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In vitro Cell Growth Inhibition by Metallocene Dichlorides

Petra Köpf-Maier[1], Waltraud Wagner[1,2], and Hartmut Köpf[2]

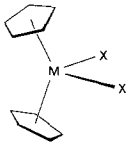
[1] Institut für Anatomie der Freien Universität Berlin, Königin-Luise-Straße 15, D-1000 Berlin 33

[2] Institut für Anorganische und Analytische Chemie der Technischen Universität Berlin, Straße des 17. Juni 135, D-1000 Berlin 12

Summary. *The in vitro growth-inhibiting potencies of titanocene dichloride (TDC), zirconocene dichloride (ZDC), hafnocene dichloride (HDC), vanadocene dichloride (VDC), and molybdocene dichloride (MDC) against Ehrlich ascites tumor (EAT) cells cultured in vitro as permanently growing suspension cultures were determined. The most striking growth-suppression activity was detected for VDC. A VDC concentration as low as $5 \cdot 10^{-6}$ mol/l effects a highly significant diminution of cell proliferation. TDC and MDC inhibit cellular growth only in concentrations of $5 \cdot 10^{-4}$ or 10^{-3} mol/l, respectively, whereas ZDC and HDC, which are ineffective against EAT cells in vivo, require higher concentration levels. The growth inhibition is caused by a cytotoxic action of the metallocene dichlorides, as is demonstrated in the case of VDC and TDC by differentiation of live and dead EAT cells by means of the dye lissamine green.*

Introduction

Metallocene dihalides $(C_5H_5)_2MX_2$, a class of organometallic complexes (formula I), have recently proved to be potent tumor-inhibiting agents against EAT in mice [3–5, 7–9]. Thus, the metallocene dichlorides of titanium, vanadium, or molybdenum effect cure rates of 100% in optimum doses. In contrast, the analogous complexes containing tantalum or tungsten as central atoms only sporadically evoke tumor inhibition and, finally, the zirconium and hafnium derivatives show no antitumor activity against the same tumor system.



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Reprint requests should be addressed to: P. Köpf-Maier

Preliminary investigations concerning the mechanism of action indicate that titanocene dichloride is able to interfere with the metabolism of DNA and RNA [6]. In this connection the question arose as to whether the cancerostatic action of metallocene dihalides in vivo is paralleled by a corresponding cytostatic or cytotoxic effect in vitro. Therefore we undertook a comparative study of the behavior of metallocene dichlorides (X = Cl), including in vivo highly active (M = Ti, V, Mo) and inactive (M = Zr, Hf) representatives, against in vitro-cultured Ehrlich ascites tumor cells.

Materials and Methods

Antitumor Agents. Titanocene dichloride (TDC), zirconocene dichloride (ZDC), hafnocene dichloride (HDC), vanadocene dichloride (VDC), and molybdocene dichloride (MDC) were prepared and purified as described in the literature [1, 12, 13].

Cell Culture Techniques. Cells utilized in this investigation were harvested from a female CF1 mouse bearing for 8 days an Ehrlich ascites tumor (EAT). About $5 \cdot 10^5$ cells per milliliter medium were inoculated into minimum essential medium (MEM) with Hank's salts and 0.35 mg/ml $NaHCO_3$. Every liter MEM had been supplemented before use with 125 ml fetal calf serum, 11 ml 0.2 *M* L-glutamine solution, and 11 ml solution of antibiotics, containing 10 mg streptomycin/ml and 10^4 IU penicillin/ml (medium and ingredients from Seromed, Munich). The cells were kept without agitation as permanently growing suspension cultures in siliconized glass bottles.

Exposure to Agents. The metallocene dichlorides TDC, ZDC, HDC, VDC, and MDC were each applied in final concentrations of 10^{-7}, $5 \cdot 10^{-7}$, 10^{-6}, $5 \cdot 10^{-6}$, and so on up to $5 \cdot 10^{-2}$ mol/l. Amounts of metallocene dichlorides corresponding to these concentrations were dissolved in 0.3 ml dimethyl sulfoxide

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(DMSO; Serva, Heidelberg, FRG) by ultrasonic treatment for 5 min. These solutions were diluted with 10 ml supplemented MEM, adjusted to pH 7.1–7.2 with 1 *M* $NaHCO_3$ solution (Pfrimmer & Co., Erlangen, FRG), and added to 10 ml supplemented MEM containing 10^5 cells/ml. Four bottles were prepared in this manner for every concentration stage. A further eight cell populations per metallocene compound served as untreated controls. The appropriate bottles were equipped in the same manner with medium and cells plus an admixture of 0.3 ml DMSO. In all cases including controls, after a treatment time of 90 min the cells were collected by centrifugation and resuspended in 20 ml fresh, warmed medium. This washing procedure was repeated to ensure that any excess chemical was removed. After the final resuspending of the cells in fresh medium no further renewal of medium was performed.

Determination of Cell Growth Inhibition. Beginning immediately after removal of the antitumor agent, every 24 h up to 120 h, 0.5 ml cell suspension was gathered from each bottle. Cells per milliliter were counted with the aid of an electronic particle counter (model Dn, Coulter Electronics, Krefeld, FRG). The increase in cell number per milliliter, expressed as a percentage of the cell number per milliliter measured immediately after removal of the antitumor agents, was determined for each cell population. For statistical analysis we used the H-test according to Kruskal and Wallis.

Determination of Cytotoxicity. Cell populations in separate bottles were exposed to $5 \cdot 10^{-5}$, $5 \cdot 10^{-4}$, and $5 \cdot 10^{-3}$ mol TDC/l or to 10^{-6}, 10^{-5}, and 10^{-4} mol VDC/l, in the manner described above, additional control cell populations being prepared in an analogous manner. Immediately after removal of the agent and again 24, 48, and 72 h later, 2 ml cell-containing medium from each bottle was centrifuged and the pellet was added to one drop of lissamine green solution (1% in saline; Chroma, Stuttgart, FRG). Five minutes later the proportion of dead cells was determined as a function of the applied TDC and VDC concentrations by counting the numbers of stained (dead) and unstained (live) cells in totals of 1,000 cells.

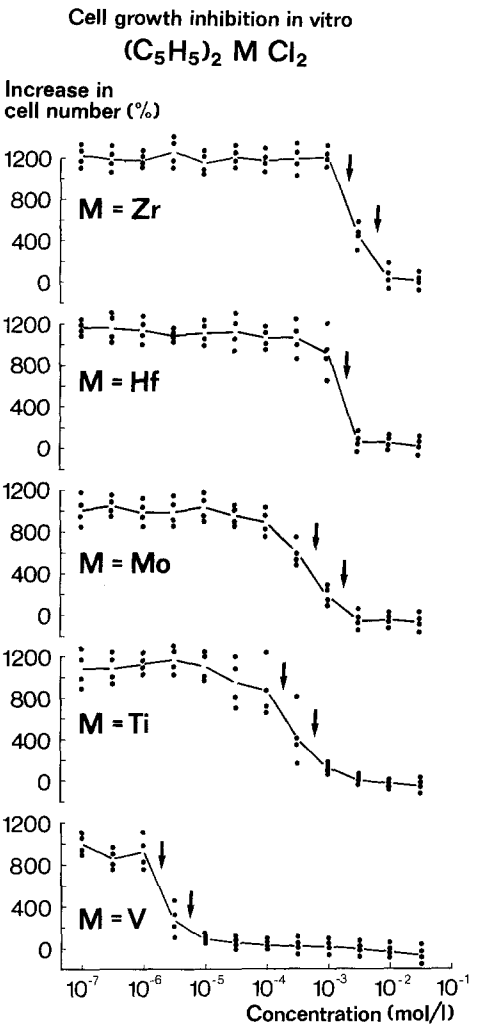

Fig. 1. Effect of a 90-min treatment with various concentrations of ZDC, HDC, MDC, TDC, and VDC on the increase in cell number, determined 72 h after removal of the agent. *Arrows* indicate highly significant differences ($\alpha < 0.1\%$) between proliferation rates of neighboring groups

Results

The effect of a 90-min exposure to metallocene dichlorides in various concentrations on the proliferation behavior of EAT cells growing in vitro as a permanent suspension culture is summarized in Fig. 1 in relation to a given time after treatment, and exemplified for TDC and VDC in Figs. 2 and 3 in relation to various intervals after treatment. Cell growth inhibition is expressed in Fig. 1 by a descent of the increases in cell number and in Figs. 2 and 3 by flattened slopes of the curves during the logarithmic proliferation period. Whereas the control populations show an intense cell growth activity characterized by average cell-cycle times of 18–22 h, the treatment with TDC, ZDC, HDC, VDC, or MDC causes a diminution of cellular proliferation, the critical concentration levels differing considerably between the single compounds.

VDC, the most potent inhibitor of cellular growth, is able to influence the proliferation already in a concentration of $5 \cdot 10^{-6}$ mol/l in a highly significant ($\alpha < 0.1\%$) manner. At 10^{-5} and $5 \cdot 10^{-5}$ mol/l the effect becomes more pronounced, until it results in a complete cessation of cell growth at concentrations of 10^{-4} mol/l and higher.

To evoke similar suppression of cellular proliferation for TDC and MDC, 100-fold higher concentration stages are required. In this way highly significant diminution is attained with TDC or MDC concentrations of $5 \cdot 10^{-4}$ or 10^{-3} mol/l, respectively, whereas complete suppression of proliferation is effected by $5 \cdot 10^{-3}$ mol/l of both substances.

ZDC and HDC are able to reduce cellular growth to a highly significant extent only at concentrations of $5 \cdot 10^{-3}$ mol/l or higher. With these compounds, the range between nonsuppressing and totally cell

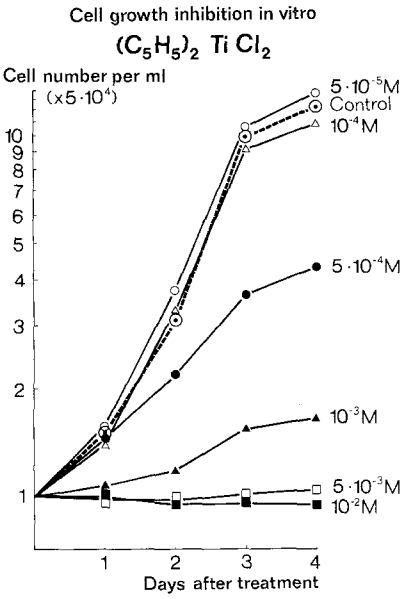

Fig. 2. Effect of a 90-min treatment with various concentrations (given on the *right*) of TDC on the cell number per milliliter as a function of the time after treatment. Values given are mean values obtained from four cell populations each

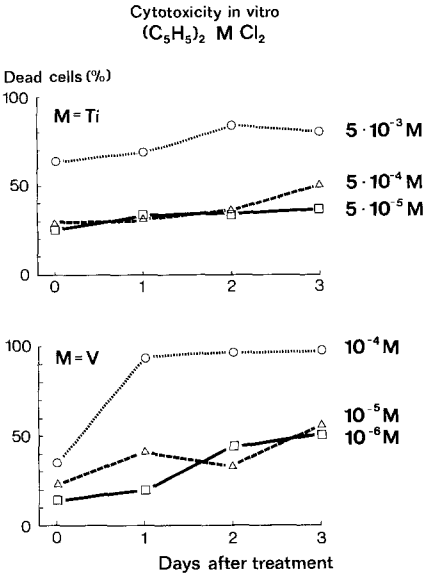

Fig. 4. Effect of a 90-min treatment with various concentrations (given on the *right*) of TDC or VDC on the vitality of EAT cells as a function of the time after treatment

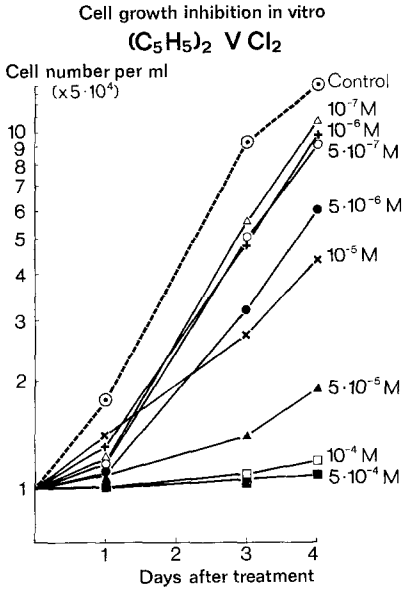

Fig. 3. Effect of VDC (see Fig. 2)

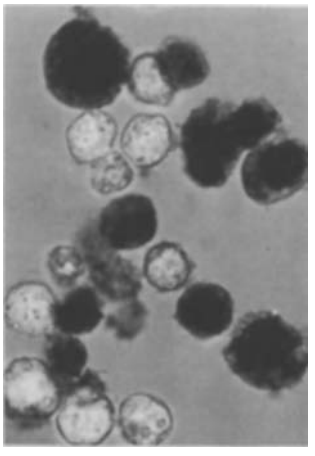

Fig. 5. In vitro cultured EAT cells immediately after exposure for 90 min to $5 \cdot 10^{-3}$ mol TDC/l. Staining of dead cells with lissamine green. Cytoplasmic blows on the cellular surface are an additional sign of cellular damage. (× 960)

growth-suppressing concentrations is comparatively narrow. After addition of the amounts of ZDC and HDC corresponding to final concentrations of $5 \cdot 10^{-3}$ mol/l and higher flocculent precipitations appeared in the medium and did not vanish during the 90-min exposure period.

In a separate experiment the dye lissamine green was used to differentiate live from dead cells at various intervals after the exposure to varying concentrations of TDC and VDC (Figs. 4 and 5). Whereas control cell populations treated only with DMSO always contain far less than 1% dead cells, about 25 or 15% of all cells are found to be irreversibly damaged after application of $5 \cdot 10^{-5}$ mol TDC/l or 10^{-6} mol VDC/l, respectively, i.e., at concentration levels at which the proliferation of the

cells is not yet influenced in a significant manner. The proportion of dead cells rises, firstly, when the highest concentration is applied at a given interval, and secondly at a given concentration stage when the interval after removal of the cytotoxic agent is increased. Thus, about 80 or 95% of the cells are dead 3 days after treatment with $5 \cdot 10^{-3}$ mol TDC/l or 10^{-4} mol VDC/l, respectively.

Discussion

When the in vitro growth-inhibiting potencies of TDC, ZDC, HDC, VDC, and MDC against EAT cells are compared, three groups of graduated activity can be distinguished. The highest activity is observed with VDC, which begins to inhibit cellular growth at low concentrations similar to those required of the prominent cytostatic drug *cis*-diamminedichloroplatinum(II) [2, 10].

Forming a group with intermediate in vitro activity, TDC and MDC begin to suppress cellular growth only in concentrations about 100 times those of VDC. This result is surprising, because TDC and MDC as well as VDC achieve 100% cure rates in vivo (ED_{50} values 20, 55, and 70 mg/kg, respectively) on application 24 h after transplantation of EAT cells on mice [3–5, 7, 8]. When a more advanced murine EAT is treated on day 6 after transplantation TDC effects even more cures than VDC (unpublished results). This discrepancy between the in vivo and in vitro behavior may be explained by additional factors in favor of TDC and MDC in vivo, such as metabolic activation or, perhaps, immunologic support.

ZDC and HDC, finally, being inactive against EAT in mice, are distinguished from TDC and MDC in vitro by requiring concentrations ten times as high again to suppress cellular growth. The appearance of precipitations suggests that even this inhibition may be an unspecific effect caused by a reaction of the substances with the nutritive medium and that ZDC and HDC on their own do not have specific cytostatic properties. All the metallocene dichlorides under discussion show comparable toxicity in vivo, with LD_{50} values of 75 (ZDC), 100 (TDC), 110 (VDC), 175 (MDC), and 220 mg/kg (HDC).

The pattern of cytostatic activity observed here is in agreement with the supposition that the cytostatic effect of the metallocene dichlorides is based on an intrastrand cross-linking of DNA similar to that postulated for *cis*-diamminedichloroplatinum(II) [11], and that the formation of these bifunctional chelating links depends on the nonbonding Cl–Cl distance ('bite') within the dichlorometal moiety [7]. In fact, the value for this bite of 3.30 Å in VDC is closer to the value of 3.35 Å found in *cis*-diamminedichloroplatinum(II) than the values of 3.47 and 3.24 Å in TDC and MDC, respectively. The values of 3.66 and 3.60 Å for ZDC and HDC are much further away from the value for *cis*-diamminedichloroplatinum(II), thus probably exceeding a critical limit for the set-up of stable intrastrand cross-links with DNA.

This mechanistic suggestion is further supported by incorporation experiments showing that TDC is able to depress the DNA and RNA synthesis in an extensive and persistent manner [6], and by UV-spectroscopic evidence for a direct interaction between TDC and nucleic acids in vitro (P. Köpf-Maier et al. 1980, unpublished work).

Therefore the nucleic acids are possibly the target of the cytotoxic attack, demonstrated by the lissamine green staining technique, exerted by the metallocene dichlorides. Besides a direct cell-killing activity recognizable immediately after treatment, TDC and VDC also obviously have the ability to damage the cells so that these die some hours or days later, thus leading to further increases in the proportions of dead cells at increasing intervals.

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