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In Vitro Anti-Rhinovirus Activity of Some Picornavirus Replication Inhibitors

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Abstract

The effect of several antiviral substances with different mode of action on the replication of human rhinovirus 14 (HRV-14) is the topic of the present study. Monolayer cultures of human cervical carcinoma (HeLa Ohio-I) cells in 96-well tissue culture plates were used. The antiviral effect against three different viral inoculation doses was tested by the neutral red uptake procedure in a CPE-inhibition setup. The following compounds were tested: ribavirin, arildone, disoxaril, S7, PTU-23, HBB and oxoglaucine (a newly characterized in this laboratory compound efficient against enteroviruses). Two of the compounds, HBB and oxoglaucine, showed the highest activity with a selectivity ratio (CC_{50}/IC_{50}) above 100 for the lowest viral inoculation dose of 100 $CCID_{50}$. Ribavirin and disoxaril occupied intermediate position according to their antiviral effect, while the effect of arildone, PTU-23 and S-7 was not significant.

Key words: Rhinovirus H14; Ribavirin; Oxoglaucine; HBB; Disoxaril

Резюме

Предмет на настоящето проучване е ефектът на серия антивирусни вещества с различен начин на действие върху репликацията на човешки риновирус 14 (HRV-14). Използвани бяха монослойни култури на клетки от човешки цервикален карцином (HeLa Ohio-I) в 96-ямкови тъканно-културални плаки. Антивирусният ефект на три различни вирусни дози бе изпитан по метода на инхибиране на цитопатичния ефект чрез процедурата на поглащане на неутрално червено. Тестирани бяха следните съединения: рибавирин, арилдон, дизоксарил, S7, PTU-23, HBB и оксоглауцин (ново съединение, охарактеризирано в нашата лаборатория като ефикасно срещу ентеровируси). Две от съединенията, HBB и оксоглауцин, показаха най-висока активност с индекс на избирателност (CC_{50}/IC_{50}) над 100 при най-ниската вирусна инокулационна доза 100 $CCID_{50}$. Рибавирин и дизоксарил заеха междинна позиция според антивирусния им ефект, докато ефектът на арилдон, PTU-23 и S7 бе статистически недостоверен.

Introduction

Picornaviruses are the most common cause of viral illness worldwide (Rotbart and Hayden, 2000). This family of small single-stranded RNA viruses currently comprises nine genera, including *Enterovirus* and *Rhinovirus*. In 2004, a proposal was submitted to the International Committee on Taxonomy of Viruses to merge the genera *Rhinovirus* and *Enterovirus* into a single genus, *Enterovirus*, with the species in it remaining intact and under the banner of a new virus order, *Picornavirales* (Le Gall *et al*., 2008). Human rhinoviruses (HRVs) comprise over 150 different virus serotypes. HRVs are the predominant cause of viral upper respiratory tract infections and particularly of common cold (Arruda *et al*., 1997; Couch, 2001; Turner, 2001)

These infections are often mild and self-limiting; nevertheless, they have a significant socioeconomic impact (Patick, 2006). Increasing evidence also describes the link between HRV infection and more serious medical complications like acute otitis media, sinusitis, pneumonia and bronchiolitis in infants and young children. Rhinoviral infection commonly causes exacerbations of the pre-existing airways disease in those with asthma, chronic obstructive pulmonary disease or cystic fibrosis, (Pitkaranta and Hayden, 1998; Bardin, 2004; Gern, 2004; Hayden, 2004; Tan, 2005; Khetsuriani *et al.*,

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2007). Among elderly people, infants and highly immunocompromised hosts, HRV infections are associated with morbidity of the lower respiratory tract and rarely mortality (Pitkaranta and Hayden, 1998; Hayden, 2004) .

To date no effective antirhinoviral chemotherapy has been approved for clinical use and the treatment of these infections is limited to symptomatic therapy only (Savolainen *et al*., 2003; Turner, 2005; Patick, 2006).

In this study we describe the results of cell culture studies on the anti-rhinovirus activity of seven picornavirus replication inhibitors: HBB (2-α-hydroxybenzyl-benzimidazole), oxoglaucine, ribavirin, disoxaril, arildone, PTU-23 (N-phenyl-N'-3-hydroxyphenylthiourea) and S-7 (ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate).

Materials and Methods

Cells

Human cervical epithelioid carcinoma (HeLa Ohio-I) cells were a kind gift of Dr. D. Barnard (Utah State University, Logan, USA). The cells were grown in minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), sodium hydrogen carbonate at a final concentration of 25 mM, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 50 IU of penicillin, 50 μg/ml of streptomycin and 50 μg/ml of gentamicin in a 5% CO_2 incubator HERA cell 150 (Heraeus, Hanau, Germany).

Virus

Human rhinovirus type 14 (strain 1059) (HRV-14) was used for the experiments described. The virus was purchased from the American Type Culture Collection (Manassas, VA, USA). HRV-14 stocks were prepared in HeLa Ohio-I cells in a maintenance medium. The maintenance medium was like the above described growth medium except for the serum which was reduced to 2 %. Stock virus titer was $10^{6.5}$ $CCID_{50}$/ml.

Compounds

Seven compounds with different mode of action were tested. These were HBB (2-α-hydroxybenzyl-benzimidazole), (a gift from Dr. T. Dmitrieva, Moscow State University, Moscow, Russia); oxoglaucine (Dr. S. Philipov, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria); ribavirin (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide), (a gift from Prof. R. W. Sidwell, Utah State University, Logan, USA); disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy] heptyl]-3-methylisoxazole; WIN 51711), (Sanofi Winthrop, Inc., PA); PTU-23 (N-phenyl-N'-3-hydroxyphenylthiourea), originally synthesized by Prof. G. Vassilev (Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia); arildone (4-[6-(2-chloro-4-methoxy-phenoxy)-hexyl] -3,5-heptanedione), (Sterling Research Group, Sterling Drug, Inc., USA); and S-7 (ethyl 2-methylthio-4-methyl-5-pyrimidine, carboxylate), (a gift from Dr. P. La Colla, University of Cagliari, Cagliari, Italy). All drugs, with the exception of ribavirin and PTU-23, were prepared as stock solutions in dimethyl sulfoxide (DMSO) and then diluted in the maintenance medium to the required concentrations. Ribavirin was dissolved directly into the medium and PTU-23 was initially dissolved in ethanol and then in the test medium. In the experiments 0.5 log_{10} dilutions of the compounds were used.

Virus assay

The virus titer was quantified in a 50% cell culture infectious dose ($CCID_{50}$) assay following the endpoint dilution design (Reed and Muench, 1938). HeLa Ohio–I cells were seeded into 96-well tissue culture microplates at 4 x 10^4 cells/well in Minimal Essential Medium (MEM) followed by overnight incubation at 37°C. The growth medium was then removed and serial 10-fold dilutions of virus were added (100 μl/well; eight wells per dilution). After 2h of adsorption at 33°C, excess virus was removed and 0.1 ml of maintenance medium was added to each well. The virus titer was presented as log_{10} of 50% cell culture infectious dose ($CCID_{50}$/ml) by visual recording of the virus cytopathic effect (CPE) following 72h of incubation at 33°C.

Antiviral tests

The end-point dilution method in the multi-cycle cytopathic effect (CPE) inhibition set up was used to assess the antiviral effect of the compounds. Confluent cell monolayers of HeLa Ohio-I in 96-well tissue culture microplates were infected with three viral inoculation doses 100, 1000 and 10 000 $CCID_{50}$ per well or mock infected with maintenance medium only. After 2h of adsorption at 33°C, excess virus was removed and medium containing 0.5 log_{10} dilutions of the test compounds or medium only for the toxicity control was added. Each drug concentration was assayed in quadruplicate. After 3 days of incubation at 33°C, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and then stained with neutral red. The plates were incubated for 3 h at 33°C in the dark

to allow the cells to absorb the dye. After rinsing and drying steps, ethanol/acetic acid solution was added to each well and plates were shaken on a microtiter plate shaker for 10 min until neutral red was extracted from the cells and formed a homogeneous solution. Absorbance at 540 nm was read with a microplate reader (Organon Teknika Reader, Anthos Labtec Instruments GmbH, Salzburg, Austria).

Cytotoxicity assay

Cytotoxicity tests were done in the same plate simultaneously with the antiviral tests. After formation of the cell monolayer, the growth medium was discarded and 0.1 ml containing 0.5 $\log_{10}$ dilutions of the test compounds diluted in maintenance medium was added. The results were read after 72 hour by neutral red uptake procedure.

Statistical analysis

Absorbance values obtained from the antiviral and cytotoxicity tests were expressed as percentage of untreated or uninfected controls and the 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentrations (CC_{50}) were calculated by regression analysis. Selectivity index (SI) was calculated using the formula $SI = CC_{50}/IC_{50}$.

Trials were carried out in quadruplicate in three to five independent experiments. Virus titers were calculated according to Reed and Muench (1938). Mean values and standard deviations, as well as IC_{50} and CC_{50} were calculated by regression analysis accomplished with Origin 7.5 computer program.

Results and Discussion

The cytotoxic concentrations 50 (CC_{50}) of all seven compounds tested for HeLa Ohio–I cell line were determined. Fig.1 represents the amount of viable cells as percent of the control depending on the concentration of each compound. As seen, S-7, HBB and ribavirin possess lowest cytotoxicity (CC_{50} values exceeding 4 mM), PTU-23 occupies intermediary position and disoxaril, arildone and oxoglaucine have CC_{50} values below 55 μM.

The activities of all tested compounds against HRV-14 are expressed as 50% inhibitory concentrations (IC_{50}). The results are determined from the dose-response curves obtained by the CPE inhibition test for each compound at three viral inoculation doses. The data are summarized in Table 1.

To estimate the selectivity of the antiviral action of tested compounds, the data for inhibitory concentration and cytotoxicity of each compound and viral inoculation dose are used. The calculated selectivity ratio is displayed in Table 2.

2-(α-hydroxybenzyl)benzimidazole (HBB) is a compound known for decades as a selective picornavirus inhibitor (Tamm and Eggers, 1962; Dmitrieva and Agol, 1974). It is known that this substance, at a concentration nontoxic to cells, inhibits the synthesis of viral RNA (Eggers and Tamm, 1961; Dmitrieva and Agol, 1974). Tamm and Eggers (Tamm and Eggers, 1962) have recognized the considerable variation in the susceptibility of different picornaviruses to the action of HBB. Thus, most enteroviruses are found to be sensitive to inhibition by HBB, whereas many rhinoviruses are found insensitive (Tamm, 1972). More recent studies on the mechanism of action of HBB have revealed that the compound interacts directly or indirectly with the nonstructural protein 2C (Hadaschik *et al.*, 1999). Although many rhinoviruses are found insensitive to it, HRV-14 is one of the few, which is inhibited by 200-220 μM of HBB (Gwaltney, 1968). In our study the effect of the compound against human rhinovirus 14 is confirmed but using a different approach, namely the neutral red uptake assay. The data presented in Table 1 show that concentrations of 44 μM, 128 μM and 179 μM are sufficient to protect 50% of the cells from 100, 1000 and 10 000 $CCID_{50}$, respectively. The inhibition depends on the concentration of the compound as well as on the viral inoculation dose. Compared to the results obtained for the other tested compounds, the effect of HBB is well pronounced and comparable to the data for the effect of oxoglaucine.

Oxoglaucine is an aporphinoid alkaloid isolated from the aerial parts of the plant *Glaucinum flavum* Cranz (Kuzmanov, 1992). It can be obtained synthetically from the main plant alkaloid (Philipov *et al*., 1998). This compound has been found to be active against a panel of 16 enteroviruses with some variations in the sensitiveness of the different enteroviruses (Galabov *et al.*, 1995; Nikolaeva-Glomb *et al*., 2008). The results summarized in Table 1 reveal the marked inhibitory effect of oxoglaucine on the replication of human rhinovirus 14. The compound inhibits all three virus inoculation doses tested. The values of selectivity ratio calculated for oxoglaucine and HBB (data are shown in Table 2) are very similar. The comparison between the antiviral effects of HBB and oxoglaucine allows the statement that oxoglaucine can be situated among the most effective antipicornaviral compounds.

Ribavirin is a potent antiviral agent active against different viruses. In some cases, this inhibition has transferred into clinical applications. Five distinct mechanisms have been proposed to explain

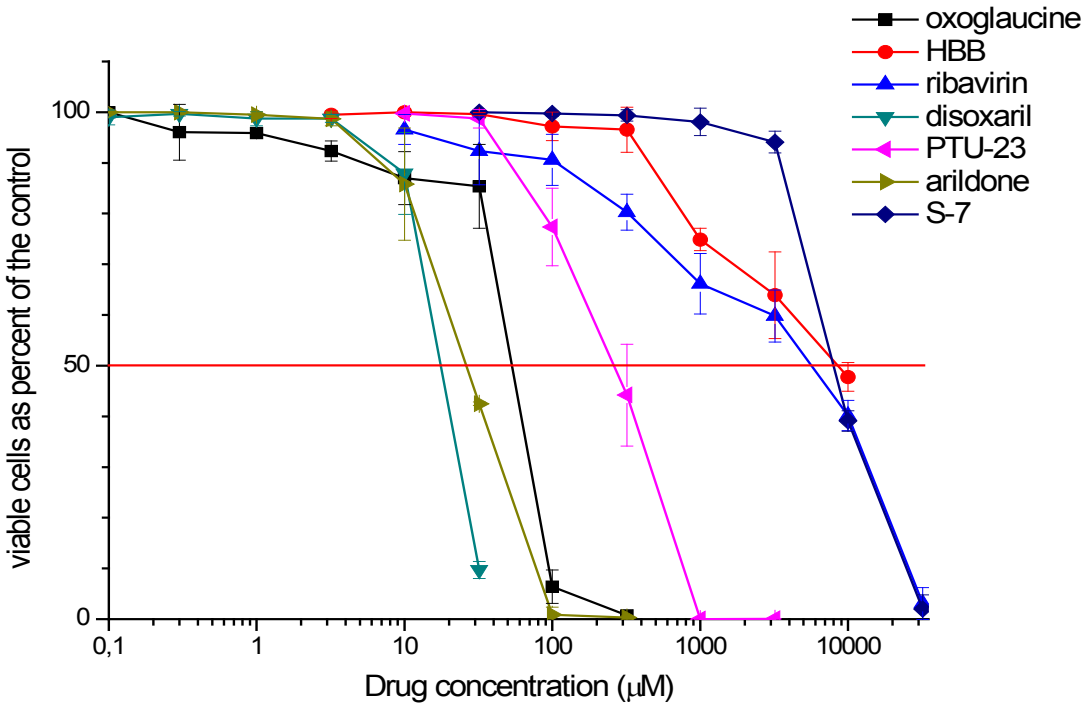

Fig.1. Cytotoxicity of HBB, oxoglaucine, ribavirin, disoxaril, PTU-23, arildone and S-7 determined according to the neutral red uptake procedure

Table 1. Anti-rhinovirus activity of seven antiviral agents

Viral inoculation dose	Antiviral activity (IC_{50} [μM])[a] of:						
	HBB	Oxoglaucine	Ribavirin	Disoxaril	PTU-23	Arildone	S-7
100 $CCID_{50}$	44	0.390	116	1.538	50	9.770	2484
1000 $CCID_{50}$	128	0.516	168	2.091	126	21.770	8516
10 000 $CCID_{50}$	179	1.234	841	3.334	407	12.121	-
Cytotoxic Conc.[b]	8345	53.461	4542	20.973	254	27.638	8590

[a] Each IC_{50} was the mean of two to four experiments
[b] The 50% cytotoxic concentration (CC_{50}) evaluated according to the neutral red uptake procedure

Table 2. Selectivity of antiviral action

Viral inoculation dose	Selectivity ratio (CC_{50}/IC_{50}) of:						
	HBB	Oxoglaucine	Ribavirin	Disoxaril	PTU-23	Arildone	S-7
100 $CCID_{50}$	189.66	137.08	39.16	13.64	5.08	2.83	3.46
1000 $CCID_{50}$	65.20	103.61	27.04	10.03	2.02	1.27	1.01
10 000 $CCID_{50}$	46.62	43.32	5.40	6.29	0.62	2.28	-

the antiviral properties of this compound. It seems likely that ribavirin does not have one universal mechanism of action, and inhibit different viruses in different ways (Parker, 2005; Graci and Cameron, 2006). Some authors have reported values of 10-100 μg/ml as minimal inhibitory concentrations for some rhinoviruses like types 1A, 2, 13 and 56 (Sidwell *et al.*, 1972; Huffman *et al.*, 1973). Our results for the effect of ribavirin on the replications of human rhinovirus 14 are very similar. The inhibitory concentration 50% varies between 100 and 800 μM depending on the virus inoculation dose. Compared to other compounds tested, ribavirin reveals a moderate anti-rhinoviral effect.

Disoxaril and arildone are WIN compounds which inhibit virus uncoating by direct insertion into the hydrophobic canyon within the VP1 capsid protein (Zeichhardt *et al*., 1987; Eggers and Rosenwirth, 1988). The effect of disoxaril on the replication of entero- and rhinoviruses is well known. Generally, the rhinoviruses are less sensitive to disoxaril than the enteroviruses (Otto *et al.*, 1985; Fox *et al.*, 1986). The data presented in our paper are coincident with the results reported by Otto (1985). Compared to HBB, oxoglaucine or ribavirin, disoxaril shows rather weak antirhinoviral effect. The antirhinoviral effect of arildone is not significant.

PTU-23 inhibits the synthesis of viral 37S RNA as a result of suppression of the synthesis of a viral protein with regulatory functions in the replicative cycle (Galabov, 1979; Galabov and Dmitrieva, 1983; Galabov *et al.*, 1983). In primary tests PTU-23 exhibited borderline activity against rhinovirus H-17 (Galabov *et al*., 1977). In our study the effect of this compound was tested against human rhinovirus 14. The effect observed was close to the results of the team of Galabov, and PTU-23 showed too weak effect on the replication of the tested virus.

S-7 is known to prevent uncoating by direct interaction with the enterovirus particle (Lonberg-Holm *et al.,* 1975). No activity of this compound against HRV-14 was registered in our study.

In conclusion, the aporphinoid alkaloid oxoglaucine reveals a well pronounced inhibitory effect on the replication of human rhinovirus 14. The IC_{50} evaluated according to the neutral red uptake procedure varies from 44 to 179 μM depending on the viral inoculation dose. From the data above it can be considered that oxoglaucine, possessing a selectivity index above 100, is a prospective anti-rhinoviral substance and a candidate for further preclinical and clinical trials.

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