INHIBITION OF HERPES SIMPLEX VIRUS TYPE 1 INFECTION BY SOME SELECTED ANTITUMOR TITANIUM(III)-BASED COORDINATION COMPOUNDS

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ABSTRACT

Using a standard plaque assay and clinical strains of Herpes Simplex virus type 1 (HSV 1), we have tested the ability of two titanium (Ti) – content complexes – Titanocene Dichloride (Cp_2TiCl_2) and Titanocene Y. Virus was treated by incubation at 37°C with both complexes in cell culture media and was then diluted and plated onto human embryonic lung fibroblast cells MRC-5 for detection and quantitation of remaining infectious virus. Of one randomly chosen clinical HSV 1 (TM strain), sensitive to acyclovir (ACV), was inactivated > 98% by treatment in vitro with 0.01µM Titanocene Y for 2h. The effect was concentration dependent. With an HSV 1 strain, 0.01µM Titanocene Y or Cp_2TiCl_2 caused 99% inactivation, 0.001µM caused 98 to 99% inactivation, and 0.0001µM caused 63 to 86% inactivation. Short (5 min) treatments of selected isolate with Titanocene Y or Cp_2TiCl_2 yielded inactivation rates of 0 to 55%. We assumed also that Titanocene Y or Cp_2TiCl_2 could be able to affect negatively not only virus DNA synthesis but also another virus target. The data obtained show that: (i) the 300bp relatively stable region during a short period of viral replication corresponding to ReIV is amplified in non-treated viral control, but not in mock cells and in negative control and (ii) the region was not amplified in Titanocene Y treated samples. The data show that the effect of the suppressive therapy of virus replication by Titanocene Y could be due to the suppression of immediate early Us1 and Us12 genes encoding essential for virus replication products α22 – the protein affecting virus ability to replicate, and α47 – the protein inhibiting MHC I antigen presentation.

Keywords: *Herpes Simplex virus (HSV),* inhibition, Titanocene Dichloride (Cp_2TiCl_2), Titanocene Y

Introduction

Herpes Simplex virus (HSV) causes diseases ranging from "fever blisters" and sexually transmitted disease to encephalitis and serious infections in neonates and immunocompromised hosts (1 – 4). Acyclovir (ACV) is a prodrug and it is the first nucleoside-based therapeutic effective for the treatment primary and recurrent HSV infections (5). ACV has to be phosphorylated by the viral thymidine kinase (TK) and subsequently by cellular kinases in order to inhibit competitively HSV DNA polymerase and to terminate the viral DNA chain elongation. The two most common causes of resistance are mutations in thymidine kinase (*tk)* gene, approximately 95 to 96% of ACV-resistant (ACV^R) HSV isolates are thymidine kinase (TK) deficient (TK^N) or TK-partial (TK^P) and the remaining isolates are usually TK-altered (TK^A) mutants unable to phosphorylate the pro-drug but not the thymidine (6). The problem for effective treatment of HSV infections is still open, since the resistance to ACV and the cross resistance to other nucleoside analogues increases with relatively high frequency.

Over the past 30 years metal coordination compounds had been widely studied for their broad spectrum of human pathogens, herpes viruses included (7 – 12). Moreover, because of the link existing between herpes viruses and malignant transformation, many metal compounds also exhibit antitumor properties, which are less toxic than the platinum–based drugs (13 – 16). For instance only Titanocene dichloride (Cp_2TiCl_2) has reached Phase I clinical

trials so far (17). Compared to standard antineoplastic agents such as *cis*-platinum, vinblastine, doxorubicin, Cp_2TiCl_2 was found to exhibit higher cytotoxicity in renal cell carcinoma (18). The Cp_2TiCl_2 was found to exhibit more efficacies in a human ovarian cancer xenograft model than *cis*-platinum (19). Recently some derivatives of Cp_2TiCl_2 showed enhanced anti-cancer activity (20). Now scientists all over the world are seeking new titanium-based complexes of potent antitumor effects with a different mode of action in the hope of adding new chemotherapeutic agents to the arsenal of weapons used against the world's most life threatening disease, *i.e.*, infectious diseases and cancer.

Therefore, the aim of this study was to determine the antiviral and cytotoxic properties of Cp_2TiCl_2 and newly synthesized titanium(III)-based coordination complex.

Materials and methods

Compounds

Solvents were purified and dried according to the standard procedures. Titanocene Y and Titanocene Dichloride (Cp_2TiCl_2) were first dissolved in dimethylsulfoxide (DMSO, Serva) to a concentration of 1mM (stock solutions). Serial tenfold dilutions (0.00001 – 100 μM) were made from them in cells growth medium DMEM (Gibco, USA). All compound solutions were prepared *ex tempore*.

Cells and viruses

Continuous human embryonic lung fibroblasts cells (MRC-5) were grown as monolayer culture in DMEM supplemented with 5% to 10% fetal calf serum (FCS, BioWhiteker, Belgium), 50mU/ml penicillin and 50μg/ml streptomycin (Balkanpharma, Bulgaria). The culture was maintained at 37°C in a humidified CO_2 incubator. For routine passages monolayers were detached using a mixture of 0.05% trypsin (Gibco, USA) – 0.02% ethylendiamino tetraacetic (EDTA, Gibco, USA). Antiviral experiments were done on the following two herpes viruses: one wild (*wt*) strain TM (HSV 1), sensitive to ACV and one ACV^R mutant R-100 (TK^A, HSV 1). Viruses were grown in MRC-5 cell monolayers. Cultures were harvested at full cytopathic effect (CPE), freeze thawed and stored at -70°C.

Methods of determining cell growth, cell viability, maximal non-toxic concentration (MNC) and concentrations, required cell viability by 50% (CC_{50})

Cells were seeded into 96-well tissue culture plates (Grainer, Germany) at a concentration of 5 x 10^4 cells/well and cultured at 37°C in a CO_2 atmosphere. On the 24h cells from confluent monolayers were washed and covered with media containing the compounds tested in concentrations from 0.00001 – 100 μM. Cells grown in compound-free medium served as a control. After 24h and 48h the viability of cells was read by microscopy of unstained cell monolayers and by the MTT-assay and expressed as % from the cells control taken as 100%. Each experiment was done in triplicate. The MNC and CC_{50} for each compound were calculated from dose-response curves. The maximal concentration, which altered neither the morphology of monolayers nor the cell survival rate, was recognized as MNC.

Assay of antiviral activity

Assay of antiviral activity of the compounds tested was done on the basis of their effects on the infectious of different strains HSV 1 titers. MRC-5 cells grown in 96-well plates were infected with HSV 1 strains in serial tenfold dilutions. After 1.5 hr of virus adsorption, infected cells were incubated with the compounds tested in serial dilutions (starting from ½ MNC) at 37°C for 48 hrs. CPE and virus titer were determined for each well. The virus titer was expressed as log TCID/0.1 ml. The antiviral activity was expressed as % inhibition of virus titer as compared to that of control (infected cells incubated in compound-free medium). Dose-response relationships were constructed by linearly regressing compound concentrations against % inhibition values derived from virus titrations.

Direct PCR for determination of the effect on the expression of the immediate early (IE) reiterating region IV (ReIV)

Infected and mocked infected cells cultured in compound-free medium served as controls. PCR amplification 22bp primers (Applied Biosystems, USA) were designed to amplify 300bp Us1 ReIV region of HSV-1 genome positions 132333-132634 (21). The sequence (5′→3′) of the primer were: ReIVUs1F - 5'TCCGACGACAGAAACCCACC3' and ReIVUs1R - 5'GTCCCGGAGGACCACAGTGG3'). PCR was performed in a Ready-To-Go-PCR beads thermo cycle (Amersham-Pharma Biotech, USA). A 2 μl sample of DNA suspension was added to the reaction mixtures and overlaid with 25μl of mineral oil (CinnaGen Inc.). PCR amplification was carried out as follows: an initial denaturation step of 94°C for 5min followed by 35 cycles of alternating denaturation (94°C for 30sec), primer annealing (60°C for 60sec) and primer extension (72°C for 60sec). A final extension step of 5min at 72°C was included. The PCRs were performed in 10μl volume. Briefly, 2μl of the each sample were added to a separate tube containing 100μl of lysis buffer (Applied Biosystems, USA) and stored at -20°C over night. After centrifugation at 12 000rpm for 5min the lysis

buffer was removed, pallets were re-suspended in nucleolysis buffer (300 μl phenol (Sigma, USA), pH7.8; 300μl chloroform: isoamyl alcohol = 24 : 1 (Sigma, USA) and centrifuged at 12 000 rpm for 5min. DNA was extracted by re-suspending the pallets in 10% SDS (Sigma, USA) 10mg/ml proteinase K (Pharma Biotech, USA), 10mM Tris (Sigma, USA) and 0.1mM EDTA (Sigma, USA) at pH 7.4 and centrifuged at 12 000rpm for 5min. A volume of 2μl of supernatants containing 50 – 100ng of the resulting DNA suspension was used per PCR mixture. The reaction mixture contained 5U/μl cloned recombinant thermo stable STS DNA taq polymerase (Applied Biosystems, USA), corresponding primers at a concentration of 20μl/ml each and 5mM/μl deoxynucleoside triphosphate (Pharma Biotech, USA). Amplicons were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Results and Discussion

The first obligatory step in antiviral experiments is evaluation of their cytotoxicity. Both MNC and CC_{50} were evaluated simultaneously by morphological and by cell survival criteria – MTT-assay. When a microscopic observation of the morphology of monolayer was carried out at intervals of 24h and 48h of the treatment with Titanocene Y and Cp_2TiCl_2 in a concentration ranges respectively from 100 – 10μM to 2.5 – 1μM a typical cytopatology characterizing the toxically effect was registered. This typical cytopatology was expressed in a round form of the cells and their grouping in the "islands" isolated from the surface of the cells. We found some nuclear morphology changes. When a treatment with lower concentrations was performed, no essential change was registered in the monolayer in comparison with cell control. When MNC was defined, no essential changes were established in the values according to the time of cultivation. During experimentations it was found that the cytotoxicity of both compounds increased when the concentration increased too. Cp_2TiCl_2 and Titanocene Y were also active against MRC-5 cells in a dose-dependent manner. It was found that the cell surveillance depended on both complex and cell specificities. *In vitro* cytotoxicity data for the tested Titanocene compounds were summarized in **Table 1.**

Preliminary data presented here show that the tested compounds expressed a different degree of cytotoxicity in MRC-5 cell line. The less cytotoxic compound according MNC was Cp_2TiCl_2, whose MNC values were 10 and 70 times lower than those of Titanocene Y. With the prolongation of action on MRC-5 cells the cytotoxicity of Cp_2TiCl_2 decreased up to 7 times, while the cytotoxicity of Titanocene Y was independent on the duration of action. According to CC_{50} values the investigated compounds at 24h and 48h treatment can be arranged as follows: Titanocene Y > Cp_2TiCl_2. In addition, the cytotoxicity of both tested compounds increased with the prolongation of treatment, the induction of this are the correspondingly 2 and 7 times higher values of CC_{50} at 48h versus those at 24h of action.

TABLE 1.

Cytotoxic effect of Cp_2TiCl_2 and TitanoceneY at 24h and 48h

Compound	MNC, μM		CC_{50}, μM	
	24h	48h	24h	48h
Cp_2TiCl_2	0.00001	0.00007	2	1
TitanoceneY	0.000001	0.000001	0.07	0.01

In the second group of experiments the antiviral activity of Cp_2TiCl_2 and Titanocene Y were evaluated against two different strains HSV 1 in cultured cells. Furthermore, the data showed that both of tested compounds haven't (Cp_2TiCl_2) or have very less (Titanocene Y) effects against the HSV 1 strain R-100, resistant to ACV *in vitro*. Both of tested compounds inhibited the strain TM growth and manifested a structure-activity relationship. The dose-response effect of Cp_2TiCl_2 was manifested by concentrations under maximal non-toxic (0.001μM), while very less inhibition was obtained at a concentration 0.0001μM. With an HSV 1 strain, 0.01μM Titanocene Y or Cp_2TiCl_2 caused 99% inactivation, 0.001μM caused 98 to 99% inactivation, and 0.0001μM caused 63 to 86% inactivation.

The clinical HSV 1 (TM strain), sensitive to acyclovir (ACV), was inactivated > 98% by treatment *in vitro* with 0.01μM Titanocene Y for 2h. Short (5 min) treatments of selected isolate with Titanocene Y or Cp_2TiCl_2 yielded inactivation rates of 0 to 55%.

We assumed also that Titanocene Y or Cp_2TiCl_2 could be able to affect negatively not only virus DNA synthesis but also another virus target, which is possible to be the essential structure proteins from the β(E) and γ(L) kinetic groups, whose synthesis is impossible without α, IE proteins. The expression of the immediately earliest α, IE genes by means of a direct PCR was studied. A direct multiplying was used with PCR by a primer, determining region 300bp, corresponding to ReIV region of Us1. The data obtained show that: (i) the 300bp relatively stable region during a short

period of viral replication corresponding to ReIV is amplified in non-treated viral control, but not in mock cells and in negative control and (ii) the region was not amplified in Titanocene Y treated samples.

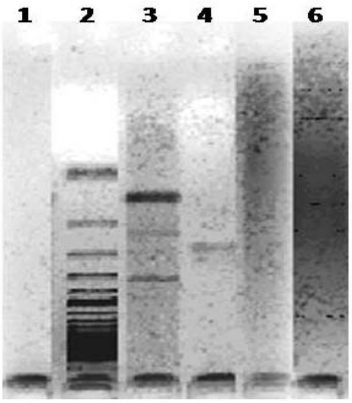

Fig. 1. Amplification of ReIV containing region within *wt* HSV 1 genome. 1 – Negative control (lane 1); 2 – Markers (lane 2); 3 – Cell control (mock infected cells cultured in compound-free medium) (lane 3); 4 – Positive control (HSV 1, strain TM infected cells cultured in compound-free medium) (lane 4); 5 – Titanocene Y (lane 5) and 6 – Cp_2TiCl_2 (lane 6).

In conclusion, HSV 1 strains, sensitive and resistant to ACV can be efficiently inactivated by treatment with Titanocene Y than Cp_2TiCl_2 *in vitro*. The suppressive therapy of virus replication by Titanocene Y could be due to the suppression of immediate early Us1 and Us12 genes encoding essential for virus replication products α22 – the protein affecting virus ability to replicate, and α47 – the protein inhibiting MHC I antigen presentation.

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