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Study of antiviral activity of a new plant origin preparation neoflazidum on a model of the hepatitis c virus

Yu. I. Porva[1], S. L. Rybalko[1], L. I. Palchykovs'ka[2], V. P. Atamanyuk

[1] Gromashevsky L. V. Institute of Epidemiology and Infection Diseases, NAMS of Ukraine
5, Amosova Str., Kyiv, Ukraine, 03038

[2] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680
epidemics@ukr.net L.Palchykovska@ukr.net

This paper presents some results concerning investigation of antiviral activity of a new plant origin preparation – neoflazidum, a form of proteflazidum – using a model of hepatitis C virus-producing *Jurkat* cell culture (*Jurkat*^{HCV}) which has been previously transfected with complementary DNA preparations isolated from different types of hepatitis C virus (HCV). To obtain these preparations, HCV RNA preparations were isolated from viral material of HCV-diseased patients, the transfecting agent *Turbofect* being used. The cytotoxic dose (CD_{50}) was shown to be 37.2 μg/ml, the effective dose (ED_{50}) being 0.46 μg/ml for the HCV type 3a; this dose, however, was as high as 3.7 μg/ml when neoflazidum had been tested for HCV type 1b. Therefore, the selectivity index for the HCV type 3a reached 80.8 in the *Jurkat*$^{HCV\text{-}3a}$ cell system, being as low as 10.0 for the HCV type 1b. Using a model system RNAP T7, the antiviral neoflazidum activity was demonstrated to be realized due to interferons α- and γ-induction and RNA synthesis inhibition.

Keywords: antiviral activity, neoflazidum, hepatitis C virus, transfecting, interferons α- and γ-induction, model system RNAP T7

Introduction

The victims of the hepatitis C virus (HCV) are about 130–170 million people worldwide, i.e. about 2.2-3.0 % of all our planet population, 3–4 million becoming infected every year[1]. In 55–85 % of all cases the infection becomes chronic, leading usually to serious liver damages. During the last 20 years the chronic hepatitis C causes the liver cirrhosis in about 4–20% of the patients. The patients with identified cirrhosis are known to have a frequent risk of liver decompensation (18–29 %) during next 10–20 years and a risk of the hepatocellular carcinoma development (10–30 %) during next 20 years. Nowadays, the chronic hepatitis C patients are usually treated with the pegylated interferon preparations (Peg-IFN) combined with rybavirin (RBV). Such a combination permits to reach the permanent absence of HCV RNA (i.e. steadfast virological response, SVR) in 40–52 % of the patients with chronic hepatitis C caused by the genotype 1 [2–4].

According to the last estimations concerning the HCV-caused mortality, 50 thousand patients worldwide die directly as hepatitis victims, and 300 thousand persons perish due to the HCV-caused liver malignancies, 800 thousand persons being killed by cirrhosis; these levels are thought to become higher [5].

There are several HCV genotypes, the genotype 1 is thought to be the most spread in the world. This virus genotype is found in more than 70 % of the chronic HCV patients in Europe and the USA; it is

not only the most spread, but is also the most treatment-resistant to the up-to-date standard Peg-IFN/ RBV therapy. In almost 50 % of the persons with chronic hepatitis due to the HCV genotype 1b, the Peg-IFN/RBV therapy leads to the SVR; in 24 weeks following the end of the treatment it becomes impossible to evaluate the level of viral RNA. The SVR is a generally accepted treatment index [6].

There are no effective therapeutic approaches for the patients who have not reached the SVR after the standard treatment. It was reported that the refresher courses of Peg-IFN/RBV treatment of the chronic patients infected by the HCV type 1b led to the SVR in 4–21 % of the previously non-responder patients as well as in 23–31 % of relapsing ones. Thus, we do need more effective therapy approaches with a shorter duration of the treatment course.

Telaprevir is a powerful, reverse, selective, linear peptidomimetic inhibitor of the HCV-specific serine protease NS3-4A; it is an additional antiviral drug used together with Peg-IFN/RBV. In the chronic patients infected by the HCV genotype 1b the telaprevir use combined with the Peg-IFN/ RBV during 24 to 48 weeks, is significantly accompanied by a higher SVR frequency comparing to 48-week-long Peg-IFN/RBV therapy only. The increased therapy efficacy was found in both the patients having not been previously treated and the patients after previous unsuccessful therapy with non-decreased virus level as well as in a lot of different patients including the persons with continuously low SVR following standard Peg-IFN/ RBV approach [7–9].

Nowadays there are promising antiviral preparations of plant origin. Plants and plant extracts had been traditionally used during centuries long before different active products were isolated as a result of science and technology progress. The WHO specialists calculated that about 80 % of the world population still use traditional medicine approaches for the infectious diseases therapy [10].

Natural flavonoids of plant origin are known since 1940; however, the problems concerning the study of their antiviral activities became actual only 25 years ago. In the late 1990s, pharmaceutical companies took interest in these compounds and initiated preclinical studies and clinical trials of flavonoid-based preparations. Some compounds with promising raw material bases, such as, for example, quercitin and dehydroquercitin, are now investigated on the largest scale [11, 12].

Flavonoids are, however, labile unstable compounds, their production being accompanied by certain technological difficulties. Organic synthesis and/or modification of substances of easy production are among the most important approaches permitting to obtain highly effective flavonoids of specific formulations and specifically directed action. An example of such successful design of medicinal substance is the obtaining of the synthetic drug 6,4-dichlorflavan [13]. However, a lot of synthetic medications possessing a high antiviral activity *in vitro*, during clinical trials were shown to be significantly less effective. That is why numerous intensive investigations are now carried out in this field.

A research group of Iry Jacobson (Cornwell University) studied the sofosbuvir therapeutic effect in combination with ribovirin for the therapy of patients with chronic hepatitides infected with the HCV types 2 and 3, the standard therapy being futile. The use of this new drug combination permitted to obtain the SVR (no virus RNA was found in blood) in 78 % of patients in 3–4 months of treatment.

Another specialists' group led by E.Lavitz (Texas State University) studied sofosbuvir, ribavirin, and pegylated alpha-2a interferon combinations for the therapy of previously non-treated patients with chronic hepatitis caused by all HCV subtypes including the most spread genotype 1 HCV. In three months of treatment the SVR was reached in 90 genotype 1-infected patients. No serious side effects were seen in both cases. Sofosbuvir belongs to the RNA-polymerase inhibitor class oppressing the HCV reproduction [14].

It goes without saying that the researches concerning the inhibition efficacy of the hepatitis C virus reproduction do need an experimental model of infection; numerous attempts aiming to obtain

such a model were unsuccessful during a very long time, the HCV replication levels *in vitro* being extremely low.

There are publications showing that the transformed human hepatome cells Huh-7 carrying a luceferase reporter construction (pGL3) is an up-to-date cell model for the HCV reproduction. Other standard models used by foreign researchers are MT-4 and Daudi cells as well as HeLa cells transformed by a recombinant plasmid (with the pBK-CMC-HCV-replicon) containing structural HCV genes [15].

The HCV reproduction in cell cultures is not a stable process, the virus kept at 70 °C, looses its infectivity. To maintain this virus, we used complementary HCV DNA, the virus having been isolated from the hepatitis C patient. The transfection of MT-4 and RHNN cell lines by complementary DNA (cDNA) preparations permitted to obtain the HCV-producing cell cultures [16].

The aims of this paper were to obtain the HCV-producing cDNA-transfected *Jurkat* cells and to study the anti-HCV activity and the mechanism of neoflazidum action.

Materials and Methods

Preparations:

Neoflazidum

It is a mixture of flavonoids obtained from wild natural flavonoid-rich gramineous plants – *Calamagrostis epigeios L.* and *Deschampsia caespitosa L*. The concentration obtained is 3.72 mg/ml.

PolyI-polyC, reference interferon inducer (Calbiochem, USA).

Cell cultures:

L929. Murine established cell line was received from the collection of Cell Culture Museum of the D.I.Ivanovski Institute of Virology (RAMS, RF). These cells grow at 37°C in the RPMI-1640 (Sigma-Aldrich, USA) medium supplemented with heated fetal serum (12 %), the cell density being (15–20) $x10^4$ cells/ml.

Jurkat. The established cell line of human origin was obtained from a patient with T-lymphoblastoid leukemia at the Institute of Immunology (RAMS, RF. These cells grow in suspension at 37°C in the RPMI-1640 medium supplemented with 2 % glutamine and 10 % heated fetal serum, the CO_2 concentration in the thermostat air being 5 %. The cell density is usually (3–9) $x10^5$ cells/ml.

RHNN. The established cell line, epithelioid monolayer-forming culture obtained from a rat Heiser node neurinoma was induced by transplacental injection of ethylnitrozourea [17, 18]. It grows in the EMEM medium supplemented with 10-20% of calf embryo serum, received from the Human Morphology Research Institute (RAMS, RF).

Virus preparations:

The vesicular stomatitis virus (VSV) of the Indiana strain was received from the Museum of Viruses of the D.I.Ivanovski Institute of Virology (RAMS, RF), it infectious titer in cultured cells L41 reaching 4.0–5.0 lg $TCID_{50}$/ml.

Animals:

White non-inbred mice (14–18 g) were grown in standard conditions in the vivarium of the state enterprise “Lev Gromashevski Institute of Epidemiology & Infectious diseases, Academy of Medical Sciences of Ukraine”.

Model cultures:

HCV-producing cells *Jurkat*, splitting suspension cultures of lymphoblastoid origin, transfected by different types of this virus.

As the HCV source, the non-diluted blood plasma samples of HCV-infected persons were used with different virus load; such plasma contains HCV RNA. All viral RNA preparations were isolated using a kit “RIBO-sorb” (RF). The HCV complementary DNA (cDNA) was synthesized by the reverse transcription reaction using a kit «Reverta-L» (RF). 10 μl of RNA samples were added to 10 μl of prepared reaction mixture (lyophilized plant preparation, 125 μl of the RT-mix solution and 6 μl of the

murine leukemia virus (MMLv) revertase; the transcription was carried out at 37^0C during 30 min, and cDNA was obtained.

Transfection process using the Turbofect

Cell cultures *Jurkat* grown in suspension were transfected using a transfecting reagent *Turbofect.* The transfection was carried out according to the standard protocol using *Turbofect* (Thermo Scientific, Lithuania). To realize a transfection process, the cell density is to reach $5x10^4$ (for established line cells) and $5x10^5$ (for cells grown in suspension) per 1 ml of nutrient medium. A cDNA preparation (1 μg) was diluted in 100 μl of serum-free RPMI-1640 medium, the tracfecting reagent being added during stirring (by aid of a pipette or a vortex). Following pipetting or stirring in the vortex, the sample was incubated at room temperature (15-20 min). Then 100 μl of a mixture of DNA and transfecting reagent were dropped into each cell culture-containing well and incubated in a CO_2-thermostat at 37 °C. The virus detection was carried out using the PCR approach on the second passage (9^{th} day of cultivation) and on the fifth one (17^{th} day of cultivation). All the cDNA-transfected *Jurkat* cultures produced the HCV both on the 9^{th} and 17^{th} day of cultivation.

Measurement of HCV load in clinical samples

Quantitative estimation of the HCV RNA in clinical samples was carried out using the real-time PCR with hybridization and fluorescent detection. For this aim, a reagent kit "AmpliSense HCV-Monitor-FRT" (Russia) and an apparatus "Rotor-Gene 3000/6000" ("Corbett Research", Australia) were used.

In vitro transcription reaction.

The effect of the preparation investigated on the RNA synthesis was evaluated in the transcription system RNAP T7 using available reagents of the firma «Fermentas» (Lithuania). The transcription was carried out using 20 μl of reaction mixture containing 0.5 μg of linearized DNA of the plasmid *pTZ19R* carrying the RNAP T7, ribonucleoside triphosphates, concentration of each one being 2 mM, 20 active units of the RNAse inhibitor RiboLock™, 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiotreiitol (DTT), and 12 active units of the T7 RNA-polymerase. These substances were solved in DMSO (1 mg/ml). The DMSO concentration in control and test samples (2.5 %) has no influence on the level of the RNA synthesis. The reaction mixture was kept at 37 °C during 45 min, the reaction being stopped by cooling (up to – 20°C). The reaction products obtained were detected by gel electrophoresis in 1% agarose supplemented with ethidium bromide (0.3 μg/ml). The RNA transcripts were visualized using a UV-transilluminator. The intensity of RNA bands on photos were measured by a densitometry method using the program Scion Image. The IC_{50} value (inhibitor concentration necessary for a decrease of the enzyme activity by 50 %) was calculated from the plot describing the dependence of activity on the enzyme concentration.

Therapeutic neoflazidum activity against the HCV

To study the antiviral activity, the preparation of different concentrations was put intothe HCV-producing *Jurkat* cultures, the virus level being evaluated for each sample in 5 days using the PCR.

Neoflazidum IFN-inductive activity in vivo

The IFN-inducing activity of the preparation *in vivo* was studied on non-inbred white mice, their mass being 14–18 g. The preparation was injected intraperitoneally. In 24 h the mice were killed by euthanasia; the IFN activities were determined in murine blood sera and in different animal organs according to a generally accepted approach – the CPE inhibition being caused by the vesicular stomatitis virus in L929 cell cultures [20].

Data analysis

All experiments were performed in triplicates. The antiviral activity of the neoflazidum was expressed as the $\log_{10}$ reduction of the viral titer by comparison

with untreated controls (inhibition of infectious titer). The standard deviation in the reduction of virus titer was about 0.5 $\log_{10}$. The neoflazidum was considered active only when the virus yield decreases $\geq 2 \log_{10}$, at the effective dose ED_{50}.

Results and Discussion

To obtain the HCV-producing cell culture, the HCV RNA preparation was isolated; the HCV-RNA complementary DNA preparation was used for the *Jurkat* cultures transfection followed by the PCR analysis (Table 1).

With the transfecting reagent *Turbofect* the HCV cDNA-transfected *Jurkat* cell cultures with stable HCV production (*JurkatHCV*) have been originated. The viral load in all variants of the HCV-transfected cells progressively increased with the passages of the culture. In the cells transfected with the HCV 1b subtype, the viral load was the highest and exceeded three-fold that in the cells transfected with the HCV 3a subtype. Taking into account that HCV 1b and 3a are the most prevalent in Ukraine, *JurkatHCV-3a* and *JurkatHCV-1b* as HCV-producing cultures were used in further experiments for the ED_{50} assessment.

To evaluate the neoflazidum cytotoxicity concentration (CC_{50}), the RHNN cells were taken. The range of neoflazidum concentrations was from 186 to 4.6 μg/ml. Every dilution was analyzed in 10 replicates, the results being presented in the Table2.

As estimated in our studies, the cytodestructive cell changes were found for the concentrations of 186, 93 and 47 μg/ml, the neoflazidum CC_{50} value was shown for the concentration of 37.2 μg/ml.

To evaluate the ED_{50}, the neoflazidum preparation was put into the virus-producing *JurkatHCV-3a* and *JurkatHCV-1b* cultures in doses: 18.6, 9.3, 4.6, and 2.3 μg/ml and incubated at 37^{0}C. On the 5th day of cultivation the virus reproduction was determined by the PCR method, the quantity of HCV genome-equivalents being a reproduction marker (Table 3).

The results obtained for neoflazidum with the *JurkatHCV-3a* and *JurkatHCV-1b* cultures show the virus load-decreasing effects to be quite different: it

Table 1. **Results [of] HCV load in transfected cells *Jurkat* for different HCV types**

Passage following transfection	HCV load in transfected cells (genome/equivalents) for different HCV types			
	1	2 – 3a	3	4 – 1b
2nd passage	2537	4485	2512	13598
5th passage	3150	5400	3120	14100

Table 2. **Evaluation of the neoflazidum CC_{50}**

Preparation	Concentration of preparation (μg/ml)						
	186	93	47	37.2	18.6	9.3	4.6
	Cytodestructive changes						
neoflazidum	10/10	10/10	10/10	0/10	0/10	0/10	0/10

Table 3. **Neoflazidum ED_{50} for the HCV**

Preparation concentration, μg/ml	HCV 3a		HCV 1b	
	virus load, genome/equivalent	inhibition, %	virus load, genome/equivalent	inhibition, %
7.44	120	61	0	100
3.72	0	100	100	54.4
1.86	0	100	279	0
0.93	56	81.8	305	0
0.46	125	59.3	302	0
Virus control	307		219	

Table 4. **CC_{50}, ED_{50}, and SI for neoflazidum in *Jurkat^{HCV-3a}* and *Jurkat^{HCV-1b}* cultures**

Genotype HCV	CC_{50}, μg/ml	ED_{50}, μg/ml	SI
Jurkat^{HCV-3a}	37.2	0.46	80.8
Jurkat^{HCV-1b}	37.2	3.72	10.0

Table 5. **Interferon level in organs of mice treated with the neoflazidum**

Organs studied	IFN activity (U/ml) following injections of :							
	the neoflazidum in doses:						saline	polyI-polyC
	37.2 μg/kg		7.4 μg/kg		3.72 μg/kg			
	- pH	+ pH	- pH	+pH	-pH	+pH		
Blood	320	0	160	0	320	20	2	1280
Spleen	80	80	160	0	0	0	10	640
Liver	40	40	80	80	160	160	10	640
Lungs	320	0	160	0	320	0	10	320
Heart	80	80	20	20	40	10	10	320
Kidneys	160	160	20	20	320	0	10	320

was 0.46 μg/ml in the first culture and eight times lower for the second one.

The results of the CC_{50}, ED_{50}, selectivity index (SI) evaluation are given in the Table 4.

The IFN-inducing neoflazidum activity *in vivo* was studied on the model of white non-inbred mice; the preparation (0.1 ml) was injected into peritoneum, the concentrations being 37.2 μg/kg, 7.4 μg/kg, and 3.72 μg/kg. In 24 h the mice were killed by euthanasia; the IFN activities were determined in murine blood sera and in different animal organs according to a generally accepted approach – the inhibition of the CPE being caused by the vesicular stomatitis virus in homologous L929 cell cultures.

The IFN type was determined according to its acid sensibility. The supernatant fluids from the suspensions of organs as well as blood sera were divided in two samples. The fluid pH of one sample was adjusted to 2.0 using 4N HCl solution and kept the sample at 4^0C during 24 h; the fluid pH value was then restored up to 7.2 with the aid of 4 N NaOH. The results of this experiment are given in the Table 5.

We analyzed the results of α- and γ-IFNs activities testing (taking into consideration such marker as their acid sensitivity) in different organs of mice treated by different neoflazidum doses. Notably, the neoflazidum was an active IFN inducer, the α- and γ-IFN induction being dose-dependent. At a higher neoflazidum concentration (37.2 μg/kg) α- and γ-IFN are induced, at lower concentrations, 7.4 μg/kg and 3.72 μg/kg, we detect mostly the γ-IFN induction. Interestingly, the blood sera and lung tissue contain γ-IFN without any dose-dependence.

The neoflazidum effect on the in vitro RNA synthesis in the transcription system RNAP T7

The transcription is a key process for cell functioning and reproduction being simultaneously among the most important targets of antiviral and antimicrobial therapy. The organic compounds which are able to modify the transcription process are used as potential drugs – the inhibitors of viral and bacterial reproduction [9].

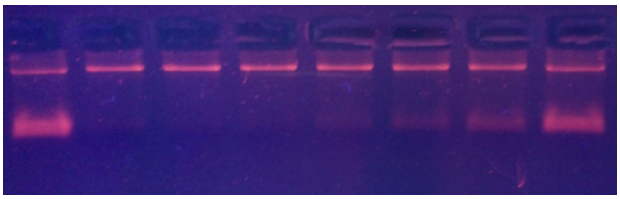

Fig. 1. Inhibition effect of neoflazidum (25, 12.5, 6.25, 3.125, 1.56, and 0.78 μg/ml) on the RNA synthesis. IC_{50} = 0.78 μg/ml.

3D-structures of different DNA- and RNA-polymerases are known to possess a high degree of similarity as well as to contain the same structural domains and conservative motifs necessary for the elongation of nucleic acid chains. That is why we chose an easy-to-use and productive model, the RNAP T7 transcription system [13], for both selection of RNA synthesis inhibitors and detection of effective antimicrobial or antiviral drugs.primary investigation of the neoflazidum in the model system RNAP T7 was carried out with preparations of 25 μg/ml concentration. All substances tested block completely the RNA synthesis. Two-fold dilutions were made to understand the effect dependence on the drug concentration (Fig. 1).

The data obtained testify a high neoflazidum inhibitory effect on the RNA synthesis. The plant extract has been shown to be an active inhibitor for the transcriptio-nal complex RNAP T7, its IC_{50} value being 0.78 μg/ml.

Conclusions

The data concerning a new plant origin preparation, namely a new proteflazidum form – neoflazidum, show its cytotoxic concentration to be 37.2 μg/ml. The effective neoflazidum dose (ED_{50}) found on the model of virus-producing transfected cells *Jurkat* is 0.46 μg/ml for the HCV type 3, being, however, eight times higher (3.72 μg/ml) for the HCV type 1b; it is a cause of a lower proteflazidum efficacy of the therapy of the hepatitis C type 1b.

It has been demonstrated that the neoflazidum antiviral effect is realized through the RNA synthesis inhibition and the α- and γ-interferon induction.

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Вивчення антивірусної активності препарату рослинного екстракту протефлазіду на моделі вірусу гепатиту С

Ю. І. Порва, С. Л. Рибалко, Л. І. Пальчиковська, В. П. Атаманюк

Мета. Вивчення антивірусної активності нової форми рослинного екстракту Протефлазіду (РЕП) – **Методи.** Моделі продукуючої культури Jurkat кДНК ВГС. **Результати.** Одержані продукуючі ВГС культури клітин Jurkat методом трансфекції комплементарної ДНК до виділеної РНК ВГС від хворих гепатитом С за допомогою трансфекуючого реагента Turbofect. Цитотоксична доза (CD50) дорівнювала 37,2 мкг/мл, ефективна доза (ED50) в системі продукції вірусу гепатиту С 3а типу дорівнювала 2 3 мкг/мл, а в системі репродукції вірусу гепатиту С 1в типу – 18,6 мкг/мл. Тому індекс селективності для РЕП в системі продукуючих ВГС клітин 3а типу дорівнював 16,2, а для ВГС 1в типу – 2. **Висновки.** Механізм антивірусної дії РЕП відбувається за рахунок індукції α- та γ-інтерферону та інгібіції синтезу РНК в модельній системі РНКП Т7 – інгібуюча концентрація (IC50) – 0,78 мкг/мл.

Ключові слова: antiviral activity, neoflazidum, hepatitis C virus, transfecting, interferons α- and γ-induction, model system RNAP T7

Изучение противовирусной активности препарата растительного экстракта протефлазида на модели вируса гепатита С

Ю. И. Порва, С. Л. Рибалко, Л. И. Пальчиковська,
В. П. Атаманюк

Цель. Изучить антивирусную активности новой формы растительного экстракта Протефлазида (РЭП). **Методы.** Модель продуцируемого культуры Jurkat кДНК ВГС. **Результаты.** Полученные продуцирующие ВГС культуры клеток Jurkat методом трансфекции комплементарной ДНК к выделенной РНК ВГС от больных гепатитом С с помощью трансфекуючого реагента Turbofect. Цитотоксическое доза (CD50) равнялась 37,2 мкг / мл, эффективная доза (ED50) в системе продукции вируса гепатита С 3а типа равнялась 2 марта мкг / мл, а в системе репродукции вируса гепатита С 1в типа – 18,6 мкг / мл. Поэтому индекс селективности для РЭП в системе продуцирующих ВГС клеток 3а типа равен 16,2, а для ВГС 1в типа – 2. **Выводы.** Механизм антивирусного действия РЭП происходит за счет индукции α- и γ-интерферона и ингибирования синтеза РНК в модельной системе РНКП Т7 – ингибирующая концентрация (IC50) – 0,78 мкг / мл.

Ключевые слова: antiviral activity, neoflazidum, hepatitis C virus, transfecting, interferons α- and γ-induction, model system RNAP T7

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