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Izvleček

Namen: *V predhodnih raziskavah smo dokazali, da so de novo sintetizirani derivati fluoro-glukopiranozil ketonukleotidov skupina obetajočih antivirusnih substanc proti RNA virusom. Zato je bil cilj tega dela preučiti, ali so te substance prav tako učinkovite v boju proti DNA virusom v primerjavi z komercialnim zdravilom Ganciclovir.*

Metode: *Uporabili smo model Pseudorabies virusa (NIA3) in prašičje ledvične epitelijske celice (celična linija PK-15).*

Rezultati: *Rezultati so pokazali, da je 1-(6-O-acetil-3,4-dideoksi-3-fluoro-β-D-glicero-hex-3-enopiranozil-2-uloza)-N4-benzoil citozin (substanca 2) močan inhibitor replikacije pseudorabies virusa z močnejšim učinkom kot ga ima Ganciclovir.*

Abstract

Purpose: *We have previously shown that newly synthesized fluoro-glucopyranosyl derivatives of ketonucleosides represent a group of antiviral drugs potent against RNA viruses. The aim of the present study was to ascertain whether these compounds are equally efficient in combating DNA viruses in comparison with the commercially available drug ganciclovir.*

Methods: *As a study model, the pseudorabies virus (NIA3 strain) and pig kidney cells (PK-15) were used.*

Results: *Our results indicated that 1-(6-O-acetyl-3,4-dideoxy-3-fluoro-β-D-glycero-hex-3-enopyranosyl-2-ulose)-N4-benzoyl cytosine (compound 2) is a potent inhibitor of pseudorabies replication, with higher antiviral activity than was found for ganciclovir.*

Zaključek: *Substanca 2 je močan inhibitor replikacije Pseudorabies virusa v okuženih celicah.*

Conclusion: *Compound 2 has strong antiviral activity against the DNA virus pseudorabies.*

INTRODUCTION

Viral infections, such as HIV-infection and influenza, are estimated to cause the highest healthcare burden in Europe of all infection diseases [1]. There is a continuing need for the development of new antiviral drugs, drug combinations and other antiviral strategies with no or mild side effects through methodical and scientific exploration of the enormous pool of synthetic, biological and natural products [2]. Nucleosides and their analogues have a very important place in medicinal chemistry and provide a structural basis for the development of antiviral and antitumor agents. Nucleoside chemistry has facilitated efficient routes to the development of effective agents for the treatment of acquired immune deficiency syndrome (AIDS), herpes, viral hepatitis, cytomegalovirus (CMV) and rotavirus. We have previously shown that newly synthesized derivatives of ketonucleosides represent a group of antiviral drugs potent against RNA viruses [3-6].

In spite of the proven activity of nucleoside derivatives, limited data are available to show their mechanism of action. Natural nucleosides are usually converted inside the cell to the corresponding triphosphates, which are incorporated into DNA by kinases and polymerases and these enzymes are involved in the mode of action of antiviral nucleosides such as ganciclovir [7]. Ganciclovir (9-[(1,3-dihydroxy-2-propoxy)methyl]guanine) is an example of a potent inhibitor of viruses of the herpes family (including CMV) that are pathogenic to human and animals. The action of ganciclovir against CMV is via its metabolite, ganciclovir-5'-triphosphate (ganciclovir-TP), which inhibits viral DNA replication.

We have previously shown that newly synthesized fluoro-glucopyranosyl derivatives of ketonucleosides represent a group of antiviral drugs potent against RNA viruses. The aim of the present study, therefore, was to investigate whether these compounds are equally efficient in combating DNA viruses in comparison with the commercially available drug ganciclovir. As a study model, the pseudorabies virus (NIA3 strain) and pig kidney cells (PK-15) were used.

MATERIALS AND METHODS

Cells

Pig epithelial kidney cells (PK-15) were obtained from American Tissue Culture Collection ATCC and maintained in Minimum Essential Medium Eagle - MEME (Cambrex, Belgium), supplemented with 10% (v/v) heat inactivated foetal bovine serum - FBS (Cambrex, Belgium), penicillin 100 IU/mL, streptomycin 100 µg/mL (Fluka, Switzerland) and L-glutamine 2 mmol/L (Sigma, USA) in 25 cm^2 plastic flasks (Corning, USA) at a temperature of 37°C in a 5% CO_2 humidified atmosphere until confluence. The cell culture medium was regularly changed and cells were split approximately twice per week. Viability of the cells was determined with a trypan blue (Sigma, UK) exclusion assay [8].

Virus propagation

Pseudorabies virus (NIA3 strain; Jouy-en-Josas, France) was propagated in PK-15 cells. Supernatant containing the virus was collected from the flasks when a cytopathic effect (CPE) was observed (24 hrs at 37°C) and clarified by centrifugation. Virus

was stored at –70°C until further use. For the virus reduction assay, stock solution of the virus with 6,5 $\times 10^8$ PFU/mL was used.

Fluoro-glucopyranosyl derivatives of ketonucleosides

Ketonucleoside derivatives were screened as potential antivirals against pseudorabies virus. Compounds were synthesized as previously described [3-6]. After promising preliminary results during an initial screening process, 3-fluoro-2-keto-β-D-glucopyranosyl derivatives of N^4-benzoyl cytosine [6] were used in the experiments.

Stock solution of each individual compound (Fig. 1) was freshly prepared in sterile dimethyl sulfoxide (DMSO) in a concentration of 5 mg/mL. On the day of the experiment, the compounds were diluted in MEME to the final concentration indicated (Table 1) and applied to the virus-infected cell monolayer. The final concentration of DMSO in the cell culture medium was less than 0.1%. All solutions used were protected from the light.

Commercially available ganciclovir (Calbiochem, U.S.) was used as a positive control and was prepared in a same way as the tested compounds.

Cytotoxicity assay

In order to determine the cytotoxic effect of the tested glucopyranosyl derivatives, the viability of PK-15 cell was measured 24 hrs post-treatment by crystal violet assay as previously described [9]. Briefly, PK-15 cells (1×10^6 cells/plate) were seeded in 96-well flat-bottomed tissue culture plates (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, U.S.A.) in the absence or presence of the individual compounds diluted in MEME medium at 37°C for 24 hrs in a 5% CO_2 humidified atmosphere. The initial concentration of each individual compound applied to the PK-15 monolayer was $2*10^{-1}$ mg/mL. The

Table 1. *The fifty percent cytotoxic concentration (CC_{50}) of glucopyranosyl derivatives of ketonucleosides to the PK-15 cells. Ganciclovir was used as a positive control.*

Compound	CC_{50} (mg/ml)
1	0,1
2	0,1
3	0,1
Ganciclovir	0,1

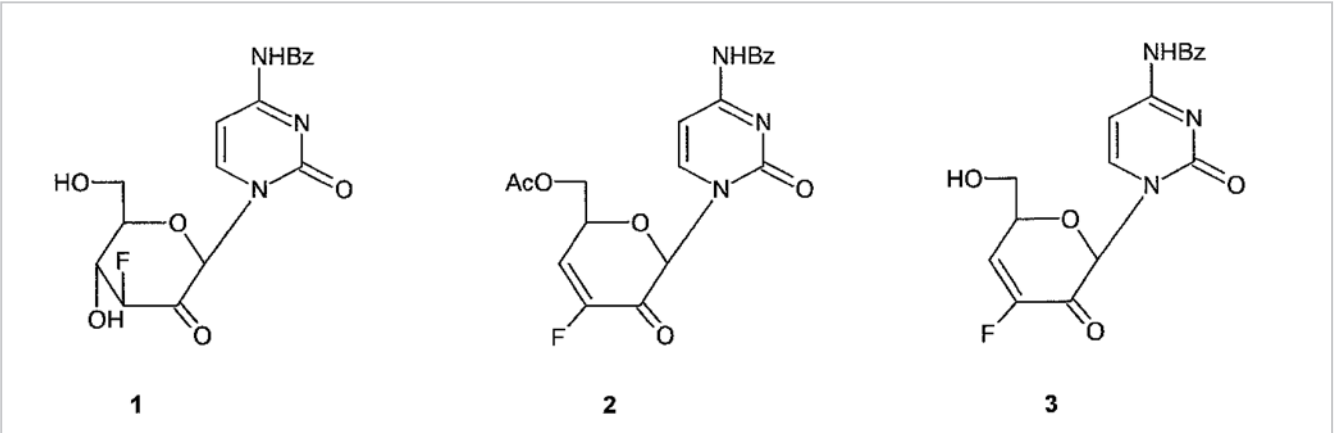

Figure 1. *3-fluoro-2-keto-β-D-glucopyranosyl derivatives of N4-benzoyl cytosine possessing the fluoro group in 3´-position and the keto group in 2´-position of the sugar moiety (Manta et al., 2007b). Compounds* ***2*** *and* ***3*** *have a carbon-carbon double bound in α,β disposition to the keto group of the sugar moiety instead of a free hydroxyl in 4´-position that is present in compound* ***1****.*

compounds were diluted twofold to a concentration of $1,56*10^{-3}$ mg/mL and tested at each individual dilution. At the end of the incubation period, the medium was discarded and the cells stained with 1% crystal violet. After a 2 min incubation at room temperature, the plates were washed with tap water and de-stained with 10% (v/v) acetic acid. Absorbance of each well at 590 nm was measured by an automatic microplate reader (Multiskan, Thermo Electron Oy, Vaanta, Finland). The absorbance of the control wells, which contained no compound, was regarded as 100%, and the percentage absorbance for each well that contained compound was calculated. Cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}), which is the lowest concentration of a tested compound capable of inhibiting the growth of PK-15 cells by 50%.

Antiviral assay

An antiviral assay was used to determine the reduction of virus propagation after incubation of virus-infected PK-15 cells in the presence of the fluoro-glucopyranosyl derivatives. PK-15 cells were seeded onto a 24-well plate ($14,4*10^6$ cells/plate). After 24 hrs of incubation, a confluent monolayer was obtained which was then washed with MEME only. After that, pseudorabies virus stock diluted in MEME was added to achieve a multiplicity of infection (MOI) of 10 per cell. Following the adsorption step (1 hr at room temperature) to allow virus to attach to the cells, non-bound virus was removed by additional washing of the cell monolayer with MEME. Then, each individual compound or ganciclovir (diluted to the initial concentration of 0,1 mg/mL in MEME containing 10% FBS) were applied to the individual infected monolayer. Wells containing virus only and wells with culture medium only were used as controls.

At the zero point (t=0 hrs), after the washing step and the addition of media with/without the tested compound, an aliquot of cell supernatant from each well was collected and stored at −70°C for future detection of any remaining virus particles. The plates were further incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 hrs. After 24 hrs (t=24 hrs), cell supernatants were collected from each well to determine the potential inhibition of virus propagation (antiviral activity) by limited dilution assay. Virus titer reduction was calculated in comparison with the virus titer in the nontreated wells (virus-infected cells only)

Endpoint dilution assay

The antiviral activity of the glucopyranosyl derivatives and ganciclovir was evaluated by the modified endpoint dilution assay described by Kärber [10]. Briefly, 10-fold dilutions in MEME medium (10^{-1} to 10^{-11}) of each collected cell supernatant (t=0 hrs and t=24 hrs) were made and added to the PK-15 cells seeded in a 96-well plate 4 hrs before the experiment at a concentration of 3×10^6 cell/10 mL in the presence of MEME containing 20% FBS. Three days of incubation took place at 37°C in a 5% CO_2 humidified atmosphere. Next, the medium was discarded and the plates stained with crystal violet as described above.

RESULTS

Cytotoxicity of fluoro-glucopyranosyl derivatives of ketonucleosides

In order to find fluoro-glucopyranosyl derivative concentrations that were not toxic to cells for use in the virus reduction assays, cytotoxicity assays were performed. When the cells were treated at concentrations from 0,1 mg/mL to $7,8125x10^{-4}$ mg/mL for 24 hrs, the ketonucleoside fluoro-glucopyranosyl derivatives showed low cytotoxicity even at the highest concentration (Table 1). Therefore, the initial concentration of 0,1 mg/mL was used in antiviral assays for all tested compounds, including ganciclovir.

Antiviral activity against pseudorabies virus

To investigate the reduction of the virus propagation

in infected PK-15 cells in the presence of glucopyranosyl derivatives of ketonucleosides or ganciclovir (CC_{50} = 0,1 mg/mL), limited dilution assays were performed. As shown in table 2, compound **2** had the highest potential to reduce virus propagation in the PK-15 cells at a concentration of 0,1 mg/mL. A 32-fold reduction in viral titer was observed after the 24 hr incubation period (Figs. 2-4). Ganciclovir, which was used as a positive control, showed much less antiviral activity (Table 2). Interestingly, the reductions in viral titers were observed for compound **2** and ganciclovir at the t=0 (Table 2). Antiviral activity was observed after 24 hrs of treatment, at which point compound **2** reduced viral DNA synthesis by 1,7 fold compared to the control (virus-infected cells only) and ganciclovir (data not shown).

DISCUSSION

We studied the potential inhibitory effect of fluoroglucopyranosyl derivatives of ketonucleosides on DNA viruses. We have shown in our previous studies that derivatives of ketonucleosides are potential antiviral drugs against the RNA viruses [3-6]. Moreover, we reported that a number of new generation unsaturated ketopyranosyl nucleoside derivatives proved to be more efficient in rotavirus inhibition and antitumor growth compared to AZT and to 5FU, respectively [3-6].

We therefore wanted to investigate whether these compounds also act against DNA viruses. In our model, pseudorabies virus was used as the DNA virus and the pig kidney epithelial cell line (PK15) was used as the host cell, having been chosen for its high susceptibility to viral infection and propaga-

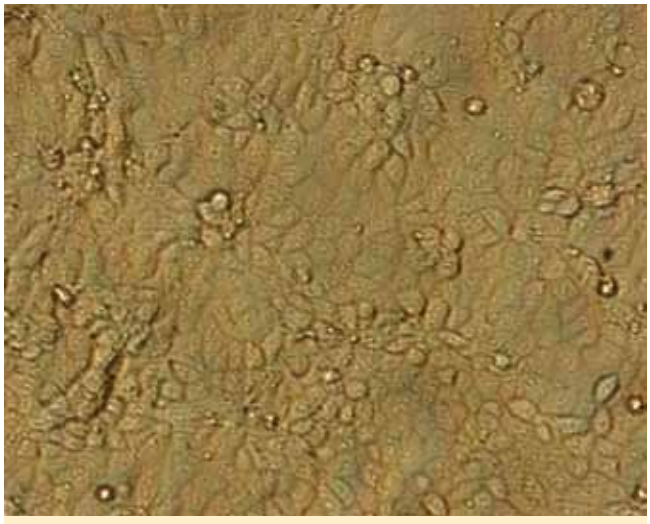

Figure 2. *Non-infected PK-15 cells (control) after 24h incubation. Magnification 100X*

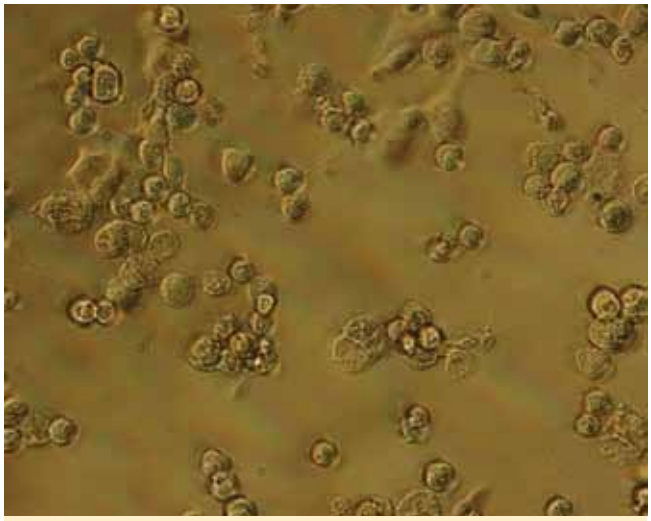

Figure 3. *PK-15 cells infected with Pseudorabies virus at MOI 10; 24 post infection. Magnfication 100X.*

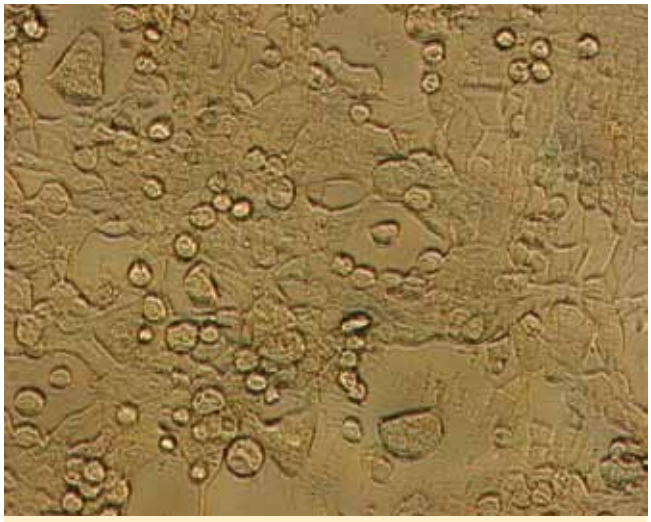

Figure 4. *Antiviral activity of substance 2 (c= 0.1 mg/ml). Cells are protected from Pseudorabies virus infection. Almost no lytic effect can be observed after 24 h period.post infection. Magnfication 100X.*

Table 2. *Virus titer reduction.*

compound	MOI 10* + compound at t= 0h (U/ml)	n-fold virus titer reduction	MOI 10* + compound at t= 24h (U/ml)	n-fold virus titer reduction
Virus infected cells only	1*10 (4)	0	3,16*10 (8) U/ml	0
1	1*10 (4)	0	3,16*10 (7)	1*101
2	3,16*10 (3)	3,16	1*10 (7)	3,16*101
3	1*10 (4)	0	1*10 (9)	0
Ganciclovir	3,16*10 (3)	3,16	3,16*10 (7)	1*101

MOI10* = Multiplicity of infection

tion (Fig.2,3). PK-15 cells were infected with pseudorabies virus at a multiplicity of infection of 10 (MOI10) and treated with fluoro-glucopyranosyl derivatives of ketonucleosides or ganciclovir (0,1 mg/mL) for 24 hrs. The antiviral effect, determined by the inhibition of virus replication, was determined by endpoint dilution assay.

It was clear that 1-(6-*O*-acetyl-3,4-dideoxy-3-fluoro-β-D-glycero-hex-3-enopyranosyl-2-ulose)-N^4-benzoyl cytosine (compound **2**) had antiviral activity, and a reduction in virus titer was observed after a 24 hr incubation period (Table 2, Fig. 4). There was more virus reduction with compound **2** than with ganciclovir, a known potent inhibitor of the herpes viruses, including CMV (Table 2). To ascertain whether the mechanism of antiviral action of the fluoro-glucopyranosyl derivatives of ketonucleosides included inhibition of viral DNA synthesis, quantification of de-novo synthesis of viral DNA in infected cells was performed (data not shown). We found that 0,1 mg/mL of compound **2** caused a significant decrease in the amount of viral DNA (Table 3) compared to the control (virus-infected cells only) and to ganciclovir.

It could well be that the presence of the carbon-carbon double bond in α,β disposition to the keto group of the sugar moiety enhances activity and that derivatives with 6′-CH_2OR are better able to inhibit viral DNA synthesis than derivatives with free CH_2OH. Although decreased synthesis of DNA suggests that one antiviral mechanism of action of compound **2** is interference with viral DNA synthesis, other mechanisms may be involved, as significantly higher reductions in virus titer were observed by endpoint dilution assay (Table 2). The mechanism of action of ganciclovir against CMV is inhibition of viral DNA replication of by ganciclovir-TP (De Clercq, 2001). Ganciclovir is metabolised in the cell to the triphosphate form by deoxyguanosine kinase, and this is induced by CMV infection, guanylate kinase and phosphoglycerate kinase. The selective antiviral response associated with ganciclovir treatment is achieved because of its much weaker actions on cellular DNA polymerases [11]. We may speculate that similar mechanisms are involved in the actions of compound **2**, and that activity is enhanced by its specific structure. Surprisingly, ganciclovir stimulated the synthesis of viral DNA in infected cells after 8 hrs of treatment (Table 3); it therefore seems that other pathways may be switched on in favor of DNA synthesis of pseudorabies virus in PK-15 cells, pathways that take advantage of ganciclovir's intracellular metabolites.

We can conclude that of the compounds we tested, including ganciclovir, 1-(6-*O*-acetyl-3,4-dideoxy-3-fluoro-β-D-glycero-hex-3-enopyranosyl-2-ulose)-N^4-benzoyl cytosine (compound **2**) was the most potent inhibitor of pseudorabies virus replication in PK-15 cells. Its mechanisms of antiviral activity may include direct inhibition of virus DNA synthe-

sis but other mechanisms, most probably involving proteins in the virus replication cycle (e.g. cyclin-dependent kinases), may be involved [12].

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