CENTRAL EUROPEAN JOURNAL OF PUBLIC HEALTH

No. 3/Vol. 10 (JHEMI vol. 46) September 2002

Published by the National Institute of Public Health, Prague, in cooperation with Tigis Ltd.

ISSN 1210-7778
INDEXED IN EMBASE / Excerpta Medica, MEDLINE / Index Medicus, CHEMICAL ABSTRACTS
EXCERPTED IN BIBLIOGRAPHIA MEDICA ČECHOSLOVACA

TIGIS

ENTEROVIRUSES IDENTIFICATIONS AND DIFFERENTIATION ON THE BASIS OF THE SELECTIVE GROUP - SPECIFIC INHIBITORY EFFECT OF CHEMICAL COMPOUNDS

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SUMMARY

Two new enteroviruses (EV) inhibitors with the selective group-specific effect were detected and studied representing the products of the original chemical synthesis. One of them - nifan (arylfuran derivative) inhibits poliomyelitis virus replication, the other one - belvtazide (synchonic acid derivative) blocks non-poliomyelitis EV (ECHO and Coxsackie B) replication.

The study of the reference strains of poliomyelitis virus type 1-3, twenty-three ECHO virus types (from the 1st to the 33rd), Coxsackie B virus type 1-6 and 288 primary EV isolates did not reveal type or strain specific variability in the inhibitors effect. Nifan and belvtazide suppress the replication of both EV monostrains and their mixtures. The isolates of mixed nature are inhibited by the mixture nifan + belvtazide. At the same time neither separate chemicals nor their blend affects viruses from other families (Adenoviridae, Orthomyxoviridae, Herpesviridae etc.). The mechanism of nifan and belvtazide action is intracellular EV replication inhibition (they do not affect the process of virus adsorption and penetration into the cell), suppression of de novo virus synthesis by 7.0 - 2.25 lg (tissue culture infective dose 50 per cent) $TCID_{50}$/ml and of virus-induced RNA synthesis. The drugs feature is high selectivity (90 - 91%) regarding RNA polioviruses (nifan) and RNA non-poliomyelitis EV (belvtazide).

Nifan and belvtazide antiviral effect selectivity allows unknown cytopathic agents (CPA) belonging to the EV to be established with the high degree (over 98%) of reproducibility at the stage of primary identification with the differentiation of poliomyelitis and non-poliomyelitis viruses.

Key words: enterovirus, antiviral drugs, differentiation, cell culture

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INTRODUCTION

Enteroviruses can cause a spectrum of infections including aseptic meningitis, epidemic myalgia, meningoencephalitis, epidemic haemorrhagic conjunctivitis, acute respiratory and gastrointestinal infections often in the form of local outbreaks, not infrequently with epidemic spread. A special place among these viruses belongs to the poliomyelitis virus, whose spread in the past caused epidemics of the severe paralytic forms of poliomyelitis with the tendency to the pandemic course. Implementation of mass immunization stopped this process (1).

In the 1990's EV resumed to attract attention. Their increasing role in the epidemic outbreaks occurrence was proved. Major meningoencephalitis outbreaks with hundreds of cases were reported to be caused by the rare ECHO 30 (2, 3, 4). Since 1988 many countries are involved in the WHO Global Poliomyelitis Eradication Program. Important components of the Program are EV circulation monitoring, strict polioviruses detection and prediction of the emergence of the potentially dangerous EV infection epidemics strains (1, 5, 6, 7, 8).

The neutralization test with the specific antienteroviral serum still remains the chief method of the EV identification. This method employing the highly specific sera is specific, however, very laborious. The elaboration of the more cost-effective, "express-like" methods is an actual task.

The purpose of the present work was to study the EV inhibiting properties of the chemical compounds - nifan and belvtazide (commercial names) - in order to find out the perspective of their use as the means of primary identification of the cytopathic viral agents of unknown origin. Both compounds were produced through the directed virus-controlled synthesis of the arylfuran (nifan) (9, 10) and synchonic acid (belvtazide) (11, 12, 13) derivatives. Their group specific selectivity is characterized by the high level (98%) of poliomyelitis (nifan) and ECHO and Coxsackie B viruses (belvtazide) replication inhibition (9, 12, 14).

MATERIALS AND METHODS

Viruses. In our study we used the reference strains of ECHO 1 - 33, Coxsackie B 1 - 6, poliomyelitis 1 - 3 viruses from the Virus Museum of the Institute of the Poliomyelitis and Viral Encephalitis of the RAMS (Moscow), 288 local EV strains isolated in Belarus in 1960-1982 from the patients with CNS disease, healthy (contact) persons and environmental samples

(sewage) as well as 15 reference virus strains from 14 other families from the Virus Museum of the Ivanovsky Virology Institute of the RAMS (Moscow). Sensitive cell lines were used for the viral cultures.

The titers of the examined virus made up 5.0 - 7.0 lg $TCID_{50}$/ml.

Cell cultures. We used the re-inoculated cell lines FL (human amnion cells), Hela (cervix cancer cells), BGM (African green monkey kidney cells), HESMC (human embryo skin muscle cells), HEF (hen embryo fibroblasts) produced at the Research Institute for Epidemiology and Microbiology, Minsk.

Suspending medium. MEM, DMEM, hemohydrolysate 5.0 and 2.5% with the embryonic calf serum and antibiotic (Sigma, "Dialek" Republic Belarus) added.

Chemical compounds. Nifan was synthesized at the Research Chemical Pharmaceutical Ordzhonikidze Institute (Moscow) (9). Belvtazide was synthesized at the State Technology University (Samara) (12). Nifan and belvtazide were dissolved in DMSO (Sigma). The basic solutions were diluted with the suspending medium till the working concentration and DMSO level of 1% and less.

Cytotoxic properties of the drugs were determined in the monolayer cell culture with the concentration range of 1600 - 0.1 μg/ml. The maximum tolerated concentration (MTC) was determined, the presence of which in the suspending medium didn't cause morphological cell changes on the 7th day of incubation at the temperature of 36.5 °C (15).

Nifan and belvtazide antiviral properties study. We evaluated: 1. the virucidal effect (intact virus treatment); 2. the effect after simultaneous virus and drugs application for an hour with their following removal and exchange of the medium for the suspending one; 3. the effect of drug application an hour after the infection. Antiviral effect was assessed using standard method based on the registration of the virus cytopathic effect (CPE) in the cell culture. Quantitative characteristic of anti-virus effect was determined by chemo-therapeutical index (ChTI): ratio of maximum tolerated concentration to minimum inhibitory concentration (15).

Radioassay technique was used for the evaluation of the inhibitors effect on the macromolecular protein and nuclear acids synthesis both in the intact and infected with the virus cell culture (16). Radioactive RNA (^{3}H-uridine), DNA (^{3}H-thymidine) and protein (^{14}C-leucine, ^{14}C-protein hydrolyzate) synthesis precursors were provided by the firm "Isotope" (St-Petersburg). Virus-specific RNA synthesis was studied under the conditions of the one-cycle virus replication with actinomycin D (Sigma) added.

Virus type identification. Neutralization test (NT) was performed according to the standard technique with the standard kits of diagnostic anti-ECHO, anti-Coxsackie B and anti-poliomyelitis sera produced at the Institute of the Poliomyelitis and Viral Encephalitis (Moscow) and at the National Health Institute (Bilthoven, the Netherlands) (17). We designed a method of enterovirus differentiation with nifan and belvtazide based on the CPE inhibition (18).

RESULTS AND DISCUSSION

A high degree of selective anti-virus effect of inhibitors was found. All three poliovirus types - 1,2,3 were sensitive to nifan. Belvtazide demonstrated high inhibiting activity as regards twenty-three ECHO virus Serotypes (from the 1st to the 33rd) and Coxsackie B virus type 1 - 6. In a tolerated concentration range 200-10 μg/ml the substances blocked completely CPE of test-viruses. In minimum active concentration nifan (0.25 μg/ml) and belvtazide (2.25 μg/ml) reduced reproduction level of viruses on 2.25 and 3.75 lg $TCID_{50}$, accordingly. The effect of both nifan and belvtazide was characterized by high ChTI (Table 1). Table 1 shows that neither nifan inhibited ECHO or Coxsackie B viruses nor belvtazide inhibited poliomyelitis virus. Group-specific selectivity of nifan and belvtazide activity was confirmed by the results of the differentiation of the

Table 1. Belvtazide and nifan effect on the reference EV strains

Virus type		Strain	Drugs inhibitory effect, ChTI	
			Belvtazide	Nifan
ECHO	1	Farauk	40	0
	2	Cornelis	80	0
	3	Morriscy	80	0
	4	Du Toit	40	0
	5	Noyce	80	0
	6	D'Amori	80	0
	7	Wallace	80	0
	8	Bryson	200	0
	9	Hill	80	0
	10	Gregory	80	0
	12	Travis	80	0
	13	Del Carmen	200	0
	14	Tow	80	0
	17	CHHE-29	80	0
	19	Burke	200	0
	20	IV-1	200	0
	24	De Camp	200	0
	25	IV-4	80	0
	26	Coronel	200	0
	29	IV-10	200	0
	30	Bastiani	80	0
	32	PR-10	200	0
	33	Toluka	80	0
Polioviruses	1	Mahoney	0	100
	1	Lugovskoy	0	200
	1	LSc 2ab	0	100
	2	MEF-1	0	100
	2	Smirnov	0	100
	2	Ovchinnikov	0	100
	2	P 712 Ch2ab	0	100
	3	Saukett	0	>200
	3	Leon 12 ab	0	100
Coxsackie B	1	Connecticut-5	40	0
	2	OHCO-1	200	0
	3	Nancy	40	1
	4	IVB	200	0
	5	Foulkner	200	0
	6	Shmidt	200	1

Table 4. Belvtazide and nifan effect on RNA synthesis in the infected cell lines

Cell culture, virus type, drug	Time, hours	3H-uridine insertion, imp./min	Synthesis suppression, %
HESMC + ECHO 6	2	2166	-
HESMC + ECHO 6 + belvtazide	2	275	87.3
HESMC + ECHO 6	4	2421	-
HESMC + ECHO 6 + belvtazide	4	213	91.2
Hela + polio-2	2	3221	-
Hela + polio-2 + nifan	2	390	87.9
Hela + polio-2	4	3534	-
Hela + polio-2 + nifan	4	451	90.1

Table 5. Activity spectra of 2-(α-oxybenzyl)benzimidazole (OBB), guanidin, belvtazide, nifan

Virus type	Antiviral effect			
	Guanidine	OBB	Belvtazide	Nifan
Poliomyelitis 1, 2, 3	+	+	-	+
Coxsackie B 1, 2, 3, 4, 5	+	+	+	-
ECHO 1, 2, 6, 7 9, 11, 12, 13, 15	+	+	+	-

"+" - positive effect; "-" - no effect.

shows the results of identification of the EV experimental mixtures. The effective doses (10 μg/ml) of nifan and belvtazide applied simultaneously were shown to inhibit CPE of EV in the mixture.

The findings of the experiments showed that the stage sensitive to the nifan and belvtazide inhibitory effect in the reproduction cycle of ECHO and poliomyelitis virus was the stage of intracellular synthesis (Fig. 1). No drugs effect on the cell monolayer was found at the stage of virus adsorption and penetration (after simultaneous virus and drugs injection into the culture medium for an hour with the following exchange of the medium). The inhibitors action resulted in the complete or considerable (to the trace titers) virus synthesis suppression both on the first and the following (5^{th}) days of the experiment.

The radioassay technique in the intact Hela and HESMC cell cultures demonstrated that nifan and belvtazide did not inhibit cellular macromolecular synthesis. The intensity of the cellular DNA, RNA, and protein synthesis in the intact cells corresponded to the synthesis level registered during 24, 48 hours of contact with belvtazide and exceeded a little the synthesis level with nifan (with the chemicals concentrations of 25.0 - 100.0 μg/ml).

At the same time nifan in the Hela cells infected with poliomyelitis virus type 2 and belvtazide in the HESMC cells infected with ECHO virus type 6, dynamically inhibited RNA synthesis by 87.9 - 90.12% and 87.3 - 91.2% correspondingly (Table 4). These results prove that both nifan and belvtazide block specific RNA synthesis and de novo virus production, thus explaining the mechanism of their selective antiviral activity.

The selectivity of the nifan and belvtazide group-specific activity was confirmed in the comparative experiments with the well-known EV inhibitors - 2(α-oxybenzyl) benzimidazole (OBB) and guanidine which possess more universal anti-EV mechanism of action (19, 20). Table 5 shows that both poliomyelitis virus and ECHO and Coxsackie B viruses are sensitive to the OBB and guanidine while nifan and belvtazide demonstrated the precise group-specific effect.

The findings of our study show that nifan and belvtazide can be used for the primary EV express-identification and differentiation of the poliomyelitis and non-poliomyelitis EV (ECHO, Coxsackie B).

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