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Pharmacokinetic Study and Toxicity of Leukovir: A New Combined Drug for the Treatment of Multiple Sclerosis

Elena N. Kalinichenko*, Irina V. Ponteleeva, Marina B. Golubeva

State Scientific Institution "Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus", Minsk, Belarus
Email: *kalinichenko@iboch.by

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Abstract

Leukovir, an enteric-coated tablet, is the original drug product for internal use. The well-known nucleosides cladribine and ribavirin are the active ingredients of the drug product leukovir. Pharmacokinetic parameters of the drug product for the internal use of leukovir active ingredients have been established. The cladribine half-absorption period was $t_{1/2a}$ = 49.5 h, C_0 = 276.4 μg/ml, C_{max} = 6.0 μg/ml. Distribution and accumulation parameters (V_d, V_{ss} and AUC) have indicated that the drug distribution between the blood cells and blood plasma takes place in the same way, irrespective of the dosage form. Cladribine half-life period is $t_{1/2e}$ = 0.62 hours. The molecule total clearance and average lifetime in the body in the case of subcutaneous drug administration are approximately the same. Ribavirin is characterized by a half-absorption period of $t_{1/2a}$ = 0.71 h, C_0 = 115.6 μg/ml and C_{max} = 75.5 μg/ml. Ribavirin total volume of distribution (V_d = 1.3 l/kg) and stationary volume of distribution (V_{ss} = 1.64 l/kg) were practically similar to leukovir when administered subcutaneously. The AUC value = 504.2 μg h/ml, which is 2.5 times less than that in the case of drug form administration. Leukovir was regarded as slightly toxic in an acute toxicity study. The risk of cumulation for this drug product is low.

Keywords

Leukovir, Cladribine, Ribavirin, Multiple Sclerosis, Pharmacokinetics, Toxicity

1. Introduction

Multiple sclerosis is a chronic progressive (relapsing or progressive-relapsing, depending on the type of clinical course) inflammatory-degenerative disease of the central nervous system, clinically manifested by disseminated organic neu-

rological symptoms, in a pathomorphological aspect: foci of inflammation and demyelination with subsequent formation of sclerotic plaques in the white matter of the brain and/or the spinal cord [1]. In the treatment of multiple sclerosis, the main target is to achieve stable remission. The main principles of pharmacotherapy in multiple sclerosis are based on a reduction in the frequency of exacerbations, the severity of neurological symptoms, stabilization of the patient's health condition and an effect on the pathological process to prevent the occurrence of further possible exacerbations.

The anti-inflammatory effect of immunomodulators traditionally used to treat multiple sclerosis leads to a significant reduction in the number of multiple sclerosis exacerbations [2]. However, irreversible neurological deficits are a consequence of not inflammatory but neurodegenerative processes in the central nervous system. Therefore, neuroprotective properties in the spectrum of pharmacological activity of the drug proposed for multiple sclerosis therapy is a significant advantage.

The developed drug product cladribine (MAVENCLAD®; Merck Serono Europe Ltd.) for oral therapy has been demonstrated to be effective in recurrent multiple sclerosis therapy in placebo-controlled phase III trials [3] [4] [5] [6]. In comparison with the parenteral route, the enteric route of pharmacological substance administration is preferable for a number of reasons and, first of all, due to the simplicity of the patient's adherence to the regimen and reduction in the cost of therapy, including the cost of medical care. In addition, it has been found that the side effects observed in patients with chronic progressive multiple sclerosis after oral therapy with cladribine were less pronounced than those registered in the case of intravenous (i/v) infusion of cladribine [7]. One of the significant side effects of oral MAVENCLAD is a decrease in immunity with the subsequent progression of infections, including those caused by *herpes simplex*, *varicella zoster* and *cytomegalovirus*.

Previously, we evaluated the immunotropic effect of cladribine and ribavirin combination and individual components in a cellular immune response model (RGZT), as well as the protective potential of the oral formulation leukovir based on this combination in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE) [8] [9]. It was confirmed that the cladribine and ribavirin combination had an immunosuppressive effect on the cellular immune response manifested in the inhibition of antigen-specific T lymphocyte clone formation and proinflammatory cytokines in F1 (CBA × C57Bl/6) hybrid mice. The effectiveness of the cladribine and ribavirin combination was superior to the effectiveness of its individual components.

Therapy with leukovir (70.5 mg/kg, intragastric route, 7 days) resulted in successful control of EAE development in guinea pigs, both in preventive and therapeutic courses of therapy. In the preventive course of therapy with leukovir, the proportion of animals with EAE and mortality were reduced significantly, making it possible to delay the first symptoms of the disease and weaken the

clinical manifestations of the modeled pathology. Inhibition of neurological disorders, reduced mortality and a significant increase in animals' life span after immunization have been observed during the therapeutic course of treatment with leukovir.

In this study, the acute and cumulative toxicity of leukovir was determined, and its pharmacokinetic parameters were evaluated.

2. Materials and Methods

2.1. Materials

All chemicals, solvents and plastic were obtained from commercial suppliers (Thermo Fisher Scientific and Acros Organics). Cladribine, ribavirin and leukovir were received from the SPC "ChemPharmSynthesis", The Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus, licensed for drug product manufacturing.

Oral toxicity and pharmacokinetics were assessed with the use of leukovir administered in the form of whole tablets (when used in rabbits) or crushed tablet masses (when used in mice). One enteric-coated tablet of leukovir contains 1 mg of cladribine and 100 mg of ribavirin as an active ingredient and lactose monohydrate, white sugar, polyvinyl pyrrolidone (povidone), magnesium stearate and corn starch as excipients. The composition of the enteric coating was methacrylic acid and ethyl acrylate copolymer (1:1), propylene glycol, titanium dioxide and talc. Acryl-EZE 93A18597 White (Colorcon®) was also used for the enteric coating. The drug dosage was estimated according to the total content of active ingredients (APIs).

Toxicity studies and pharmacokinetics in parenteral routes of administration were carried out with a cladribine-ribavirin combination (in a ratio of 1:100) dosed according to the total content of active ingredients.

2.2. Animals

Acute toxicity and cumulative toxicity studies were carried out with outbred ICR mice weighing 20 - 25 g each at 3 - 4 months old. A pharmacokinetic study was performed in chinchilla rabbits with a weight of 2.5 - 3.0 kg, 5 - 6 months old.

Laboratory animals were kept in standard vivarium equipped in accordance with the requirements of sanitary-epidemiological and veterinary legislation at the State Scientific Institution "Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus". They were fed granulated feed and grain and had free access to water.

All experiments were performed according to the national Rules of Good Laboratory Practice (TCP 125-2008; 020040), harmonized with the International Rules of Good Laboratory Practice of the Council of the Special Program for the Control of Chemical Products (OECD Principles on Good Laboratory Practice. C (97) 186 Final). All procedures with animals were carried out in accordance with the protocol (No. 01-2014) approved by the Commission on Bioethics of

the State Scientific Institution "Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus".

2.3. Ethical Standards for the Treatment of Animals

In the experiments, sedatives, analgesics, anesthetics were not used. Short-term manipulations (taking blood samples, subcutaneous ones, etc.) do not cause severe pain and suffering to the animals. All test participants have appropriate (medical, biological) education, experience in conducting experiments on animals, without causing them pain, suffering, or inconvenience. To remove the animals from the experiment after its completion, sacrifice was used by introducing (quickly) a solution of magnesium sulfate 25% into a vein.

2.4. Pharmacokinetic Studies

Two experimental groups were formed to study pharmacokinetics (6 animals/group; 6 males) as follows: some of the animals received a single oral dose of leukovir at a dose of 150 mg/kg, and the rest were infected subcutaneously with a similar dose of cladribine and ribavirin combination.

Blood was taken from the rabbit's marginal ear vein every 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, and 10.0 hours following drug administration (irrespective of the route of administration). The blood samples for cladribine and ribavirin determination were carried out by HPLC.

2.5. Blood Samples Preparation

Silicone centrifuge tubes with 4% sodium citrate solution were used for blood sample preparation. Then, 0.6 ml of blood taken from the punctured ear vein was transferred into test tubes. The blood was carefully mixed with sodium citrate. Before centrifugation, the tubes were placed in ice for a short time period (10 - 20 min). Then, blood samples were centrifuged at 3000 rpm for 10 min. The resulting plasma was transferred into 200 μl plastic microtubes and immediately frozen at −20˚C.

2.6. Cladribine and Ribavirin Assay (Quantification)

0.25 Mg of internal standard and 2 ml of acetonitrile were added to 200 μl of blood plasma sample obtained by the method stated above to precipitate plasma proteins. Uridine and 2-fluoro-arabinoadenosine were used as the standards. The samples were centrifuged, the supernatant was separated and evaporated at a temperature of 30˚C, and then 100 μl of water was added to the dried sediment. As a result, the analyzed sample was concentrated 2 times. When extracted with acetonitrile, the extraction coefficient for ribavirin was 95%; for cladribine, it depended on concentrations from 85% at high doses and up to 58% at small doses.

The samples were analyzed by HPLC in a "Waters HPLC system" (Waters, USA) with a UV detector. Chromatographic separation was carried out at a

temperature of 30˚C with a C18 Nova-PakWaters analytical column of 3.9 × 300 mm. The absorption wavelength was 210 nm.

A mixture of 2% methanol in a monopotassium phosphate 0.05 M solution was used as an eluent for ribavirin concentration determination. Volumetric elution rate was 0.7 ml/min. The retention time for Ribavirin was 4.5 minutes. A mixture of acetonitrile 7% solution in monopotassium phosphate 0.1 M solution was used as an eluent for cladribine concentration determination. The retention time of cladribine was 14.9 minutes.

Formulation assays (quantitative determination) were performed with the use of a calibration method. Calibration curves for ribavirin and cladribine were plotted in a system of coordinates, test drug concentration in μg/ml, area response ratio (ratio of the drug peak area to the internal standard area).

Upon analyte concentration data obtained in the blood samples, the following pharmacokinetic parameters were calculated: AUC (μg*h/ml), C_{max} (μg/ml), C_0 (μg/ml), MRT (h), $t_{1/2a}$ (h), $t_{1/2e}$ (h), V_d (l/kg), V_{ss} (l/kg), k_a (h^{-1}), k_e (h^{-1}), Cl (ml/h) and f(%).

2.7. Acute Toxicity Studies

Acute toxicity studies were carried out in healthy outbred ICR mice using a single dose of leukovir administered orally. Eighty-four mice divided into 7 groups were designed for the study of acute toxicity via the oral route. Each group of 12 mice (6 males and 6 females) received a single oral dose of 3500, 3800, 4000, 4500, 5000, and 5500 mg/kg body weight leukovir in the form of a crushed tablet mass (in a natural gum 2% solution), while the control group was treated with distilled water. All studied samples were administered intragastrically (i/g) using a special probe.

Additionally, acute intraperitoneal (IP) toxicity studies were carried out in healthy outbred ICR mice using a single dose of cladribine and ribavirin combination. Seventy-two mice, divided into 6 groups, were designed for the study of acute toxicity via the IP route. Each group of 12 mice (6 males and 6 females) received a single oral dose of 500, 800, 1000, 1500 and 2000 mg/kg body weight of composition, while the control group was treated with distilled water.

The general behavior of the mice and signs of toxicity were observed continuously for 24 h after oral or IP treatment. The mice were further observed once a day for up to 14 days for the following treatment for behavioral changes and signs of toxicity and/or death. The median lethal dose (LD_{50}) value and its 95% confidence intervals (CI) were determined according to the Litchfield and Wilcoxon method [10]. The maximum nonlethal dose (MNLD) value was determined as the highest dose that did not cause death [11].

The Hodge and Sterner Scale was used to determine the toxicity rate of the studied samples [12].

2.8. Cumulative Toxicity Studies

Thirty-six healthy mice (21 - 25 g) were randomly divided into six groups, in

which 6 males were included at a dose of 170. Mice from groups 1 - 3 were administered Leukovir orally daily in the form of a crushed tablet mass at a dose of 170 mg/kg that demonstrated activity in a model of EAE taking into account interspecies dose transfer. Mice from control groups 4 - 6 were treated similarly with 0.9% physiological saline. All studied samples were administered i/g for 5 consecutive days using a special probe.

Based on the LD_{50} results obtained, on the sixth day, mice from groups 1 - 2, 3 - 4, and 5 - 6 were administered leukovir at doses of 3700, 4000 and 4500 mg/kg, respectively, by the oral route. The mice were further observed once a day for 14 days. The total number of dead mice in each group was recorded. The LD_{16}, LD_{50} and LD_{84} values and function of the slope of the dose-response curve (S) were determined according to the Litchfield and Wilcoxon method [10].

The accumulation coefficient (K) was calculated according to the formula:

$$\frac{LD_{50_1} \times S_1}{LD_{50n} \times S_n} \tag{1}$$

$LD_{50(1)}$ and $LD_{50(n)}$, median lethal doses after a single dose administration or repeated dosing, respectively;

$S_{(1)}$ and $S_{(n)}$, a function of the slope of the dose-response curve after a single dose administration or repeated dosing, respectively [13].

$S_{(1)}$ and $S_{(n)}$ values were calculated according to the following formulas:

$$S_1 = \frac{\frac{LD_{84}^1}{LD_{50}^1} + \frac{LD_{50}^1}{LD_{16}^1}}{2} \tag{2}$$

$$S_n = \frac{\frac{LD_{84}^n}{LD_{50}^n} + \frac{LD_{50}^n}{LD_{16}^n}}{2} \tag{3}$$

The greater the value of the cumulation coefficient is from one, the more pronounced the cumulative properties of the study compositions. If $K < 1$, it indicates the development of habituation.

2.9. Statistical Analysis

The results are presented as the mean ± standard error (SE). Statistically significant differences between experimental groups were assessed using Student's t-test for the data that followed a normal distribution. The level of statistical significance was $P < 0.05$. Statistical analysis was performed using Microsoft Excel.

3. Results and Analysis

3.1. Pharmacokinetic Studies

Pharmacokinetic parameters of the drug product leukovir in the form of enteric-coated tablets in enteric administration (orally at the root of the tongue of experimental animal) were compared with the parameters of its active ingredient combination, the substance cladribine and ribavirin (hereinafter referred to as

the combination) with a parenteral route of administration (subcutaneously). The results of the pharmacokinetic study of cladribine and ribavirin in the analyzed samples are presented in Table 1.

Table 1. Pharmacokinetic parameters of cladribine and ribavirin in leukovir (oral) composition and API combination (subcutaneous).

Parameters 1	Drug product 2	API combination 3
	Cladribine	
absorption	k_a = 0.014 h^{-1}	k_a = 1.5 h^{-1}
	$t_{1/2\alpha}$ = 49.5 h	$t_{1/2a}$ = 0.46 h
	C_{max} = 6.0 mcg/ml	C_{max} = 19.0 mcg/ml
	C_0 = 276.4 mcg/ml	C_0 = 3.2 mcg/ml
Distribution and accumulation	V_d = 0.54 l/kg	V_d = 46.7 l/kg
	V_{ss} = 9.93 l/kg	V_{ss} = 7.8 l/kg
	AUC = 30.7 mcg·h/ml	AUC = 43.9 mcg·h/ml
elimination	k_e = 1.11 h^{-1}	k_e = 0.63 h^{-1}
	$t_{1/2e}$ = 0.62 h	$t_{1/2e}$ = 1.11 h
	Cl = 4.89 ml/h	Cl = 3.42 ml/h
	MRT = 2.03 h	MRT = 2.3 h
bioavailability of Cladribine (*f*) in the claimed drug product composition, 70%		
	Ribavirin	
absorption	k_a = 0.972 h^{-1}	k_a = 8.99 h^{-1}
	$t_{1/2a}$ = 0.713 h	$t_{1/2a}$ = 0.077 h
	C_{max} = 75.5 mcg/ml	C_{max} = 209.8 mcg/ml
	C_0 = 115.6 mcg/ml	C_0 = 172.37 mcg/ml
Distribution and accumulation	V_d = 1.298 l/kg	V_d = 0.870 l/kg
	V_{ss} = 1.635 l/kg	V_{ss} = 1.138 l/kg
	AUC = 504.24 mcg·h/ml	AUC = 1275.13 mcg·h/ml
elimination	k_e = 0.213 h^{-1}	k_e = 0.105 h^{-1}
	$t_{1/2e}$ = 3.25 h	$t_{1/2e}$ = 6.6 h
	Cl = 0.300 ml/h	Cl = 0.118 ml/h
	MRT = 5.45 h	MRT = 9.64 h
bioavailability of Ribavirin (*f*) in the claimed drug product composition, 40%		

Note: k_a: absorption rate constant; $t_{1/2}$: half-absorption period; C_{max}: maximum concentration; C_0: apparent initial concentration; V_d: apparent volume of distribution; V_{ss}: stationary volume of distribution; AUC: area under the pharmacokinetic curve; k_e: elimination rate constant; $t_{1/2e}$: half-elimination period; Cl: total clearance; MRT: mean retention time; *f*: bioavailability.

Based on the data presented, the cladribine kinetics of absorption when administered in tablet form at a dose of 150 mg/kg orally were characterized by a long half-absorption period ($t_{1/2a}$ = 49.5 h), which was undoubtedly due to the very low value of its absorption rate constant (k_a = 0.014 h^{-1}). After tablet intake, cladribine C_{max} became 6 μg/ml, and C_0 reached 276.4 μg/ml. Consequently, the pharmacokinetic parameters and cladribine absorption rate constant upon oral administration of leukovir demonstrated a sharp slowdown in penetration and absorption of the active substance in comparison with its absorption when administered subcutaneously as a combination drug.

Cladribine bioavailability when consumed in an oral dosage form of leukovir with calculations based on the drugs AUC value was 70% [14]. Since cladribine bioavailability when administered subcutaneously was higher than that when administered enterally and was 100% of the administered dose [15] [16], it was obvious that cladribine bioavailability when consumed in an oral dosage form of leukovir was very high.

Ribavirin kinetics of absorption at Leukovir tablet administration had a longer half-absorption period ($t_{1/2a}$ = 0.71 h) and almost one order of magnitude lower values of the absorption rate constant (k_a = 0.972 h^{-1} and 8.99 h^{-1}, respectively). In this case, ribavirin C_{max} was 75.5 μg/ml, and C_0 reached 115.6 μg/ml. Therefore, ribavirin penetration and absorption slowed down in comparison with the same process in subcutaneous administration of the API composition. The initial effective concentrations of ribavirin and C_{max} in the case of leukovir oral administration were significantly lower than the same values of the reference drug product.

3.2. Acute Oral Toxicity

Table 2 demonstrates the effects of leukovir in mice after acute oral administration. Mild signs of toxicity, including anorexia, hypoactivity and diarrhea, were observed at a dose of 3500 mg/kg. The mortality rate and acute toxicity of leukovir oral administration increased progressively as the dose increased from 3800 to 5500 mg/kg.

The mortality produced in the animals by leukovir was used to calculate the LD_{50}, which was 4050 (3649 ÷ 4496) mg/kg for male mice and 4150 (3915 ÷ 4399) mg/kg for female mice. MNLD was 3500 mg/kg.

Table 3 demonstrates the effects of cladribine and ribavirin composition in mice after acute IP administration. Mild signs of toxicity, including anorexia and hypoactivity, were observed at a dose of 500 mg/kg. The mortality rate as well as the acute toxicity of the IP-administered cladribine and ribavirin composition at doses > 800 mg/kg increased progressively in a dose-dependent manner. The mortality produced in the animals by the cladribine and ribavirin composition was used to calculate the $LD_{50,}$ which was 1100 (846 ÷ 1430) mg/kg for male mice and 1200 (896 ÷ 1608) mg/kg for female mice. MNLD was 500 mg/kg.

3.3. Cumulative Toxicity

The accumulation coefficient (K) was 1.03 ($1 < K < 3$), calculated according to

Table 2. Mortality and clinical signs of acute toxicity of leukovir administered orally to mice with ICR.

Dose of Leukovir (mg/kg)	Mortality Death/Total		Toxic symptoms
	♂♂	♀♀	
0	0/6	0/6	None
3500	0/6	0/6	Hypoactivity, anorexia, diarrhea
3800	2/6	1/6	Hypoactivity, anorexia, diarrhea, weight loss, breathing abnormalities
4000	3/6	3/6	Hypoactivity, anorexia, diarrhea, weight loss, breathing abnormalities
4500	4/6	5/6	Hypoactivity, anorexia, diarrhea, weight loss, breathing abnormalities
5000	5/6	5/6	Hypoactivity, anorexia, diarrhea, weight loss, breathing abnormalities
5500	6/6	6/6	Hypoactivity, anorexia, diarrhea, weight loss, breathing abnormalities

Table 3. Mortality and clinical signs of acute toxicity of cladribine and ribavirin composition administered intraperitoneally to mouse ICR.

Dose of Leukovir (mg/kg)	Mortality Death/Total		Toxic symptoms
	♂♂	♀♀	
0	0/6	0/6	None
500	0/6	0/6	Hypoactivity
800	1/6	1/6	Hypoactivity, decreased response to stimuli, breathing abnormalities
1000	3/6	2/6	Hypoactivity, decreased response to stimuli, breathing abnormalities
1500	4/6	4/6	Hypoactivity, decreased response to stimuli, breathing abnormalities
2000	6/6	6/6	Hypoactivity, decreased response to stimuli, breathing abnormalities

the formula (4100 × 1.13)/(3950 × 1.14). The value of the accumulation index indicated the slightly expressed cumulative properties of the drug product and reversibility of the toxic effects of leukovir when administered to mice orally for 5 consecutive days.

4. Discussion

Previous studies displayed promising results of the cladribine/ribavirin combination and the oral drug leukovir based on this combination [8] immunotropic potential, thus justifying the necessity of further development of this drug. Preclinical development programs included acute toxicity studies and cumulative toxicity studies, as well as pharmacokinetic assessments.

We have changed the approach to cladribine oral dosage form development. The active substances of the dosage form, called Leukovir®, are cladribine and ribavirin, which determine the pharmacodynamic processes of the drug product. It was suggested that the addition of a ribavirin nucleoside analog to the basic drug product cladribine, which has a very wide spectrum of activity against DNA- and RNA-containing viruses and possesses immunotropic action, will re-

sult in a more rational dosage form receipt for oral administration than monopreparation.

Limitation to cladribine administration, also as a part of combined therapy *per os* route of administration, is its low bioavailability [17] in the range of 37% - 51% [18], which is primarily based on the low stability of cladribine under acidic stomach conditions (normally, the basal level of pH secretion in the stomach corresponds to 1.5 - 2.0). Cladribine is stable in alkaline and neutral media in the temperature range of 37˚C - 80˚C; in acidic environments, it is destroyed even at temperatures within the physiological norm. At 37˚C and pH = 2 after 6 hours, only 13% of cladribine is determined in solution; at pH = 1 after 2 hours, less than 2% of cladribine is remained [19]. The developed drug in the form of enteric-coated tablets proposed for the treatment of multiple sclerosis is characterized by high bioavailability and proven neuroprotective activity in the EAE model [8], resulting in its corrective effect on the neurological component of multiple sclerosis.

The proposed composition affects the cellular immune response of experimental animals. At the same time, it should be noted that in multiple sclerosis pathogenesis, not only cellular but also humoral immunity factors play an important role [20]. Both active ingredients act as antiproliferative agents primarily on hematopoietic cells, including lymphocytes; exhibit an immunosuppressive effect on T lymphocytes and B lymphocytes; inhibit the functional activity of activated T cells and inflammatory and autoimmune processes in the body; and affect regulatory and inflammatory cytokine and chemokine expression.

Active substances are also characterized by significant pharmacodynamic differences that apparently play an important role in the beneficial drug mechanisms that affect multiple sclerosis [8].

Cladribine absorption occurs mainly with participation of the small intestine nucleoside transporter (ENT1) [21], while ribavirin absorption is provided by purine (CNT2) [22] and ENT1 intestinal transporters. Taking these data into account, the use of delivery systems that ensure the described combination of active ingredients direct transfer into the small intestine determines an increase in their bioavailability and provides an opportunity to reduce the administered doses of pharmacologically active substances.

R. Hermann noted in his article [23] that compounds requiring intracellular phosphorylation for activation, such as lamivudine, zalcitabine, ribavirin, stavudine and zidovudine, should not be taken together with cladribine in MS treatment. This statement is based on a case report of cladribine and lamivudine interaction in a patient with B-cell chronic lymphocytic leukemia, and *in vitro* study results demonstrated the ability of lamivudine to inhibit intracellular phosphorylation of cladribine, which may be associated with a risk of its treatment effectiveness reduction in lymphoproliferative diseases [24].

However, there is a suggestion that cladribine phosphorylation by deoxycytidine kinase (dCK) is not the only mechanism that may explain cladribine activi-

ty [25] [26] [27]. Prevention of cladribine phosphorylation in the case of excessive deoxycytidine does not affect the inhibition of cytokine secretion by lymphocytes observed in the case of cladribine administration [25]. These effects can be explained by the ability of cladribine, which is a derivative of adenosine, to bind to adenosine receptors [26] [28] involved in immune response modulation [29].

Our study results have shown that cladribine together with ribavirin, and possibly with other nucleosides with antiviral effects whose activation required dCK phosphorylation, demonstrated an additive effect with an increase in suppressor effect by more than 2 times in comparison with the total effect of individual ingredients [8].

The model pharmacokinetics of Leukovir® in the form of enteric-coated tablets for oral administration are characterized by a relief deceleration of cladribine absorption and gradual achievement of C_{max} = 6000 ng/ml without a sharp peak and a high degree of active substance accumulation in the body (AUC = 30,920 ng×h/ml) with tablet bioavailability 70%, which indicates a new dosage form with prolonged action. In a relatively short period of time ($T_{1/2}$ = 0.62 h), the cladribine concentration in blood plasma decreased by 50%. This period for the claimed drug product was almost 2 times shorter than that for the cladribine and ribavirin combination without tablet mass formation.

The total clearance of cladribine from the claimed drug product increased by 1.4 times, and the average lifetime of an active substance molecule in the human body practically did not change (2.03 and 2.3 h).

Enteric coatings made it possible to create long-acting (retarded) dosage forms with a delayed release of active ingredients. The coating is characterized by alkaliphilic properties that ensure tablet movement through the stomach acidic environment in an unchanged form and the release of active components after their solubilization into the intestine. This approach enables the use of active ingredients in lower doses and therefore is associated with a decrease in the manifestation of drug toxic effects.

The acute toxicity evaluation in outbred mouse ICR demonstrated in this study showed that the oral LD_{50} value of leukovir was 4050 - 4150 mg/kg and the IP LD_{50} value of the cladribine/ribavirin combination was 1100 - 1200 mg/kg. MNLD values for the oral and IP routes of administration were 3500 mg/kg and 500 mg/kg, respectively, and practically did not depend on mouse sexual dimorphism. According to Hodge and Sterner [12] and based on oral LD_{50} determination, six classes of toxicity have been classified: "Class 1 = extreme toxicity, LD_{50} < 1 mg/kg; Class 2 = high toxicity, LD_{50} at 1–50 mg/kg; Class 3 = moderate toxicity, LD_{50} at 50 - 500 mg/kg; Class 4 = low or slight toxicity, LD_{50} at 500 - 5000 mg/kg; Class 5 = practically nontoxic, LD_{50} at 5000 - 15,000 mg/kg, and Class 6 = relatively harmless with LD_{50} > 15,000 mg/kg". According to this classification and taking into account the LD_{50}, we noted that leukovir with LD_{50} values of 4050 - 4150 mg/kg could be graded in the slightly toxic category.

The accumulation coefficient (K) of orally administered leukovir was 1.03 in outbred mouse ICR, suggesting extremely low cumulative risk levels of this drug.

5. Conclusions

The preclinical acute toxicity study of a new combined drug product leukovir in the form of enteric-coated tablets, its cumulative potential after oral administration, and its pharmacokinetic parameters are described in this article. The acute toxicity results showed that the oral LD_{50} value of leukovir was lower than 5000 mg/kg, and it was considered that this drug was slightly toxic. The risk of cumulation for this drug was low.

The model pharmacokinetics of Leukovir® in the form of enteric-coated tablets for oral administration are characterized by a relief deceleration of cladribin absorption and gradual achievement of C_{max} = 6000 ng/ml without a sharp peak and a high degree of active substance accumulation in the body (AUC = 30,920 ng × h/ml) with tablet bioavailability 70%, which indicates a new dosage from leukovir with prolonged action. In a relatively short period of time ($T_{1/2}$ = 0.62 h), the cladribin concentration in blood plasma decreased by 50%. This period for the claimed drug product was almost 2 times shorter than that for the cladribine and ribavirin combination without tablet mass formation.

The total clearance of cladribine from the drug product leukovir increased by 1.4 times, and the average lifetime of an active substance molecule in the human body practically did not change (2.03 and 2.3 h).

Pharmacokinetic data indicate cladribin and ribavirin interaction in composition of Leukovir® tablets at the stages of absorption, distribution and excretion. This interaction does not prevent an effective concentration in blood plasma and contributes to a change in the nature of active substance distribution in the human body and the substance elimination rate in the direction of process optimization.

The drug product Leukovir® in the form of enteric tablets for oral administration according to the clinical study results [30] is recommended for use in clinical practice as a drug product with neuroprotective properties in a complex therapy of relapsing-remitting and secondary progressive forms of multiple sclerosis.

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Author Contributions

M.B.G. performed the experiments; I.V.P. analyzed the data, wrote the draft of

this manuscript; E.N.K. supervised the study and wrote the manuscript. All the authors contributed equally and were fully aware of submission.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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