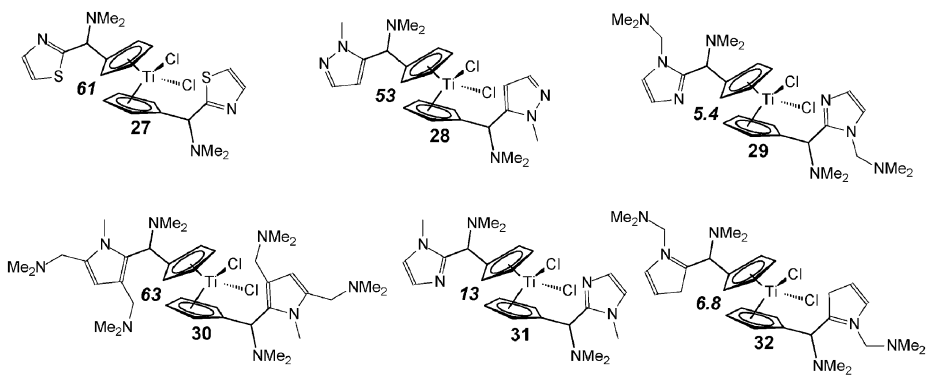


Fig. 16 Structures and IC_{50} values (italics; in 10^{-6} mol l^{-1}) of the chiral dimethylamino-functionalised titanocenes **27–32**.

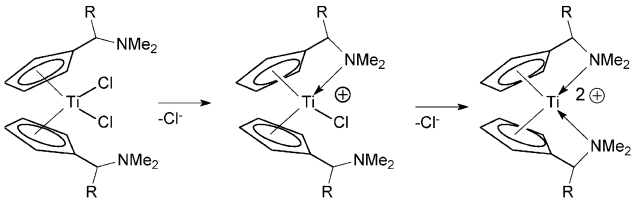


Fig. 17 Stabilisation of the mono- or dications of dimethylamino-functionalised titanocenes. (Reprinted from ref. 24 Fig. 3 with permission of Elsevier. © 2007 Elsevier.)

N,*N*-Dimethylamino-functionalised titanocenes presented within this review show in general an impressive high cytotoxicity in the range from 240 to 5.4×10^{-6} mol l^{-1}, depending on the substitution pattern. Compared to the unsubstituted Cp_2TiCl_2, which reached Phase I and Phase II clinical trials, this represents an up to 400-fold increase in cytotoxic activity and there are several compounds which reach the target area of the lower micromolar range. This increase is believed to be the result of stabilising effects in titanocene mono- or dications through intramolecular coordination from the *N*,*N*-dimethylamino group, which is visualised in Fig. 17.

Unfortunately, the carbolithiation reaction of lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation reaction leads to titanocenes with stereoisomers, as an equimolar mixture of the *R* and *S* forms of the substituted lithium cyclopentadienide is formed. Subsequently, this equimolar mixture of *R* and *S* configured substituted lithium cyclopentadienides is used for the transmetallation reaction and therefore, a mixture of 25% of the *R*,*R*, 25% of the *S*,*S* and 50% of the *R*,*S* forms of the chiral titanocene dichloride is obtained (see Fig. 18).

Unfortunately, the presence of stereoisomers does not allow this class of compounds to proceed directly into clinical trials. There is hope that future syntheses using chiral bases like sparteine may allow enantioselective preparation of certain derivatives not just in the case of ferrocenes.[29] Additionally, further reaction pathways leading to achiral titanocenes have to be explored.

Carbolithiation leading to achiral titanocenes

In order to avoid the presence of stereoisomers a further pathway including a carbolithiation reaction delivering achiral titanocene dichlorides was explored. Different aryl or heteroaryl lithium species were reacted with 6-bis-*N*,*N*-

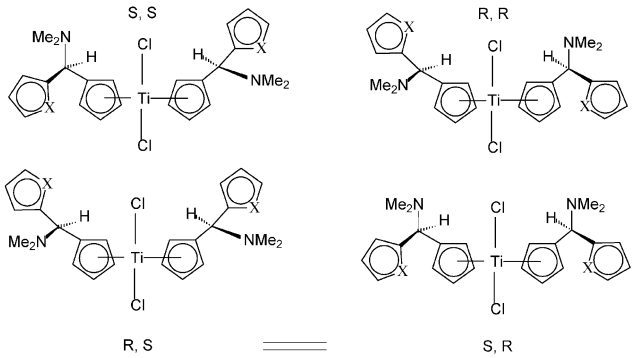


Fig. 18 Possible titanocene isomers originating from the carbolithiation reaction starting with 6-*N,N*-dimethylamino fulvene. (Reprinted from ref. 24 Fig. 1 with permission of Elsevier. © 2007 Elsevier.)

dimethylamino fulvene and therefore, the intermediate lithium cyclopentadienide and the resulting 6-bis-*N,N*-dimethylamino-functionalised titanocene dichlorides become achiral.

Recently, this was experimentally proven with *ortho*-lithiated furan, thiophene and *N*-methylpyrrole leading to the corresponding titanocenes with IC_{50} values of 270 $\times$ 10^{-6} mol l^{-1}, 240 $\times$ 10^{-6} mol l^{-1} and 36 $\times$ 10^{-6} mol l^{-1}.[30] Compared to the analogous 6-*N,N*-dimethylamino-functionalised titanocene dichlorides the new thienyl-substituted titanocene **33b** shows similar activity, but the exceptionally high activity of the furyl- and *N*-methylpyrrole-substituted 6-*N,N*-dimethylamino-functionalised titanocenes **22a** and **22c** could not be repeated (Scheme 8).

Within this review we present a second reaction pathway, which includes a carbolithiation reaction and leads to achiral substituted titanocene dichlorides also. Therefore, aryl lithium species are added to the identical substituted 6-aryl fulvenes. This leads to the formation of highly substituted, but achiral diarylmethyl-functionalised lithium cyclopentadienides, which can still be used in the transmetallation reaction with titanium tetrachloride (Scheme 9).

This method was explored for the synthesis of diheteroaryl-substituted titanocenes (**34a–c**). Cytotoxicity studies reveal IC_{50} values of 140 $\times$ 10^{-6} mol l^{-1}, 240 $\times$ 10^{-6} mol l^{-1} and 32 $\times$ 10^{-6} mol l^{-1}, respectively (Fig. 19).[31]

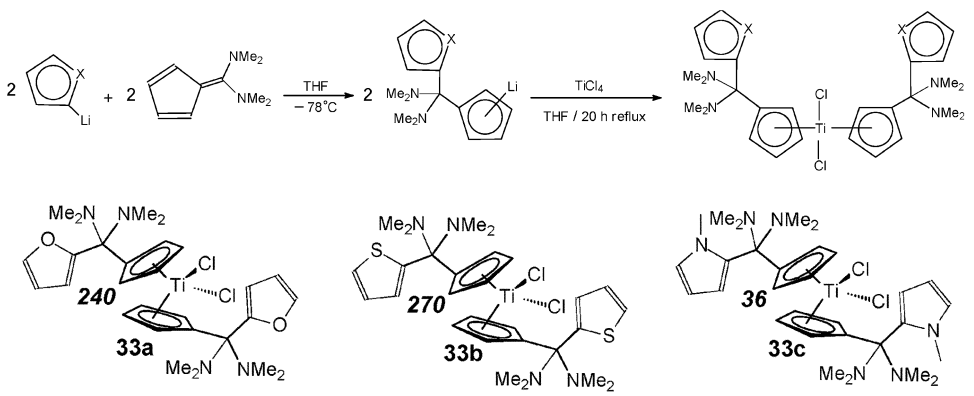
Within this review article we presented a wide variety of heteroaryl-substituted titanocenes dichlorides, with Fig. 20 summarizing the main IC_{50} values. As the graphic shows there is no main trend visible. On average, thienyl-substituted titanocenes have lower IC_{50} values. Furthermore, the two most cytotoxic titanocenes with this comparison are *N,N*-dimethylamino-functionalised titanocenes.

The corresponding diaryl-substituted titanocenes carrying 3,5-dimethoxyphenyl, *p*-*N,N*-dimethylanilyl or *p*-anisyl substituents reach IC_{50} values of 74 $\times$ 10^{-6} mol l^{-1}, 40 $\times$ 10^{-6} mol l^{-1} and 38 $\times$ 10^{-6} mol l^{-1}, when tested against LLC-PK.[32] This series of diarylmethyl-functionalised titanocene dichlorides was again chosen in analogy with previously synthesised substituted titanocene dichlorides, such as benzyl- and 6-*N*, *N*-dimethylamino-functionalised titanocenes (Fig. 21).

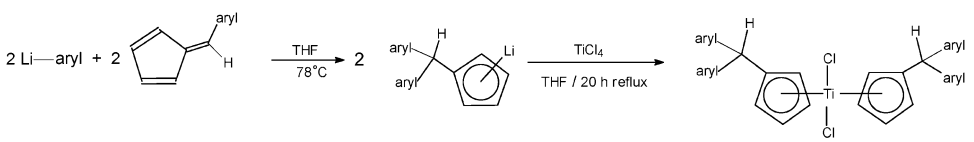
In summary this novel approach to substituted titanocene dichlorides delivered compounds with a cytotoxic activity in the range of 270 to 32 $\times$ 10^{-6} mol l^{-1}, which represents an up to 60-fold increase in cytotoxicity compared to the unsubstituted Cp_2TiCl_2. The major advantage of diarylmethyl-functionalised titanocenes is the absence of stereocentres and, with further improvements, there are potential candidates for preclinical studies with potential for clinical trials.

Biological activity of substituted titanocene dichlorides

The cytotoxicity of substituted titanocene dichlorides is highly depending on the substitution pattern and the structural elements. The most cytotoxic substituted titanocene dichlorides with IC_{50} values of 5.5 and 5.4 $\times$ 10^{-6} mol l^{-1} were synthesised using the carbolithiation method (Fig. 22).



Scheme 8 Synthesis, structures and IC_{50} values (italics; in 10^{-6} mol l^{-1}) of 6-bis-*N,N*-dimethylamino-functionalised titanocene dichlorides **33a–c**.



Scheme 9 Synthesis of diarylmethyl–functionalised titanocene dichlorides.

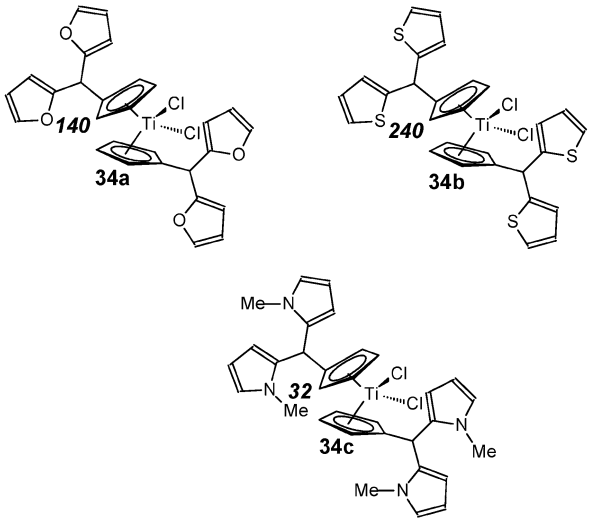


Fig. 19 IC_{50} values (italics; in 10^{-6} mol l^{-1}) showing the effect of titanocenes **34a–c** on the viability of LLC-PK cells.

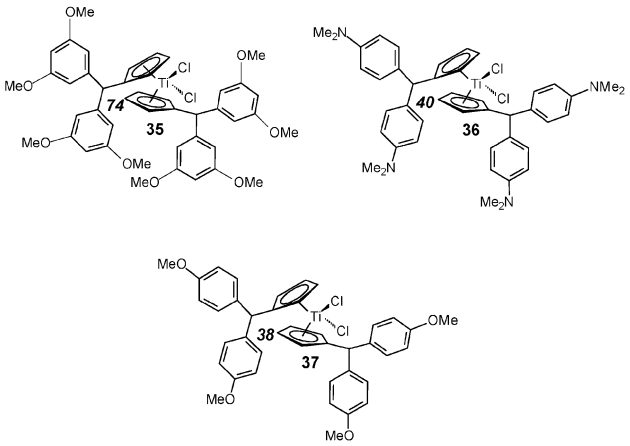


Fig. 21 IC_{50} values (italics; in 10^{-6} mol l^{-1}) showing the effect of titanocenes **35–37** on the viability of LLC-PK cells.

The *ansa*-titanocene **3** and the benzyl-substituted titanocene **14** were tested on the growth of a wide variety of tumor cells *in vitro* on a panel 36 human tumor cell lines containing 14 different tumor types investigated in a cellular proliferation assay.[33] Titanocene **14** showed a significantly higher cytotoxic activity than the *ansa*-compound and reached, on average over the whole cell panel, the activity of *cis*-platinum within a factor of 4. Nevertheless, there were three main targets for titanocene **14** identified, which are pleura mesothelioma, uterine and renal cell cancer. Here, titanocene **14** showed a significantly higher cytotoxicity than *cis*-platinum. Furthermore, within the group of ovarian, pancreas, prostate and breast cancer single cancer cell lines were identified which show comparable sensitivity for titanocene **14** and *cis*-platinum.

A series of biomedical studies including *ex vivo* experiments and a mouse model have been undertaken using the *ansa*-titanocene **3**.[34] The *ex vivo* anti-tumor effect of *ansa*-titanocene **3** was studied against a total of eight freshly explanted tumors, using an *in vitro* soft agar cloning system. A concentration-dependent anti-tumor activity was observed for all samples, except melanoma. The highest activity was observed for renal cell carcinomas and remarkable cytotoxicity was noted for breast cancer (MCF-7) (Fig. 23).

Furthermore, *ansa*-titanocene **3** was tested in a mouse model against Ehrlich's ascites tumor (EAT).[35] This study showed that the treatment with *ansa*-titanocene **3** increases the

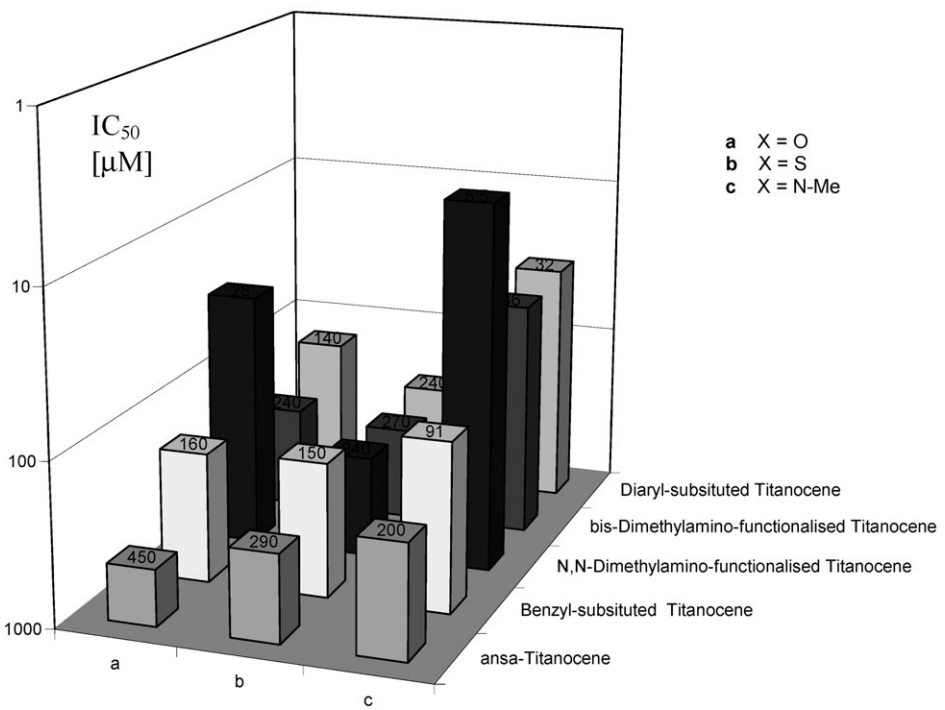


Fig. 20 Overview of IC_{50} values obtained with different classes of heteroaryl-substituted titanocene dichlorides.

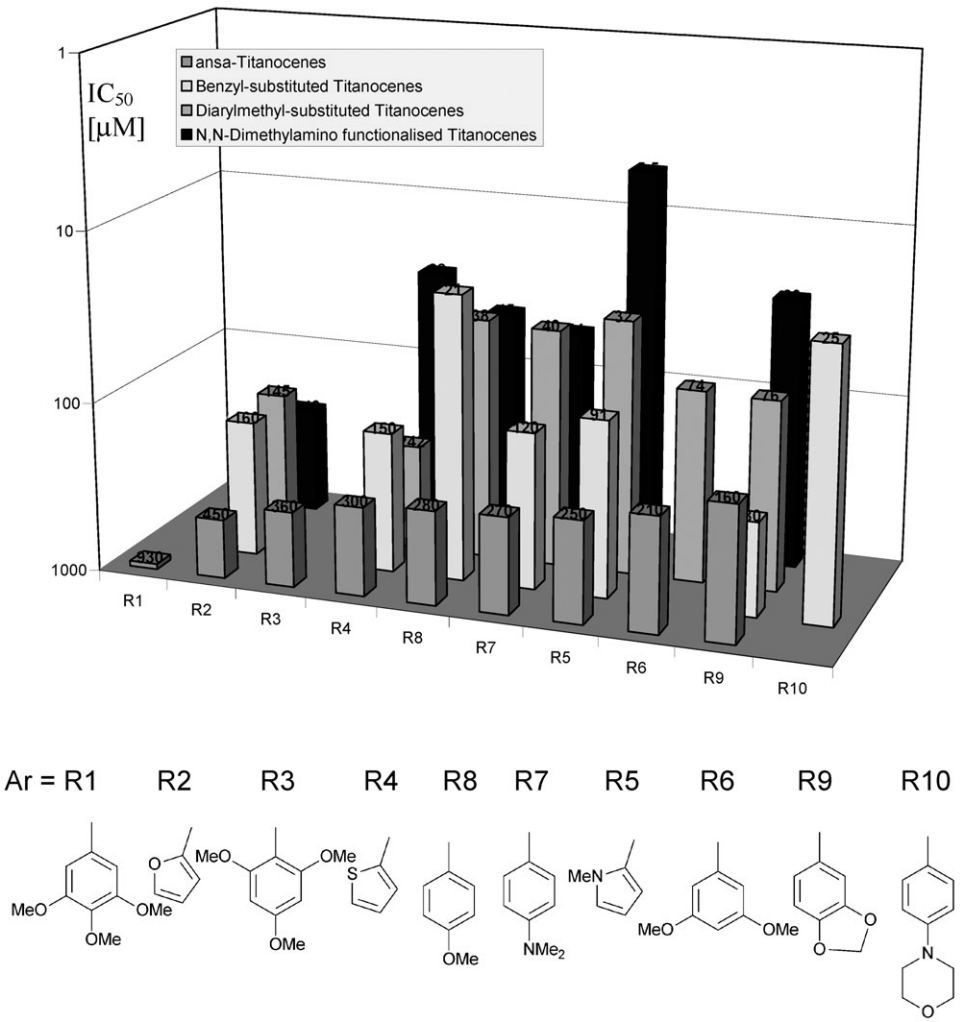


Fig. 22 IC_{50} values (LLC-PK) of titanocene derivatives obtained by reductive dimerisation, hydridolithiation or carbolithiation reactions.

lifespan of EAT-bearing mice by 25 and 50% in a dose dependent manner, and myelopoiesis (the formation of bone marrow or of blood cells derived from bone marrow) was not suppressed. Additionally, these experiments showed that *ansa*-titanocene **3** restores the natural killer cell function, which is reduced due to a dysfunction in EAT, and stimulates the natural killer cell-mediated cytotoxicity (Fig. 24).

The benzyl-substituted titanocene **14**, which showed a significant higher cytotoxicity when tested *in vitro* against LLC-

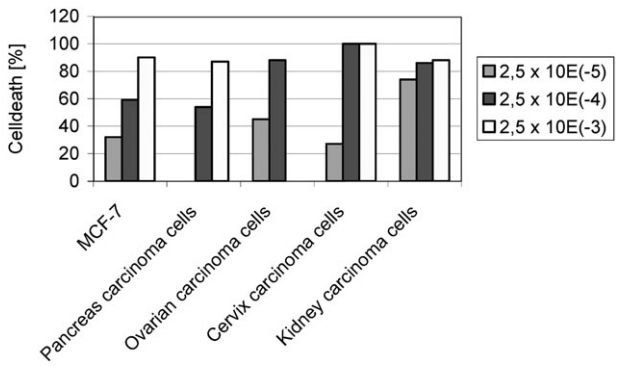


Fig. 23 *Ex vivo* studies of titanocene **3** against several freshly explanted human tumors. (After Fig. 2 from ref. 34 with permission of Lippincott Williams & Wilkins. © 2005 Lippincott Williams & Wilkins.)

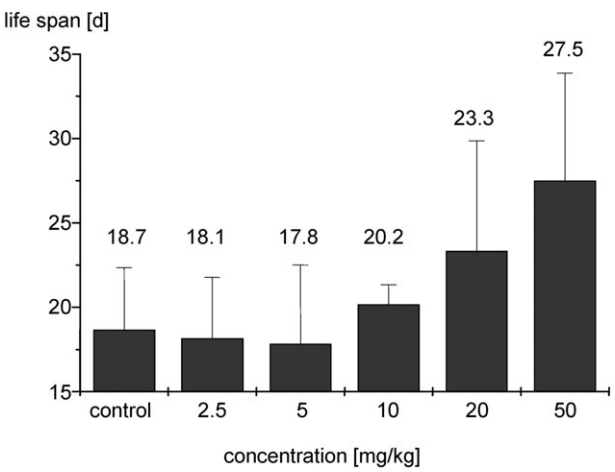


Fig. 24 Effects of different doses of titanocene **3** on the survival of Ehrlich's ascites tumor-bearing mice.

PK, was evaluated in a series of biomedical studies as well.

Watson *et al*. tested the ''open'' titanocene **14** in comparison to the heteroaryl substituted *ansa*-titanocene **6b** and the 3,5-dimethoxyphenyl *ansa*-titanocene **5c** on prostate cancer cells, as advanced prostate cancer is not curable up to now.[36] As a result, it was shown that all three titanocenes induced more apoptosis (programmed cell death, in contrast to necrosis) compared to *cis*-platinum in a dose dependent manner. Titanocene **14** had the most significant effect on the cell cycle and apoptosis.

Furthermore, the benzyl-substituted titanocene **14** was tested *ex vivo* against a range of freshly explanted human tumors, using an *in vitro* soft agar cloning system.[37] Titanocene **14** showed a remarkable high sensitivity against renal cell cancer, ovarian, non-small lung and colon cancer. The good response with non-small lung and colon cancer was especially surprising. In contrast ovarian and renal cell cancer were already predicted as good targets in the *in vitro* panel experiment performed by Kelter *et al*.[33]

Encouraged by these results the unbridged benzyl-substituted titanocene **14** was tested in two *in vivo* and additional *ex vivo* and *in vitro* experiments.

The first mouse model used, was xenografted Caki-1 tumors in mice.[38] The Caki-1 mouse model represents human renal cell cancer, one of the prime targets identified for titanocene **14**. Titanocene **14** was given *in vivo* in doses of 10, 20, 30, 40, 50 mg kg^{-1} on 5 consecutive days to Caki-1-bearing mice. The results showed a significant dose-dependent tumor growth reduction. The maximum tolerable dose (MTD) was determined to be 40 mg kg^{-1} per day and at this concentration it showed a superior activity compared to *cis*-platinum, given at 2 mg kg^{-1} per day. Additionally, the *in vitro* test showed an IC_{50} of 36 μM for titanocene **14**, when tested against Caki-1 (Fig. 25).[38]

Very recently, the caspase-dependent apoptosis triggered by titanocene **14** in epidermoid carcinoma cells A431 were shown *in vitro*. An *in vivo* study using the same cell line xenografted to mice revealed a tumor volume reduction by titanocene **14** identical to *cis*-platinum.[39]

Furthermore, titanocene **14** has been studied in a very promising mouse model, using xenografted MCF-7 tumors

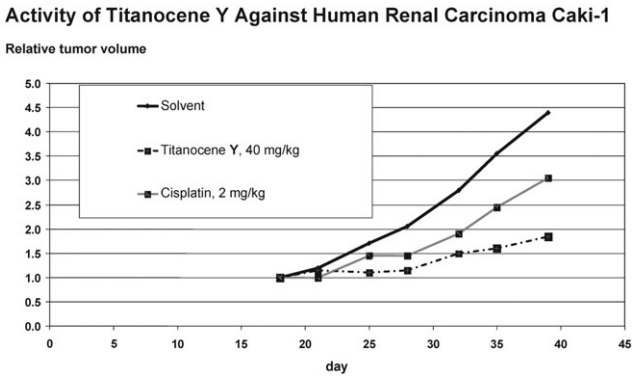


Fig. 25 Tumour growth curves comparing Titanocene **14** and *cis*-platinum in Caki-1 xenografts in nude mice. (After Fig. 3b from ref. 38 with permission of Lippincott Williams & Wilkins. © 2006 Lippincott Williams & Wilkins.)

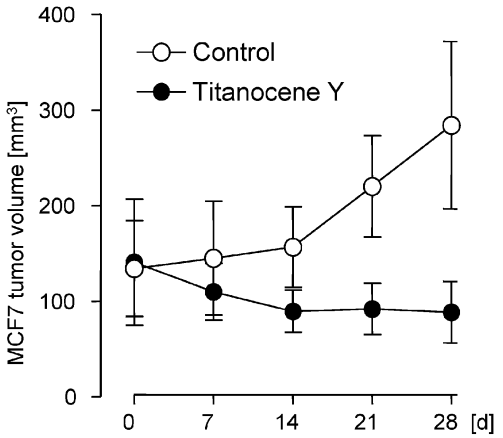


Fig. 26 Tumor growth curves of MCF-7 xenografts in nude mice comparing a titanocene **14** treated cohort against a control cohort. (After Fig. 3 from ref. 40 with permission of Lippincott Williams & Wilkins. © 2007 Lippincott Williams & Wilkins.)

in mice. In prior experiments titanocene **14** had been successfully tested *ex vivo* against freshly explanted human breast tumor cells.[40] The sensitivity of these breast tumor cells against titanocene **14** was highly remarkable over the full concentration range. Titanocene **14** showed cell death induction at the lowest concentration (2.1×10^{-6} mol l^{-1}), well comparable to *cis*-platinum. Therefore, for this *in vivo* experiment the human breast cancer MCF-7 cell line was xenografted into non-obese diabetic and severe combined immune-deficient (NOD/SCID) mice. Titanocene **14** was given for 21 days at 30 mg kg^{-1} d^{-1}, which represents 75% of the MTD and therefore for a longer period, which resulted in the reduction of the tumor volume to around 1/3 and not only the reduction of the tumor growth. This was the first time that using a substituted titanocene dichloride a shrinking of a tumor was observed (Fig. 26).

Currently, further mouse model studies using titanocene **14** and even more cytotoxic compounds are under way.

Conclusions and outlook

Reductive dimerisation of aryl fulvenes with titanium dichloride offers a general approach for the synthesis of *ansa*-titanocene dichlorides, which unfortunately show just a limited cytotoxicity. Carbolithiation of aryl or dimethylamino fulvenes followed by transmetallation delivered the most cytotoxic titanocenes up to now by reaching IC_{50} values of around 5×10^{-6} mol l^{-1}. Hydridolithiation of aryl fulvenes followed by transmetallation delivered titanocenes with IC_{50} values as low 21×10^{-6} mol l^{-1}, but they do not contain stereocentres and therefore appear to be the better candidates for further evaluation. As expected, the water solubility and cytotoxicity of titanocenes are highly dependent on the substitution pattern introduced by the fulvene. The mechanism of DNA coordination seems different with respect to *cis*-platinum and encourages treatment of *cis*-platinum resistant cancer types. No myelosuppression, the activation of the immune system and anti-angiogenesis are strongly encouraging factors for the further development of fulvene-derived titanocenes. The

substituted titanocenes show medium-high to high cytotoxicities in cell tests and are very promising in *ex vivo* renal-cell and cervical cancer experiments. *In vivo* data from EAT-bearing mice look promising and there are convincing results from A431, CAKI-1 and MCF-7 xenografts. Experiments in the near future will take the most promising candidates from the carbolithiation and hydridolithiation groups and derivatise them through anion-exchange in order to optimise their biological potential. It is believed that the chloride derivatives hydrolyse too quickly and are therefore not optimal candidates for more advanced biological studies. Then this new class of titanocenes is close to clinical studies aiming for advanced and metastatic renal-cell cancer.

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