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Application of the Agar-Diffusion Plaque-Inhibition Method for Testing Combination Antiviral Effects: PTU-23 and Guanidine Hydrochloride Interactions and Combined Effects on the Replication of Poliovirus 1

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Abstract

Original modifications of the agar-diffusion plaque inhibition method were used for testing the combination effect of anti-enteroviral substances. It was established that the anti-picornavirus compounds PTU-23 and guanidine.HCl are antagonists when applied in combination against the replication of poliovirus 1 in cell cultures. Some significant differences in their activity were found: (i) guanidine antagonists did not influence the anti-poliovirus effect of PTU-23; (ii) Cu^{++} and Zn^{++} ions significantly increased the activity of PTU-23 towards poliovirus 1, whereas Zn^{++} ions had no effect on the antiviral activity of guanidine.HCl, and Cu^{++} ions manifested a marked antagonistic effect on the guanidine activity. Evidently, the anti-enterovirus activity of these compounds is directed to different targets in the virus replication cycle.

Key words: agar-diffusion test, combination effects, poliovirus 1, PTU-23, guanidine.HCl

Резюме

Оригинални модификации на агар-дифузионния плако-инхибиращ метод са приложени за изпитване на комбинирания ефект на анти-ентеровирусни вещества. Установено е, че анти-пикорнавирусните съединения PTU-23 и гванидин.HCl, приложени в комбинация спрямо репликацията на полиовирус 1 в клетъчни култури показват отчетлив антагонизъм. Установени са някои съществени различия в тяхната активност: (i) гванидиновите антагонисти не влияят върху анти-полиовирусния ефект на PTU-23; (ii) Cu^{++} и Zn^{++} йони усилват значително активността на PTU-23 спрямо полиовирус 1, докато Zn^{++} йони са без ефект върху антивирусния ефект на гванидин.HCl, а Cu^{++} йони проявяват отчетлив антагонистичен ефект спрямо активността на гванидина. Очевидно, анти-ентеровирусната активност на тези съединения е насочена към различни мишени в ентеровирусния репликативен цикъл.

Introduction

Enterovirus infections are among the principal indications for application of chemotherapy, but the lack of clinically effective agents is due mainly to the development of drug resistance. This phenomenon is related to the unusually high mutation rate and the consequent composition of the enterovirus progeny of billions of quasispecies. The combined administration of enterovirus replication inhibitors with different mechanisms of action is considered to be a basic approach which could counteract the drug resistance (Galabov *et al*., 2012). In this connection, several various methods for studying the combination effect of virus inhibitors were introduced in the experimental chemotherapy of viral infections. The 3D spaced method of Prichard and Shipman (1990) stands out among these methods. In the field of antivirals it definitely replaced the routine isobologram method, largely used in the experimental pharmacology (Fucquier and Guedj, 2015; Tallarida, 2011).

The present work describes a completely different approach for testing the combination effects of inhibitors of enterovirus replication. It is a variant of the "classical" agar-diffusion plaque-inhibition method of Rada and Zavada (1962). Its basic advantage is the very convincing direct visualization of the combination effects of the antivirals, unattainable by other methods for testing combinations effects.

As a model enterovirus, poliovirus 1 (Ma-

honey) we used, replicated in monolayer cultures of FL or MK cells. The work with this polio 1 strain was done before its stock was destroyed in the late 1990s. In the meantime, The Third Meeting of the Advisory Committee on Poliovirus Eradication, held in October 2006, proposed the establishment of a "poliovirus antiviral initiative" and the appropriate and possibly essential development of at least two anti-polio drugs for control in the post-eradication era. This will be of great benefit for post-exposure prophylaxis and outbreak control (Workshop Report, Committee on Development of a Polio Antiviral and its Potential Role in Global Poliomyelitis Eradication, 2006; Chumakov *et al.*, 2007).

The combination effect of two selective inhibitors of enterovirus replication was investigated, namely guanidine.HCl and PTU-23. PTU-23 (N-phenyl-N'-3-hydroxyphenyl thiourea) was selected and characterized in a series of publications within the period 1972 – 1999. PTU-23 has been characterized as a strong and specific inhibitor of picornavirus replication with a very broad antiviral spectrum, embracing polioviruses 1-3, CVB 1-6, CVA 7/IV, echoviruses 1, 3, 6-9, 11-14, 17, 19, 20, enterovirus 71, ECSO II-IV, EMCV, rhinoviruses 1B-532 and H-17, FMDV type C (Galabov, 1979).

The comparison of the anti-picornaviral effects of PTU-23 and guanidine.HCl marked several substantial similarities: (i) the highest susceptibility of viruses belonging to *Enterovirus* genus; (ii) the arrangement of polioviruses by their susceptibility: polio 2 > polio 3 > polio1; (iii) the analogous susceptibility of type C FMD virus. Nevertheless, some differences were also found: (i) PTU-23 inhibits EMC virus replication, insensitive to guanidine.HCl; (ii) PTU-23 inhibits rhinovirus 1B-632 replication, also insensitive to guanidine.HCl; (iii) PTU-23 possesses a marked protective effect in experimental picornavirus infections *in vivo* (in laboratory animals) - against Coxsackieviruses A6, A7, B1, B3 and FMD virus in newborn mice and against Coxsackievirus

A7-IV in cotton rats, whereas guanidine.HCl does not show *in vivo* activity (Caliguiri and Tamm, 1973; Galabov and Velichkova, 1974; Galabov, 1979; Herrmann *et al*., 1982; Klein *et al*., 2000).

It is of interest to compare of the mechanisms of anti-picornavirus action of the two compounds. PTU-23 and guanidine.HCl are similar in the following properties: (i) lack of virucidal effect ; (ii) they do not influence viral adsorption and penetration; (iii) the drug-susceptible period ranges from 1st to 5th hour in the picornavirus replication cycle; (iv) pronounced inhibition of infectious virus production; (v) marked inhibition of viral 37S RNA synthesis; (vi) strong inhibition of CPE development. However, several significant differences were registered: (i) PTU-23 significantly decreases the activity of the virus-specific RNA-dependent RNA polymerase in a cell-free system, guanidine.HCl being without effect; (ii) PTU-23 markedly inhibits the synthesis of the double-stranded RNA, whereas guanidine.HCl has no effect on this species of virus-specific RNA; (iii) PTU-23 does not inhibit the synthesis of RNA-dependent RNA polymerase, strongly suppressed by guanidine.HCl; (iv) there are some reasons to consider that under the effects of PTU-23 and guanidine some partial degradation of viral RNA occurs, but for PTU-23 this phenomenon supposedly occurs only at the end of the latent period; (v) PTU-23 prevents virus-induced inhibition of the host-cell protein and RNA syntheses independently of the multiplicity of infection; guanidine.HCl is without effect at m.o.i. exceeding 10; (vi) PTU-23 and guanidine.HCl do not markedly inhibit the poliovirus protein synthesis in contrast to the pronounced inhibition of the virus-specific RNA synthesis, quantitatively the guanidine effect being better expressed; (vii) guanidine.HCl effect is directed to the process of the posttranslational morphogenesis (cleavage) of the structural proteins and to the virions morphogenesis; guanidine.HCl damages the functional configuration of the structural proteins' precursor NCVP1; the data on PTU-23 effect on EMC virus protein synthesis evidenced a lack of influence on the protein cleavage process; (viii) a single passage of poliovirus in the presence of guanidine.HCl is sufficient for the resistant mutant selection; in the case of PTU-23, 6-7 passages are needed for the development of drug-resistant progeny (Caliguiri and Tamm, 1973; Galabov, 1981; Galabov and Dmitrieva, 1983; Galabov and Svitkin, 1983; Saunders *et al.*, 1985).

It was established that the target of guanidine effect on picornavirus replication is the non-structural protein 2C (Pincus *et al*., 1986; Barton and Flanegan, 1997). According to Barton and Flanegan (1997), guanidine inhibits the 2C function that is required for the initiation of RNA(-), but not RNA(+) synthesis or RNA elongation. In addition to this target, it was found that guanidine prevents the association of 2C/2BC with a host membrane structure without affecting their association with the viral RNA (Bientz *et al.*, 1990). Studies on the mode of anti-picornavirus effect of PTU-23 characterized this compound as a selective inhibitor of specific

viral 37S ssRNA synthesis as a result of suppression of the synthesis of a viral protein with regulatory functions in the replication cycle (Галабов, 1981; Galabov and Dmitrieva, 1983; Galabov and Svitkin, 1983).

The comparative analysis of the data on the anti-picornaviral effects of PTU-23 and guanidine. HCl outlines the necessity to study the interaction of these two specific inhibitors of picornavirus replication: (i) the resultant effect of the simultaneous application of the two compounds on poliovirus 1 replication; (ii) action of guanidine.HCl antagonists on the antiviral effect of PTU-23; (iii) influence of heavy metals (Cu^{++}, Zn^{++}) on the effects of PTU-23 and guanidine.HCl on poliovirus replication.

Materials and Methods

Compounds

N-phnyl-N'-3-hydroxyphenylthiourea (PTU-23), synthesized by G. N. Vassilev (Institute of Plant Physiology, Bulgarian Academy of Sciences), dissolved ex tempore in ethanol was used.

Guanidine hydrochloride was supplied by Eastman Organic Chemicals, New York, USA, dissolved in physiological saline.

Cells and media

Monolayer FL and MK cells were used. The growth medium consisted of a mixture of equal parts of medium 199 (Difco) and Hanks' saline, supplemented with 10% heated calf serum and antibiotics (penicillin 100 IU/ml) and streptomycin 100 µg/ml).

Virus

Poliovirus type 1 (Mahoney) was grown in Eagle's Minimal Essential Medium (MEM, Flow) with 5% heated calf serum and antibiotics. The stock virus titer was 2-5 x 10^9 PFU/ml.

Agar-diffusion plaque-inhibition method

Monolayer FL cells or MK cell cultures in Anumbra (Czeck Republic) Petri dishes (Ø = 90 mm) were inoculated (adsorption: 60 min at 20°C) with 1.88 ml of a virus dose, giving semiconfluent plaques after 24-48 h of incubation at 37°C. A 12.5-ml agar overlay (1% agar in MM Eagle Difco containing 10% heated calf serum, 1.65 mg/ml sodium bicarbonate, 100 IU/ml penicillin and 100 µg/ml streptomycin) was placed on the dish. One or more glass cylinders (Ø = 6 mm) were fixed in the agar overlay, in which 0.1 ml of each substance tested (usually in 0.5-4% solution) was added. A second overlay was added following incubation, containing 1.5% agar and o.o2% neutral red in physiological saline. The antiviral effect of a given compound was recorded on the basis of the size (diameter, Ø, in mm) of the plaque inhibition and the zone of cytotoxicity.

Results and Discussion

Resultant effect of the combined application of PTU-23 and guanidine.HCl

The agar-diffusion plaque-inhibition test of Rada and Zavada (1962) was applied in several experimental designs in FL cells monolayers in Petri dishes. This method gives considerable conveniences for testing the combination effects, i.e. the interaction of two or more substances applied in combination. One of the substances could be included in the agar overlay (its concentration could be varied). Depending on the size of the Petri dishes used one or more cylinders could be fixed in the agar overlay. These cylinders may be filled with substances whose combination effect with a substance included in the agar overly is tested. It is also possible for two substances to be added in one and a same cylinder and their combination effect to be tested against a third substance. By changing the distance between the cylinders in this method, the combination effects manifest themselves within a part of the overlapping diffusion zones of the two substances. It is also possible, to record the effect as a function of the time of application of the substances in the cylinders, etc. Thus, the labour-consuming preliminary procedures are avoided in the course of the cell cultures work with liquid medium including determination of the substances' individual toxicity and their combined toxic effects. In the latter case, a combination of two methods has to be used in order to obtain statistically significant results in the determination of the minimal subtoxic or CC_{50} (50% cytotoxic concentration). According to the described method, a preliminary determination of the virus inoculation dose and of the non-toxic concentration of the substance included in the agar overlay are only required.

In the initial experimental setup, PTU-23 applied at various concentrations (graded from 20 to 60 µg/ml) was included in the agar overlay in Petri dishes (with 90 mm diameter), and guanidine.HCl in a 0.5% solution (5 mg/ml) was added in a cylinder placed in the agar overlay, simultaneously with the virus inoculation . In order to increase the diffusion time of the substances in the agar overlay, FL cells cultures were initially stored for 20 hours at 4° C, and then incubated for 24 hours at 37° C.

As seen in Table 1, a marked antagonistic ef-

Table 1. Effect of PTU-23 and guanidine.HCl on poliovirus 1 (Mahoney) growth in FL cells by the agar-diffusion plaque-inhibition test of Rada and Zavada (1962)

PTU-23 concentration (µg/ml) in the agar overlay	Inhibitory effect of guanidine.HCl (5 mg/ml) in cylinder inhibition zone (Ø mm)
0	67.5
20	51.5
30	52.5
40	32.0
50	30.0
60	27.0

An analogous experiment with some modifications is shown in Figures 1A and 1B.

fect of PTU-23 towards the antiviral effect of guanidine.HCl was registered. It was expressed by diminishment of the diameter of the inhibition zone around the cylinder with guanidine, increasing with the raising of the PTU-23 concentration (up to 60 µg/ml). At higher PTU-23 concentrations a complete inhibition of plaque formation was present.

As seen in Figure 1A, the inhibition zones of PTU-23 40 mg/ml and of guanidine.HCl 1 mg/ml were of approximately equal size in "pure" agar overlay. The guanidine.HCl effect on such agar overlay was one and the same irrespective of the compound addition simultaneously with or 6 hours post the infection onset (inhibition zones with diameters 19.7 and 20.9 mm, respectively).

In Figure 1B the agar overlay contained 30 µg/ml PTU-23, a concentration selected in order to reach a moderate reduction of the plaque number and size, aiming a more clear marking of the inhibition zones of the substances applied in the cylinders. Guanidine.HCl at concentration 1 mg/ml was added in a cylinder (No 1) simultaneously with virus infection or 6 hours post incubation at 37°C of the infected cultures (immediately after infection the Petri dishes were stored for 20 hours at 4 °C). In the same Petri dish, control cylinders with PTU-23 (40 mg/ml) (No 2) and with a mixture of 1/1 v/v guanidine.HCl 1 mg/ml + PTU-23 40 mg/ml (No. 3) were placed. In all cases PTU-23 was dropped simultaneously with the infection, i.e. in one of the test groups the two compounds were applied simultaneously, and in the other test group guanidine was applied 26 hours post PTU-23: 20 hours at 4 °C (PTU-23 diffusion in the absence of virus replication) plus 6 hours at 37 °C.

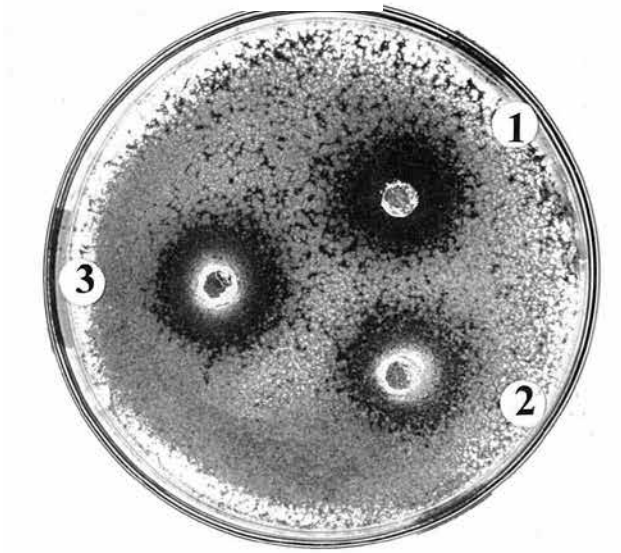
When the substances diffused in the agar overlay containing PTU-23 30 µg/ml (Figure 1B), the following was observed: (i) an additive increase in the PTU-23' inhibition zone (cylinder No 2); (ii) a markedly weakly expressed at the combination PTU-23 + guanidine.HCl in one cylinder (cylinder No 3); (iii) a decrease in the guanidine effect - a decrease in the inhibition zone diameter by 25% when the compound was applied simultaneously with the infection (not illustrated) and a complete disappearance of the guanidine inhibition zone when applied 6 hours post incubation at 37°C (cylinder 1).

It was of interest to check the opposite, i.e. how guanidine.HCl influences the antiviral activity of PTU-23 against poliovirus. An identical experimental setup was used (Table 2): the agar overlay contained guanidine.HCl of various concentrations (5 – 40 µg/ml), and cylinders with PTU-23 (40 mg/kg) and with guanidine.HCl, respectively, were placed simultaneously with the infection.. It was observed that guanidine, even at a lower degree, antagonized the effect of

PTU-23 on poliovirus replication, i.e. a two-way antagonism was registered. It was also seen that guanidine.HCl slightly stimulated the toxic effect of PTU-23.

These data convincingly prove the antagonistic interaction between the effect of PTU-23 and guanidine.HCl. towards poliovirus replication in FL cells. PTU-23 attained a decidedly higher concentration in the poliovirus infected cells and bonded with the virus-specific target before the entry of guanidine.HCl, and *vice versa*.

a



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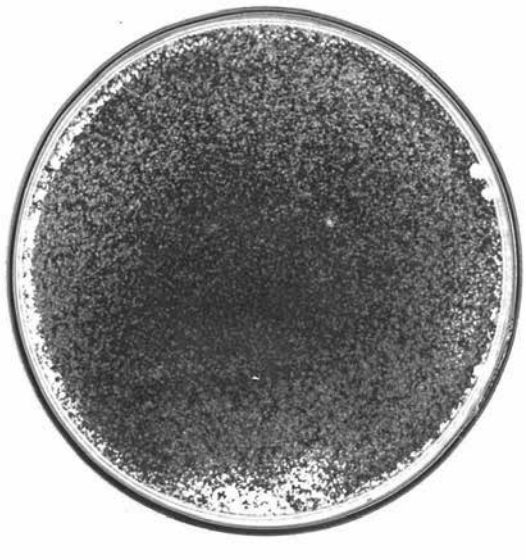

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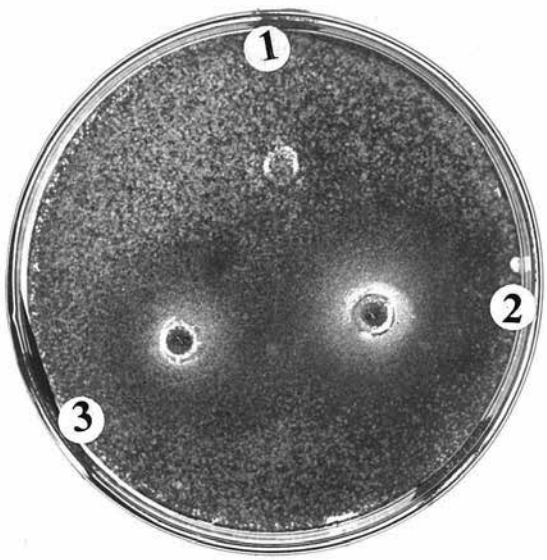

d

Fig.1A. Combination effect of PTU-23 and guanidine. HCl on replication of poliovirus 1 (Mahoney) in FL cells by the agar-diffusion plaque-inhibition test of Rada and Zavada (1962) on ”pure” agar overlay: **a.** virus control; **b.** effect of guanidine.HCl (1 mg/ml) in cylinder **1**; PTU-23 (40 mg/ml) in cylinder **2;** PTU-23 + guanidine 1:1 v/v in cylinder **3.**

Fig. 1B. Combination effect of PTU-23 and guanidine.HCl on replication of poliovirus 1 (Mahoney) in FL cells by the agar-diffusion plaque-inhibition test of Rada and Zavada (1962) on agar overlay containing PTU-23: **c.** effect of PTU-23 (30 μg/ml) included in the agar overlay; **d.** effect of guanidine. HCl (1 mg/ml) in cylinder.**1**; PTU-23 (40 mg/ml) in cylinder **2;** PTU-23 + guanidine.HCl 1:1 v/v in cylinder **3.**

Table 2. Effect of guanidine.HCl on PTU-23 effect towards poliovirus 1 (Mahoney) by the agar-diffusion plaque-inhibition test (1962)

Guanidine.HCl concentration in agar overlay (µg/ml)	Effect of compounds placed in cylinder (Inhibitory zone, Ø mm)	
	PTU-23 (40 mg/ml)	Guanidine.HCl (1 mg/ml)
0	26.0	17.5
10	26.5	34.0
20	20.0	38.0
30	0	52.0
40	0	76.0

Influence of guanidine.HCl antagonists on the PTU-23 effect

It is known that there are substances which diminish or completely cancel the antiviral effect of guanidine.HCl, the so-called guanidine antagonists (Lwoff, Lwoff, 1964; Loddo *et a*l., 1966; Philipson *et al*., 1966).

The experimenmtal setup of the agar-diffusion plaque-inhibition test of Rada and Zavada (1962) was used to test the influence of some guanidfine. HCl antagonists on the inhibitory effect of PTU-23. The following substances were applied: (i) from the first class antagonists (amino acids) - 1-methionine, l-valine, l-leucine, l-isoleucine and l-phenylalanine; and (ii) from the second class (choline-like compounds) - choline chloride. In parallel, the effect of some other substances was checked-up, namely of methanol (possessing also an anti-guanidine effect - Loddo *et al*., 1966), amino acids l-glycine and l-glutamine, and uridine (RNA precursor, in view of the pronounced inhibitory effect of PTU-23 towards virus-specific RNA synthesis).

For this study, a non-toxic for FL cells PTU-23 concentration of 60 µg/ml included in the agar-overlay was previously selected. It exerted complete inhibition of poliovirus 1 in FL cells (in Petri dish with Ø = 90 mm) during a 24-h incubation. Such properties PTU-23 demonstrated within the concentration range of 55-70 µg/ml. For the guanidine.HCl the minimal 100% concentration was ≥ 30 µg/ml.

It was establishes (Table 3) that none of the tested substances influenced the antiviral effect of PTU-23 against poliovirus 1. Among them l-methionine (135 mM), l-isoleucine (1150 mM) and choline-chloride (85 mM) markedly antagonized the guanidine effect.

In the experimental setup presented in Table 4, a "pure" agar overlay was placed on FL cells monolayers in a Petri dish with a diameter of 150 mm. Guanidine.HCl (at a relatively high guanidine concentration of 20 mg/ml) was applied in a cylinder placed in the Petri dish center, and cylinders with PTU-23 (40 µg/ml) and a series of guanidine antagonists in suitable concentrations were placed around in a 30 mm-radius circle. A marked narrowing of the guanidine inhibition zone was manifested by PTU-23 (whose antagonistic effect towards the guanidine activity was described above), along under the effects of l-isoleucine and choline chloride, and at a lower extent by l-leucine and l-valine. Evidently, only substances antagonizing the guanidine effect more strongly were selected at this experimental setup.

Walking all over the concept of Moser *et al*. (1971) antagonists shift the guanidine.HCl from its target, by binding to a receptor locus, adjacent to the locus of guanidine. The lack of effect by guanidine antagonists on the antiviral effect of PTU-23 shows that PTU-23 and guanidine.HCl bind to different receptor loci, i.e. the two enterovirus inhibitors have different targets, although different receptor loci could be situated on one and the same target. The presumable methylating effect of antagonists, according to another concept on their mechanism of action (Lwoff, 1965; Ménard *et al*., 1966)

Table 3. Influence of guanidine antagonists on the effect of PTU-23 against poliovirus 1

Substances Concentration -- in cylinder	Reduction of antiviral effect: Guanidine.HCl 0.25 mM in agar overlay	Reduction of antiviral effect: PTU-23 0.25 mM in agar overlay
l-Methionine 135 mM	+	-
l-Leucine 150 mM	-	-
l-Isoleucine 150 mM	+	-
l-Valine 170 mM	-	-
Choline-chloride 85 mM	+	-

Table 4. Effect of PTU-23 and guanidine antagonists on the antiviral effect of guanidine.HCl towards poliovirus 1(Mahoney) in the agar-diffusion plaque-inhibition test of Rada and Zavada (1962) in FL cells monolayers in Petri dishes

Compound	Conc. (M)	Inhibition zone of guanidine.HCl (20 mg/ml) (radius, mm)	Inhibition zone reduction (Δradius, mm)
Control		28.5	-
PTU-23	40 mg/ml	24.0	4.5
Leucine	$1.5x10^{-1}$	25.9	3.5
Isoleucine	$1.5x10^{-1}$	21.5	7.0
Valine	$1.7x10^{-1}$	25.0	3.5
Choline.HCl	$1.7x10^{-1}$	23.0	5.5

Guanidine.HCl (20 mg/ml) is placed in a cylinder placed in the Petri dish center.
Rest of substances are added in cylinders distanced of 30 mm from the central cylinder with guanidine.

is excluded in the case of PTU-23.

The RNA precursor uridine, taking down effects of a serious of metabolites, does not influence the PTU-23 activity.

Influence of heavy metals (Cu^{++}, Zn^{++}) on the effects of PTU-23 and guanidine.HCl on poliovirus 1 replication

The analysis of structure-activity relationship of diphenylthioureas proposed the hypothesis about the probable role of chelate bonds to their virus-specific target (Galabov *et al*., 1980). Their structure suggests they could be considered as two-centric ligands. In order to verify this probable property the influence of Cu^{++} and Zn^{++} ions on the PTU-23 effect towards poliovirus 1 growth in MK cells was studied. The modification of the plaque-inhibition agar-diffusion method describrd above was used: at a constant PTU-23 concentration (40 mg/ml) in a cylinder placed in the center of the Petri dish, and

$CuSO_4$ and $ZnCl_2$ were included in the agar overlay at concentration gradients. At that, $ZnCl_2$ was applied at concentrations below the one inhibiting poliovirus plaque formation.

It was established (Figure 2) that the combination of PTU-23 with Cu^{++} ($5x10^{-7}$M) or Zn^{++} (10^{-5}M) ions led to a marked increase in PTU-23 antiviral activity. Moreover, Cu^{++} effect was approximately 20 times stronger than the effect of Zn^{++}. Besides, it was found that PTU-23 toxicity zone remains unchanged in the presence of Cu^{++} or Zn^{++} ions. These data showed that (i) PTU-23 functions as a ligand, coordinating heavy metals (Cu^{++} and Zn^{++}); (ii) the complex PTU-23-Cu^{++} is 20-fold more active than the complex PTU-23-Zn^{++}; (iii) the target of the complex PTU-23-Me is virus-specific (the combination does not change the PTU-23 toxicity). Evidently, the receptor of PTU-23 preferably coordinates the Cu^{++} ions.

In parallel, the combination effect of Cu^{++} and Zn^{++} ions with guanidine.HCl (1 mg/ml in the cylinder placed in the Petri dish center) was tested. As seen in Figure 2, Zn^{++} ions do not influence the antiviral activity of guanidine.HCl, whereas Cu^{++} ions (>$5x10^{-7}$M) manifested a marked antagonistic effect on the guanidine activity, i.e. most probably, Cu^{++} ions form a coordination complex with guanidine receptors, interfering with the binding of guanidine with its virus-specific target.

These results support the proposed hypothesis on the mechanism of the observed antagonism between PTU-23 and guanidine.HCl in their effects on poliovirus type 1 replication: (i) attack on different non-structural protein targets, most probably in the virus RNA replicative complex; (ii) the receptors for both compounds preferably coordinate Cu^{++}; (iii) Cu^{++} ions suppress guanidine activity vs poliovirus 1 replication, and increase the antipoliovirus effect of PTU-23. Evidently, guanidine binding to its receptors most probably occurs through weaker and labile bonds, therefore the reversibility of the compound effect is considerable in speed and size.

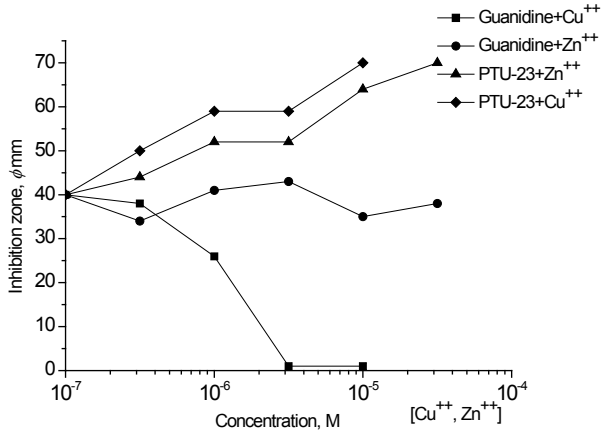

Fig. 2. Effects of PTU-23 and guanidine.HCl in combination with Cu^{++} and Zn^{++} ions on the replication of poliovirus 1 (Mahoney) in MK cells

Conclusion

The studies carried out convincingly manifested the antagonistic character of the combination effects of guanidine.HCl and PTU-23 on the replication of poliovirus 1, based on the concurrence of these two picornavirus replication inhibitors for binding to their specific targets in the viral replication cycle.

Further investigation of the guanidine.HCl and PTU-23 interactions needs to study the effect of PTU-23 on the guanidine mutants of poliovirus 1, and *vice versa*, the guanidine effect on poliovirus strains in the course of selection of PTU-23-resistance.

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