# Enhanced Antitumour Activity of Cyclopendadienyl-substituted Metallocene Dihalides in Human Breast and Colon Cancer Cells

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**Abstract.** *Metallocene dihalides, which are cyclopentadienyl complexes with the general formula $R_2MX_2$ (where R=$\eta^5$-$C_5H_5$, $\eta^5$-$CH_3C_5H_4$, $\eta^5$-$SiMe_3C_5H_4$ etc.; M=Ti, Zr, Hf, V or Nb; and X=halogen), are highly effective agents against Ehrlich ascites tumour cells and lymphocytic leukaemia. The aim of this study was to evaluate the antitumor activity of the various metallocene dihalides and particularly their effects on cell proliferation of human breast and colon cancer cells. The growth inhibition of the antitumour metallocenes ($\eta^5$-$C_5H_5)_2TiCl_2$ and ($\eta^5$-$C_5H_5)_2VCl_2$ and four ring-substituted derivatives in HT-29 (colon cancer) and MCF-7 (breast cancer) cell lines is reported. The results showed that ring-substitution of metallocenes gave similar or even better activity in cell proliferation reduction, in both cell lines, especially in HT-29 and suggested that ring-substitution may enhance the inhibitory activity of the metallocene compound family.*

Metal complexes constitute a growing field of drug design and have been considered as promising antitumour agents in recent decades. The interest in the role of metal complexes in cancer therapy was triggered by the discovery of the potent anticancer drug cisplatin and to date, cisplatin and its analogue carboplatin are currently widely used in the treatment of cancer. Since the discovery of cisplatin, metal complexes derived from a range of metals have been screened either *in vitro* or *in vivo* but only a few of them other than the platinum-based drugs have entered clinical trials along with the organometallic complex titanocene dichloride (1, 2).

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*Key Words:* Metallocene dihalides, titanocene dichloride, breast cancer, colon cancer.

The antitumour impact of an extensive range of metallocene dihalides and diacido complexes $Cp_2MX_2$ (Cp=$\eta^5$-$C_5H_5$; M=Ti, V, Nb, Mo, Re; X=halide or diacido ligand) have been investigated against a range of tumour models in mice and several xenografted human tumours transplanted into athymic mice (3). Among the metallocenes reported, titanocene dichloride has been the focus of most studies, being the only metallocene dihalide to have entered clinical trials (1, 2, 4).

In contrast to the well-characterised platinum-based antitumour drugs, the active species of metallocene dihalides responsible for antitumour activity *in vivo* has not been identified and the mechanism is poorly understood. Until now, the most complete picture of the properties and mode of action has been for titanocene dichloride, summarised in review (5).

The antitumour activity of metallocene dihalides is directly correlated to the structural properties of the molecules, which contain three elements that may be varied: the transition metal ion, the halide ligand and the cyclopentadienyl ligands. A comparison of the relative antitumour activity of the various central metal ions is currently restricted to fluid Ehrlich ascites tumours (EAT) and independent studies have shown that the results are clearly specific for different tumour models. Full screening of the neutral metallocenes $Cp_2MCl_2$ (M=Nb, Mo, Ta, W, Zr, Hf) against different tumour types under identical conditions has not been carried out and there are currently only limited studies of the ionic analogues, where M=Mo(VI), Nb(V), Re(V), against colon and breast carcinomas.

Variation of the halide ligand, or acido group in the case of titanocene dichloride, has been extensively studied and does not appear to significantly affect the antitumour properties (3, 4); provided the Ti-X bond is labile in aqueous solution, the halide- or acido-group can be modified without significant perturbation of the antitumour properties. Only limited studies have been carried out on the influence of substitution of the Cp ligands, with all to date restricted to titanocene derivatives. It has been reported that titanocene dichloride analogues with alkyl substituted Cp rings, bridged Cp rings or only one Cp ring showed significantly reduced antitumour activity compared to the parent compound (6).

The methyl groups introduce both potential steric and electronic effects on the Cp rings and the strength of the Cp-Ti bond. Steric effects, however, do not appear to be a significant contributor to the reduced antitumour activity, as substitution of the Cp rings with bulky phenyl groups gave significant antitumour properties compared to the methyl substituted derivative (3). It should be noted, however, that the introduction of alkyl and/or aryl groups reduces aqueous solubility. Thus reduced activity may be related to solubility and transport processes.

In the past, metallocenes have been considered a general class of potential drugs with similar activity and mode of action, after their extended study against fluid EAT (6). However, independent studies have shown that comparison of the cytotoxicity data between antitumour metallocenes is not straight forward. Not surprisingly, the results are clearly specific for different tumour models, such as the distinct pattern of activity of vanadocene dichloride against numerous lung and breast carcinomas compared to titanocene dichloride (3).

Although the extended research of hydrolysis and solubility and the investigation of the role of the X-ligand has resulted in an enormous amount of precious information for further research, no suggestion of the mechanism of action has been reported. In addition, limited studies have been carried out on substitution of the Cp ligands and they have been restricted to titanocene derivatives. In the past few years, interest in metallocene dihalides has increased and many studies have investigated and suggested various mechanisms of action. The current research studies are now aimed at Cp substitution and investigation of the antitumour properties.

In this study, the antitumour activity of various metallocene compounds was investigated and the effect of their Cp-substitution on the cell proliferation of breast and colon cancer cells was compared to the well-studied titanocene dichloride and the metallocene with the most profound effect on cell proliferation, *in vitro*, vanadocene dichloride.

## Materials and Methods

*Chemicals*. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), foetal bovine serum (FBS), L-glutamine, sodium pyruvate, glucose, a cocktail of antimicrobial agents (penicillin, streptomycin, gentamycin, amphoterycin B) and HEPES were all obtained from Biochrom KG (Berlin, Germany). Glucose was obtained from Sigma Chemicals (Steinhelm, Germany). All other chemicals used were of the best available grade.

*Synthesis and structure of organometallic compounds*. The following metallocene dichlorides of the general formula $\mathbf{R_2MCl_2}$ were prepared and purified according to literature methods (see also Figures 1 and 2):

**1:** **R**=$\eta^5$-$C_5H_5$ and **M**=Ti (7), titanocene dichloride
**2:** **R**=$\eta^5$-$C_5H_5$ and **M**=V (7, 8), vanadocene dichloride
**3:** **R**=$\eta^5$-$CH_3C_5H_4$ and **M**=Ti (9)

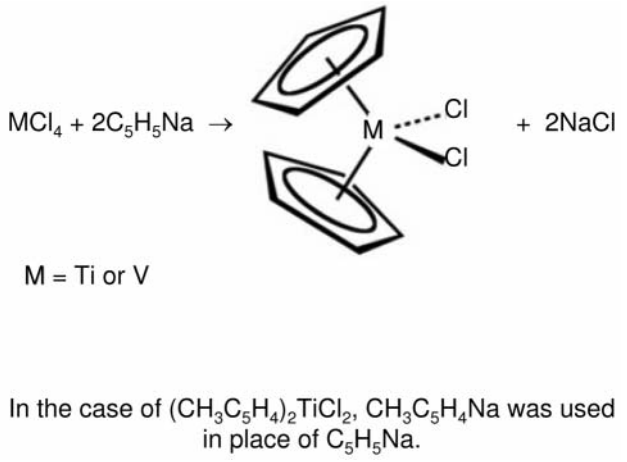


Figure 1. *General scheme for the preparation of complexes* $(C_5H_5)_2MCl_2$.

**4:** **R**=$\eta^5$-$SiMe_3C_5H_4$ and **M**=Ti (10, 11)
**5:** **R**=$\eta^5$-$tBuC_5H_4$ and **M**=Ti and (7)
**6:** **R**=$\eta^5$-$tBuC_5H_4$ and $\eta^5$-$C_5H_5$, and **M**=Ti (12)

All the compounds gave the correct elemental analyses (C, H, Ti, V) and were characterized by $^1$H NMR and infrared spectroscopy. Moreover, the crystal and molecular structure of compounds **2** and **4** were determined by X-ray diffraction studies.

*Epithelial cancer cell lines and cultures*. The colon cancer cell line HT-29 (from human epithelial orthocolic adenocarcinoma) was obtained from the American Type Culture Collection (ATCC, London, UK). The cells were maintained under a fully humidified atmosphere of 95% air and 5% $CO_2$ at 37°C, in DMEM supplemented with 10% (v/v) FBS, 1.5 mM L-glutamine and 1mM sodium pyruvate and supplemented with the cocktail of antimicrobial agents (100 IU/ml penicillin, 100 mg/ml streptomycin, 10 mg/ml gentamycin sulphate and 2.5 mg/ml amphotericin B). For the experimental incubations, cells in log-phase growth were plated at a density of $1\times10^5$ cells/ml and allowed to attach overnight. The cells were subsequently exposed to media containing the appropriate concentrations of metallocenes and maintained as described above.

MCF-7 (oestrogen receptor, ERa-positive) is a mammary breast cell line and was obtained from the ATCC and cultured as a monolayer at 37°C in a humidified atmosphere of 5% (v/v) $CO_2$ and 95% air. The cells were seeded in 75-cm$^2$ plastic tissue culture flasks and cultured in EMEM supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 0.01 mg/ml of insulin and the cocktail of antimicrobial agents. According to pilot experiments in respect to growth rate and doubling time, the medium was changed every three days. The cells were harvested after treatment with 0.25% (w/v) trypsin in PBS, containing 0.1% (w/v) $Na_2EDTA$.

*Cell proliferation and estimation of toxicity*. The proliferation of the cancer cells was determined using the WST-1 reagent, which is a water soluble tetrazolium salt. The cells were treated with various concentrations of metallocene dihalides in the presence of serum for

48h. Following a short incubation with WST-1, the absorbance of the coloured product was measured at 450 nm using an ELISA microplate reader.

*Statistical analysis*. In all the experiments, the mean values±standard deviations (SD) for six determinations in triplicate were calculated. Statistically significant differences were evaluated using Student's *t*-test. Differences were considered statistically significant at the level of $p \leq 0.05$.

## Results

As far as the HT-29 colon cancer cells were concerned, the metallocene-induced growth inhibition for compounds **2-6** was in the range 30-50%, whereas compound **1** had no statistically significant inhibition of cell proliferation (Figure 3A). Compound **2** reduced cell growth as expected with maximum inhibition of about 45%. The $-SiMe_3C_5H_4$ substituted compound **4** was also found to induce growth inhibition at about 30% for all the concentrations tested and a plateau of inhibition percentage was observed for all four concentrations tested. Finally, compounds **6** and **5** representing mono- and di-$t$BuC$_5$H$_4$ substituted metallocenes were found to induce a significant growth inhibitory effect, with maximum growth inhibition of 33 and 46%, respectively.

As far as the MCF-7 breast cancer cells were concerned, the overall inhibitory pattern of the metallocene compounds tested showed that **1-4** inhibited cell proliferation in a dose-dependent manner by up to 40%, whereas **5** and **6** did not significantly affect cell proliferation (Figure 3B). In particular, compounds **1** and **2** were found to significantly inhibit cell proliferation, with **2** being the most cytotoxic, with maximum inhibition observed at 36%. An interesting result was observed for compound **4**, the $-SiMe_3C_5H_4$ substituted metallocene, which exhibited an inhibitory effect similar to that of compound **1,** with 25% maximum inhibition. As far as compound **3** was concerned, only a mild inhibitory effect was observed, with 13% maximum inhibition at 100 μM concentration. Compounds **5** and **6** were not found to induce statistically significant growth inhibition.

## Discussion

In the present study the six metallocene dihalides tested exhibited a different profile of cell proliferation inhibition depending on the metallocene and the cell line used. As far as the HT-29 colon cancer cell line was concerned, overall inhibition ranged between 30 to 50% for compounds **2-6** whereas compound **1** did not show statistically important inhibition of cell proliferation. It is worth noting that in contrast to compound **1**, compound **3** surprisingly exhibited a growth inhibitory action. According to the literature, compound **1** and **2** have been found to inhibit various of cancer types *in vitro*, including the relatively insensitive

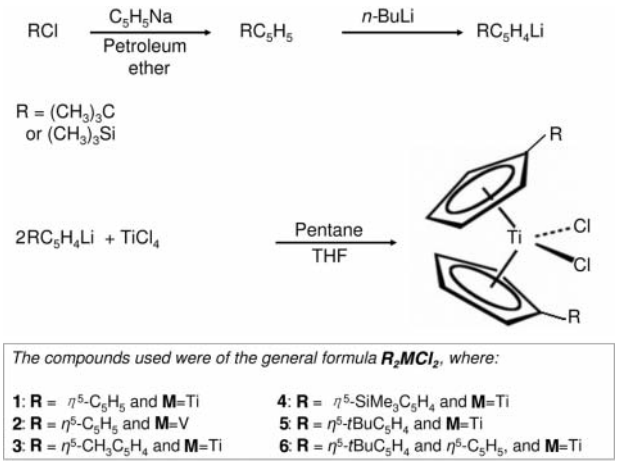


Figure 2. *General scheme for the preparation of ring substituted complexes* $(RC_5H_4)_2TiCl_2$.

colon 38 colon adenocarcinoma cell line and several human colon and lung carcinomas transplanted into athymic mice (3). In the past, titanocene dichloride showed up to 80% inhibition in the growth of colon 38 solid animal carcinoma, which was clearly more pronounced than that of cisplatin. Significant growth inhibition of up to 90% was reported against a range of xenografted human gastrointestinal carcinomas, which were much less sensitive to cisplatin, mitomycin and 5-fluorouracil, the most effective clinically used cytostatic drugs for gastrointestinal carcinoma (3, 5). Reports of synergistic activity of titanocene dichloride with the antitumour agent 5-fluorouracil *in vitro* suggested that clinical trials may involve formulations containing both antitumour agents (14). A similar inhibitory profile to that of titanocene dihalide in HT-29 cancer cells was observed in our laboratory also for 5-fluorouracil, the cornerstone of colon cancer therapy. In similar cell proliferation experiments, 5-fluorouracil did not exhibit a statistically significant growth inhibitory effect, whereas in combination with imatinib, a synergistic effect was observed (15). A recent phase II clinical trial of titanocene dichloride in patients with advanced renal cell carcinoma reported no therapeutic effect, suggesting that as with many chemotherapeutic agents, titanocene dichloride does not produce responses as a single-agent, which should be considered in clinical trials involving systemic therapy (4).

The absence of growth inhibition regarding titanocene dihalide **1** could be attributed to either poor dissolution of the agent in DMSO compared to the other compounds, or the rapid hydrolysis of the Cp ligands, since Cp hydrolysis is promoted in DMSO, leading to reduced amounts of the putative active species $Cp_2Ti^{2+}$ (16). In the present experiments, the initial stock solutions of metallocenes were prepared in DMSO and

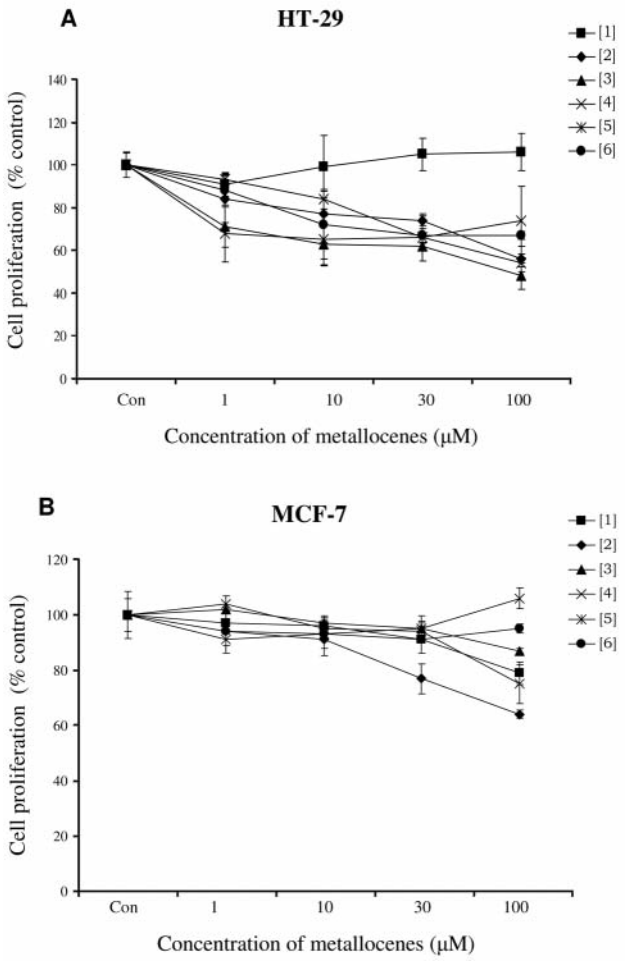


Figure 3. *Growth inhibitory effect of metallocene dihalides on HT-29 colon cancer (A) and MCF-7 breast cancer cell line (B). The compounds tested were of the general formula $R_2MCl_2$ where (1) R=$\eta^5$-$C_5H_5$ and M=Ti; (2) R $\eta^5$-$C_5H_5$ and M=V; (3) R=$\eta^5$-$CH_3C_5H_4$ and M=Ti; (4) R=$\eta^5$-$SiMe_3C_5H_4$ and M=Ti; (5) R=$\eta^5$-$tBuC_5H_4$ and M=Ti and (6) R=$\eta^5$-$tBuC_5H_4$ and $\eta^5$-$C_5H_5$, and M=Ti. The cells were incubated in serum-containing medium for 48 h in the absence or presence of the compounds. Data are representative of three individual experiments performed in six replicates.*

the desired concentrations were acquired by further dissolution in the culture medium. The lack of cell growth inhibition may be attributed to the duration of the experiment, since metallocene dichloride takes 2-3 days to show selective tumour uptake (17) and in recent reports of growth inhibition of **1** in HT-29 cells, the cytotoxic assays were performed for more than 70 h. Moreover, in the present experiments, the metallocene derivatives enhanced the antitumour activity, whereas in the literature the derivatives tested in HT-29 cells showed similar or inferior activity (18, 19). Titanocene dichloride analogues with alkyl-substituted Cp rings, bridged Cp rings or only one Cp ring showed significantly reduced antitumour activity compared to the parent compound (6). However, steric effects due to Cp substitution as well as electronic effects did not appear to be significant contributors to the reduced antitumour activity. Moreover, methyl Cp-substituted derivatives, with significantly improved aqueous solubility as compared to titanocene dichloride, confirmed that methyl substitution was detrimental to antitumour activity for reasons other than poor water solubility (20, 21).

However another aspect of Cp substitution has also been published by Mokdsi and Harding (20), showing that these complexes provided the first water soluble titanocene derivatives that could be used to prepare samples at pH 7.0 containing the Cp ligands coordinated to the Ti metal. Vanadocene dichloride significantly reduced cell proliferation as expected, since $Cp_2VCl_2$ is reported to be the most cytotoxic metallocene *in vitro*, although $Cp_2TiCl_2$ shows improved activity and selectivity in animal studies and therefore has been selected for human clinical trials (3, 5). Considering all the above regarding Cp substitution, the present study showed that Cp-substituted compounds not only were active in the colon cancer cells, but also induced significant cell growth suppression in a dose dependent manner, implying that they could be further investigated for antitumour activity for colorectal cancer.

As far as the MCF-7 breast cancer cell line was concerned, the overall inhibitory pattern of the metallocene compounds tested showed that **1-4** inhibited cell proliferation in a dose-dependent manner by up to 40% at 100 μM, whereas **5** and **6** did not significantly affect cell proliferation. Breast carcinoma is a type of cancer that has been found to be sensitive to titanocene dichloride and heterotransplanted breast carcinomas in athymic nude mice have been found to regress in size by 50-92% compared to control tumours after treatment with optimum doses of titanocene dichloride. The results of phase II clinical trials against breast cancer (22) and advanced renal carcinoma (4) agreed with those of independent chemical studies that concluded that the biological chemistry of each of the metallocene dihalides is unique (23). This can be attributed to the different mode of action of the compounds depending on the metallocene, the cell line and the experimental conditions.

Regarding metallocene complexes, it has been proposed that Cp ligands, including hydrophobic ligands, may play a significant role by facilitating transport of the metal into the cell and then by releasing an active species, or as a delivery agents to a blood transport protein such as transferrin (Tf). Considering the potential role of Tf in the mechanism of action of metallocenes it may be assumed that oestrogen dependent MCF-7 breast cancer cells may respond differently to metallocenes, compared to HT-29 colon cancer cells, since oestrogen regulation of transferrin gene expression in MCF-7 cells has been reported (24).

It has been suggested that if a species such as $Cp_2M^{n+}$ which can be produced after rapid halide hydrolysis *in vitro,* is transported into cancer cells then inhibition of topoisomerase II is possible, most probably by direct interaction with the

enzyme *via* binding to accessible coordinating groups on the surface of the protein, with resultant reduction in the activity of the enzyme (25). It has been suggested (25, 26) that this is unlikely to be the case for $Cp_2TiCl_2$, but may be feasible with the metallocenes $Cp_2VCl_2$, $Cp_2MoCl_2$ and $Cp_2NbCl_2$. The biologically inactive derivative $(MeCp)_2TiCl_2$ also showed inhibition at comparable concentrations to other metallocenes. The present results show that in HT-29 cells, where no inhibitory effect of titanocene dichloride was observed, $Cp_2VCl_2$ and $(MeCp)_2TiCl_2$ induced almost 50% inhibition at maximum concentration, allowing the speculation that topoisomerase II may be implicated in the metallocene mechanism of action in these cells. The results also paralleled the higher activity of vanadocene dichloride in inhibiting cell proliferation *in vitro*, compared to other metallocenes of type $Cp_2MCl_2$ (3) as well as inhibition of nucleic acid metabolism at a lower concentration than titanocene dichloride.

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*Received March 1, 2009*
*Revised May 8, 2009*
*Accepted May 12, 2009*