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Inhibition of adenovirus multiplication by inosine pranobex and interferon α *in vitro*

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Abstract

There are no specific antivirals designed for adenoviral infections. Due to many cases of adenovirus infections worldwide, epidemic nature of some types of adenoviruses, and growing number of patients with severe adenoviral infections resulting from dysfunction the immune system, the need for searching an effective and safe therapy is increasing. Inosine pranobex exerts antiviral effects which are both direct and secondary to immunomodulatory activity. In the present study we evaluated in vitro effect of inosine pranobex and interferon α (IFN-α) on replication of HAdV-2 and HAdV-5. The effectiveness of inosine pranobex under these conditions has not been previously reported. In conducted study we reported that inosine pranobex reduced the titer of infectious HAdV-2 and HAdV-5 in vitro. Higher concentrations of IP strongly inhibited multiplication of viruses. Combination of inosine pranobex and IFN-α display higher efficacy than either treatment alone and suggest that both agents may increase therapeutic effectiveness without augmenting toxic effects. Combination index calculations showed that inosine pranobex and INF-α synergistically inhibit HAdV-2 and HAdV-5 titers in A549 cells.

Key words: *adenovirus, antiviral drugs, inosine pranobex, interferon α.*

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Introduction

Human adenoviruses are DNA viruses that can cause a broad range of clinical syndromes, including asymptomatic viremia, respiratory tract infections, ocular disease, gastroenteritis, diarrhea, and cystitis. In contrast to a mild disease observed in normal hosts, adenoviral infections in immunocompromised people tends to disseminate, and the virus is isolated from multiple body sites, including the lung, liver, gastrointestinal tract, and urine. In individuals with an impaired immune response, life-threatening adenovirus infections are common [1-3]. There are no specific antivirals designed for adenoviral infections. In patients with a suppressed or deficient immune system, administration of cidofovir can be a therapeutic option but the agent can cause serious side effects and is also expensive. The major side effect associated with cidofovir therapy is renal toxicity, so it is reserved only for severe cases. Currently, there is no vaccine to prevent adenovirus infections [2, 4]. Due to many cases of adenovirus infections worldwide, epidemic nature of some types of adenoviruses, the growing number of patients with an impaired immune system (e.g. transplant recipients and AIDS patients), the need for searching an effective and safe therapy is increasing [1, 3, 5]. It is well known that the immune system can be manipulated specifically by vaccination or non-specifically by immunomodulation. For many years, a diverse array of recombinant, synthetic, and natural immunomodulatory preparations for prophylaxis and treatment of various infections are available. Many of them augment the anti-infectious immunity, especially cellular immune response. The immune-based therapies in combination with e.g. interferons can be one of the valuable therapeutic options [6-10]. The immunostimulating properties of inosine pranobex (Isoprinosine, 9-[3R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one: 4-acetamidobenzoic acid: 1-(dimethylamino)propan-2-ol) were well documented. Besides its immunomodulatory effect, this drug has also antiviral and antitumor properties. Inosine pranobex is a purine nucleoside that is involved in a wide variety of intracellular biochemical processes. The mechanism of action in the human body is still unclear but numerous studies have demonstrated that this drug inhibits viral replication and exhibits a pleiotropic effect [9, 11, 12]. Inosine pranobex can augment the production of cytokines such as interleukin-1 (IL-1) and interleukin-2 (IL-2). It increases the production of interleukin 12 (IL-12), interferon γ (IFN-γ) and decreases interleukin-3 (IL-3) and interleukin-4 (IL-4) production *in vivo*. Inosine pranobex normalizes the cell-mediated immunity stimulating T-cell differentiation and stimulates differentiation of B-lympho-

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cytes into plasma cells and enhances antibody production. Inosine pranobex potentiates neutrophil, monocyte, macrophage chemotaxis and phagocytosis, augments NK activity [11-15]. This immunomodulator has been found to be useful in the treatment of several viral diseases such as herpes simplex, herpes zoster, rhinovirus infections, influenza, genital warts, and EBV infection [11, 12]. In the past, there were attempts to use inosine pranobex for the treatment of immunopathological disorders, such as rheumatoid arthritis and alopecia aerate. A combination of inosine pranobex and interferon α (IFN-α) appears to be effective treatment in subacute sclerosing panencephalitis (SSPE) – the persistent measles complication of the central nervous system [11, 16, 17].

Objective

The aim of this study was to evaluate *in vitro* inhibition of HAdV-2 and HAdV-5 replication by inosine pranobex (IP) alone and in combination with IFN-α.

Material and methods

Compounds

Inosine pranobex (Isoprinosine, IP) was kindly provided by Gedeon Richter (Poland). Shortly before experiments, stock solutions of IP were prepared in culture medium (1.0 mg/ml), filtered (0.2 µm pore size) (Filtropur S 0.2, Sarstedt, Germany), and adequate volumes of the stock solution were added to cell cultures to obtain final concentrations ranging from 50 to 800 µg/ml. These concentrations of IP were found non-toxic in preliminary experiments. Interferon (IFN-α-2a) was purchased from Roche and used at final concentrations of 1000 and 2000 IU/ml. Doses of IFN-α were chosen based on studies of other authors [18].

Viruses

The viral strains used in this study were as follows: Human Adenovirus type 5 (HAdV-5); ATCC VR-5 and Human Adenovirus type 2, wild-type (HAdV-2) strain 72. Both adenoviruses were propagated on A549 cells. They were harvested when the cytopathic effect reached more than 95%, by freezing (–80°C, 10 min) and thawing (room temperature) the cell-culture flasks three times. The supernatant was cleared by centrifugation (3000 × g for 5 min) and stored at –80°C for further use.

Cell lines

Cells of the A549 cell line (human lung adenocarcinoma epithelial cells, ATCC, CCL185) and HEp-2 (human larynx carcinoma, ATCC, CCL-23) maintained in Eagle's Medium Essential Medium (Biomed, Poland) containing 10% fetal bovine serum (Gibco Life Technologies, UK) and 1% penicillin/streptomycin antibiotics (Gibco Life Technologies, UK). HEL 299 cells (primary human lung fibroblasts, ATCC, CCL-137) were cultured in Dulbecco's Modified Eagles Medium (D-MEM, Sigma-Aldrich, USA) containing 10% fetal bovine serum (Gibco Life Technologies, UK) and a mix of the antibiotic-antimycotic mentioned above. Each cell line was cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assay

This test was performed using HEp-2, HEL 299 and A549 cells. The cytotoxic effect of IP (at doses of 50-800 µg/ml) and IFN-α (at a concentration of 1000 and 2000 IU/ml) was assessed visually using light, inverted microscopy Olympus CK2 (Olympus Corp., Germany) and by the MTT colorimetric assay. The assay determines the ability of viable cells to convert a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate [19]. Cells were incubated in flat-bottom 96-microwell plates (2×10^4 cells/0.2 ml). After incubation of cells for 24 h, IP and/or IFN-α were added (at doses given above) and cultured further for 24 and 48 h. In controls, the cells were cultured without any of the tested drugs. Absorbance values of examined samples were read spectrophotometrically at a wavelength of 490 nm on a reader (Reader 230, Organon Technica Turnhout, Belgium). All experiments were performed in triplicates.

Antiviral assay

Antiviral effects of IP and IFN-α were assessed *in vitro* by phenotypic assays. The antiviral activity of inosine pranobex was tested using a series of non-toxic concentrations (50-800 µg/ml) diluted in an assay medium. Interferon α was used at final concentrations: 1000 and 2000 IU/ml. To investigate the antiviral activity, cell cultures in flat-bottom microwell plates (2×10^4 cells/0.2 ml) were infected with HAdV-2 and HAdV-5 (0.01 $TCID_{50/cell}$) for 60 min. at 37°C. After the absorption of the virus, inoculum was removed and fresh culture medium containing IP, IFN-α or both agents was added. Next, the cells were incubated for 48 h. The yield reduction assay (YRA), which evaluates the ability of the compounds to inhibit virus multiplication in cell cultures, was applied. The cytopathic effect of the virus was evaluated 48 h after infection of cultures with viruses by means of light, inverted microscopy. The Reed-Muench statistical method was used to determine the 50% end point (IC_{50}) which was the lowest concentration of the tested drugs that reduces the viral infections of the control to a 50%. The antiviral effect was estimated according to the reduction of the adenovirus titer in the presence of compounds with the controlled one. $TCID_{50/ml}$ (median tissue culture infective dose) was calculated.

$TCID_{50/ml}$ denotes the amount of a pathogenic agent that will produce pathological changes in 50% of cell virus-inoculated cultures.

Statistical methods

The Wilcoxon signed rank sum test was used to analyze differences between the IFN-α/IP-treated virus-infected cells and the control group in terms of efficacy to reduce the viral titer. A value of $p < 0.05$ was considered as statistically significant. Drug interactions were examined by the method of Chou and Talalay. The combination index (CI) was calculated to determine whether the drugs synergize. Combination index < 1 indicates synergism between the drugs, CI = 1 indicates additivity, and CI > 1 denotes antagonism [20].

Results

Cytotoxicity assay

In the present study, the cytotoxic effect of IP and IFN-α was assessed using three cell lines (A549, HEp-2, and HEL 299) exposed to different concentrations of each compound (IP: 50-800 μg/ml and IFN-α: 1000 and 2000 IU/ml) for 48 h. The cytotoxicity was evaluated microscopically and by the MTT assay. There were no morphological changes, as assessed visually, in cell cultures treated with either IP or IFN-α. MTT cytotoxicity assay confirmed microscopic observations. The viability of cells in the presence of the tested compounds was higher than 95%. Since both IP and IFN-α were nontoxic at all concentrations, TC_{50} (toxic drug concentration which caused the reduction of the viable cell number by 50%) and SI (Selectivity Index; CC_{50} to IC_{50} value) were not calculated.

Antiviral assay

The effect of IP and IFN-α on reduction of HAdV-2 and HAdV-5 titers was investigated in A549 cell culture. Inosine pranobex reduced the titer of infectious HAdV-2 and HAdV-5 *in vitro*. Higher concentrations of IP strongly inhibited multiplication of viruses. A summary of the inhibitory effects of IP on virus replication in A549 cells is shown in Fig. 1.

When 1000 IU/ml IFN-α alone was added to cultures of HAdV-2- and HAdV-5-infected cells, only a weak antiviral effect was detected. Interferon α at a concentration of 2000 IU/ml caused about 2 log reduction in titers of HAdV-2 and about 1 log reduction in titers of HAdV-5 compared to that of the control. When examined adenoviruses were exposed to 800 μg/ml of IP, reduction in virus titers was more than 1.5 log in comparison to the control (no exposure to IP). Enhanced antiviral activity was observed when infected cells were treated with IFN-α and IP simultaneously. The reduction of the average viral titers of

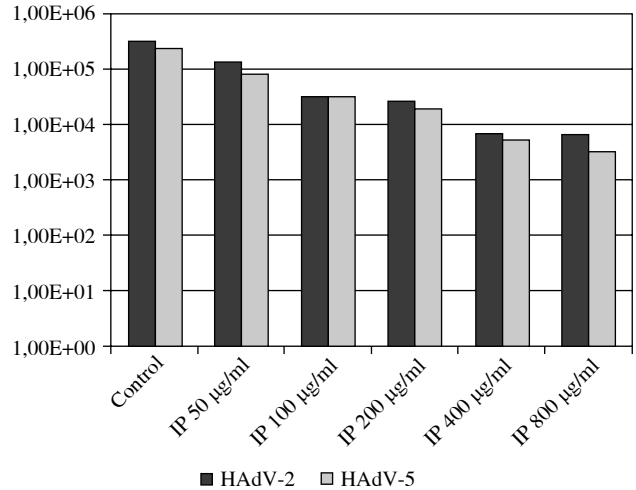

Fig. 1. Titer of the viral stock ($TCID_{50/ml}$) in cultures of HAdV-2 and HAdV-5-infected A549 cells incubated with inosine pranobex (IP)

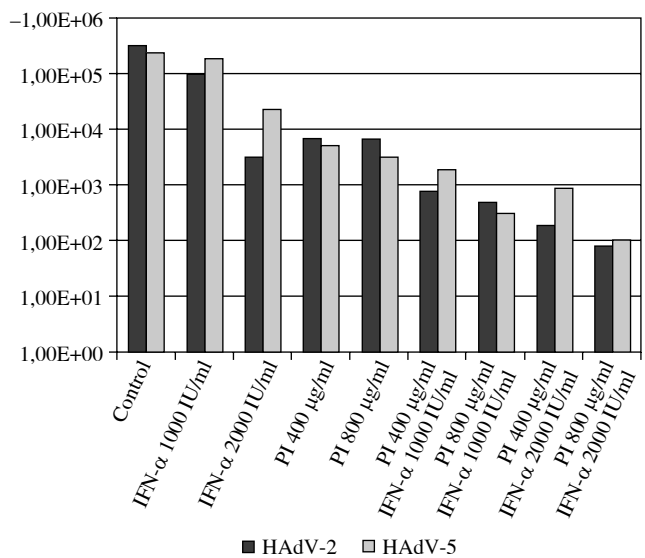

Fig. 2. Reduction of HAdV-2 and HAdV-5 titers in cultures of A549 incubated with inosine pranobex (IP) and/or interferon α (IFN-α)

HAdV-2 and HAdV-5 in A549 cell culture after applying 800 μg/ml IP and IFN-α (1000 IU/ml), in comparison to the viral titer in the control was reduced more than 2.5 log (Student's *t*, $p < 0.05$). The increase in the dose of interferon to 2000 IU/ml, still maintaining the concentration of 800 μg/ml IP, enhanced the inhibitory effect in the case of both viruses. The average HAdV-2 titer was reduced by about 2.0 log and HAdV-5 titer was reduced more than 1.0 log in comparison to the viral titer after exposure to IP (800 μg/ml) alone (Fig. 2) (Student's *t*, $p < 0.05$). Combi-

Table 1. IC_{50} values after application of inosine pranobex (IP) and interferon α (IFN-α), alone or in combination, in virus-infected cultures of A549 cells

Virus	HAdV-2	HAdV-5
	IC_{50} [μg/ml]	
IP (50-800 μg/ml)		
IC_{50}	1743.8	1304.5
IC_{50} (max.-min.)	2591.1-1264.8	1678.0-1042.0
IP (50-800 μg/ml)/IFN-α (1000 IU/ml)		
IC_{50}	963.2	694.8
IC_{50} (max.-min.)	1006.7-922.2	1050.9-474.4
IP (50-800 μg/ml)/IFN-α (2000 IU/ml)		
IC_{50}	926.1	683.1
IC_{50} (max.-min.)	1044.0-825.4	927.1-512.1

nation of IP and IFN-α display higher efficacy than either treatment alone and may increase the therapeutic effect without augmenting toxic effects. Results are statistically significant for HAdV-2- and HAdV-5-treated A549 cell cultures simultaneously with 400 μg/ml and 800 μg/ml of IP and IFN-α (Wilcoxon signed-rank test, $p < 0.05$).

The antiviral activity of the tested compounds was also analyzed on the basis of IC_{50} values (Table 1).

The IC_{50} value of IP against HAdV-2 was 1743.8 μg/ml. The IC_{50} value of IP alone against HAdV-5 was 1304.5 μg/ml. When infected cells were treated with IFN-α and IP in combination, an enhanced antiviral activity was found. Application of IFN-α (1000 IU/ml) with IP after infection of A549 cells with adenoviruses reduced the IC_{50} to 963.2 μg/ml (HAdV-2) and 694.8 μg/ml (HAdV-5). The combination of 2000 UI/ml of IFN-α and IP resulted in an even higher reduction of anti-adenoviral activity, by approximately 50%. Finally, we reported that a combination of IP and IFN-α increases its antiadenoviral potency *in vitro*. Combination index calculations showed that IP and IFN-α synergistically inhibit HAdV-2 (CI: 0.80), and HAdV-5 (CI: 0.73) titers in A549 cells.

Discussion

Common occurrence of serious viral infections in both immunocompetent and immunocompromised patients and paucity of available antiviral drugs necessitate search for new treatment options [21-24]. One of them is searching new applications for currently available drugs and co-administration of them to elicit an additive or even synergistic effect [22, 25]. The first antiviral therapies became available about 50 years ago. There are now more than 50 licensed antiviral compounds, half of them are used in the treatment of HIV infection. Hot topics in antiviral research are, besides HIV, HBV, HCV infections and influenza [21, 26]. The high cost and lengthy procedures of the drug discovery process are one of the major limiting factors in the development of new drugs. The fact that antiviral drugs are highly specific and are used for one single laboratory confirmed viral infections, significantly limits the number of diseases of commercial interest. Now, the pharmaceutical industry is not interested in the synthesis of new specific antiadenoviral drugs, so there are no possibilities for specific treatment in the near future [21, 25, 26].

Although little is known about the mechanism of action of IP, limited data have been published up to now on the bioassay and pharmacokinetics, but numerous studies have shown its ability to potentiate the cellular immune response both *in vitro* and *in vivo* experiments. Inosine pranobex was found to have a broad spectrum of antiviral activity, including reducing symptoms of human papillomavirus infection, genital herpes and some of viral respiratory infections [9, 11]. Therefore, we decided to evaluate its effect in inhibiting the replication of adenoviruses. The effectiveness of IP under these conditions has not been previously reported. The available evidence indicates that IFN severely inhibits the replication of several viruses. Interferons are widely used for the treatment of chronic viral infections, mainly caused by hepatitis B virus and hepatitis C virus [8, 17]. A combination of IFN-α and Isoprinosine has brought positive results in the treatment of a persistent and chronic encephalitis secondary to measles virus infection – subacute sclerosis panencephalitis (SSPE). Such combined therapy is still recommended because of theoretical synergistic effects [11, 16, 17]. In the conducted study we reported that IP reduced the titer of infectious HAdV-2 and HAdV-5 *in vitro*. Enhanced antiviral activity was observed when infected cells were treated with IFN-α and IP simultaneously. The combination of 2000 UI/ml IFN-α and IP resulted in reduction of anti-adenoviral activity, by approximately 50% in comparison to the control. Finally, we reported that IP and IFN-α synergistically inhibit HAdV-2 and HAdV-5 titers in A549 cells without augmenting toxic effects.

Conclusions

In the present study we demonstrated *in vitro* inhibition of HAdV-2 and HAdV-5 replication by IP and IFN-α. Our findings have shown that a combination of IP and IFN-α display higher efficacy than either treatment alone and may increase the therapeutic effect without augmenting toxic effects. The effectiveness of IP under these conditions has not been previously reported. Hence, this application of IP and interferon warrants further investigation to confirm the inhibitory effect *in vivo*.

The authors declare no conflict of interest.

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