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Anti-Influenza Virus Activity of 1-(4-Morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (Mopyridone)

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Abstract

Mopyridone inhibited the replication of influenza viruses A(H3N2), A(H2N2),) and B in MDCK and in primary calf kidney (CK) cell cultures, a higher activity been found in CK cells. The effects against A(H1N1) and A(H7N7) sybtypes strains were distinctly lower. Mopyridone at effective concentrations did not influence DNA, RNA and protein syntheses in intact cells. The compound-susceptible period in the influenza virus one-step growth cycle embraces the first 4 hours post virus adsorption. The high susceptibility of the A(H3N2) subtype to mopyridone was also manifested in embryonated eggs tests. Mopyridone was superior in comparison to rimantadine by its stronger and more selective *in ovo* effect. The compound demonstrated a marked anti-influenza activity in mice experimentally infected with influenza A(H3N2) and B viruses (even at massive virus inocula). This activity was similar to that of rimantadine by its protective rate, but a significantly higher by its selectivity: a selectivity (therapeutic) ratio value of 426 been recorded. Besides, mopyridone showed a week protective effect in the case of mouse infection with A/Puerto Rico/8/34 (H1N1) strain (drug-resistant in the *in vitro* experiments). The compound optimal treatment course was determined: 37.5 mg/kg orally daily (divided in two intakes) for 5 days from the day of infection.

Key words: influenza viruses, mopyridone, rimantadine, effect in vitro, activity in mice

Резюме

Мопиридон инхибира репликацията на грипни вируси A(H3N2), A(H2N2) и B в клетки MDCK и в първична култура от телешки бъбрек (СК), като по-висока активност бе установена в клетки СК. Ефектът спрямо щамове на подтипове A(H1N1) и A(H7N7) бе значително по-нисък. Мопиридон в ефективни концентрации не повлиява синтезите на ДНК и РНК, както и протеиновия синтез в интактни клетки. Чувствителният към съединението период в едностъпния репликативен цикъл на грипния вирус обхваща първите 4 часа след вирусната адсорбция. Високата чувствителност на щамове A(H3N2) към мопиридон се репродуцира и в тестове в кокоши ембриони. Мопиридон превъзхожда римантадин с по-силния си и по-избирателен ефект *in ovo*. Съединението показва също така отчетлива анти-грипна активност в мишки, експериментално заразени с грипни вируси A(H3N2) и B (даже при масивни вирусни инокулуми). Тази активност е подобна на активността на римантадин по индекса на протекция, но е значително по-висока по своята избирателност: селективният (терапевтичният) индекс на мопиридон е 426. Друга разлика на мопиридон от римантадин е неговия слаб протективен ефект при инфекция на мишки с щама A/Puerto Rico/8/34 (H1N1) (резистентен към мопиридон в експерименти *in vitro*). Определен бе оптималният терапевтичен курс с мопиридон: перорална дневна доза 37.5 мг/кг (разделена на два приема) в продължение на 5 дни от деня на заразяването.

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Introduction

At present two groups of antivirals effective on replication of influenza viruses are recommended for the treatment of flu: [i] M2 protein blockers rimantadine (α-kethyl-1-adamantane-methylamine hydrochloride), amantadine (1-aminoadamantane hydrochloride) and adapromine (ethyl-1-adamantatylmethylamine hydrochloride) (Zlydnikov *et al.,* 1987; Kubar, 1988); [2] viral neuraminidase inhibitors oseltamivir, zanamivir and peramivir. The great problem for the M2 blockers is the relatively quick development of drug-resistance and the lack of effect against influenza B virus.

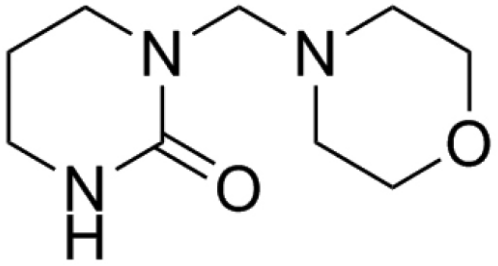

Fig. 1. 1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (mopyridone)

The present paper deals with 1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (mopyridone) (Fig. 1), a compound synthesized originally by Sidzhakova *et al*. (1982), and found to have anti-orthomyxovirus and anti-togavirus effects in a broad-scope antiviral screening carried out (Galabov *et al.,* 1984). Here we describe studies characterizing the *in vitro, in ovo,* and *in vivo* effects of mopyridone.

Matherials and Methods

Compounds

Mopyridone (MMTHP, DD-13, MCU), with molecular weight 199.25, m.p. 143-146°C, represents white fine crystals, easily soluble in water. The compound was synthesized by Dorotea Sidzhakova (Faculty of Pharmacy, Medical University of Sofia; Medical University of Plovdiv, Bulgaria).

Rimantadine hydrochloride was kindley supplied by Georgii A. Galegov (The D.I. Ivanovskii Institute of Virology, Moscow, Russia) and Vera I. Ilyenko (Research Institute of Influenza, St. Petersburg, Russia).

Cells and media

Primary chick embryo fibroblast cultures (CEF) were prepared after Porterfield (1960) by seeding cell suspension, $1.1x10^6$ cells/ml, in a growth medium Eagle's MEM (Difco) supplemented with 10% calf serum.

Primary calf kidney cultures (CK) were prepared by Bodians's (1956) method,

The cell suspension, $6\text{-}8x10^5$ cells/ml, was grown in a medium of 10% calf serum, 0.2% egg hydrolisate and 0.5% lactalbumine hydrolysate in Hanks' saline.

Madin-Darby canine kidney (MDCK) cells were cultivated in a medium of 10% foetal bovine serum (Flow) in Eagle's MEM (Flow), pH 7.5.

Embryonated eggs

Nine-to-eleven-day old embryonated eggs of Leghorn hens were employed.

Mice

White mice of the randomly bred H line, 10 g body weight, were used. Mice of the ICR randomly bred line weighing 20 g were employed in experiments with influenza virus A/Victoria/35/72 (H3N2) only.

Viruses

The following influenza virus strains were used: A/chicken/Germany/27 (FPV, Weybridge) (H7N7), A/Puerto Rico/8/34 (H1N1), A/England/333/80 (H1N1), A/Chile/1/83 (H1N1), A/Sofia/1672/86 (H1N1) [a clinical isolate of Chile/1/83), A/Taiwan/1/86(H1N1),A/Krasnodar/101/59 (H2N2), A/Hong Kong/1/68 (H3N2), A/Aichi/2/68 (H3N2), A/Victoria/35/72 (H3N2), /Sofia/897/87 [a clinical isolate of A/Philippiines/2/82 (H3N2)], A/Mississippi/1/85 (H3N2), A/Sofia/2541/87 (a clinical isolate of A/Mississippi/1/85), A/Leningrad/360/86 (H3N2), B/Lee/40 and B/Beijing/1/87. The influenza virus strains adapted to mice (A/Puerto Rico/8/34, A/Aichi/2/68, A/Victoria/35/72 and B/Lee/40) were passed serially by intranasal route in mice using lung extracts as inocula and by single alternative passages in embryonated eggs. These strains were received from the collections of the D.I.Ivanovskii Institute of Virology, Moscow (A/Aichi/2/68) and of the Research Institute of Influenza, St. Petersburg (A/Puerto Rico/8/34, A/Victoria/35/72, B/Lee/40). All other strains were cultivated through serial allantoic passages in chick embryos and were received through the courtesy of Dr Rossitsa Kotseva (National Influenza Center, National Institute of Infectious and Parasitic Diseases, Sofia).

Cytotoxicity test

The effect of the test compound on uninfected confluent cell monolayer and cellular morphology was traced for overt signs of cytotoxicity during 96-h incubation at 37°C and the maximum tolerated (nontoxic) concentration (MTC) value has being determined. In addition, quantitative assessment of

possible cytostatic effect was made by growing uninfected cells in the presence of the compound studied till reaching the stationary growth phase (48 h for CEF and MDCK cells, and 96-120 h for CK cells). The 50% cell growth inhibitory concentration ($CGIC_{50}$) value was evaluated on the basis of the average cell number counted.

Cellular DNA, RNA and protein synthesis

Uninfected monolayer CEF cultures (in the stationary phase), grown in 20-ml scintillation vials, were treated with the compound tested for 18 h. ^{3}H-Thymidine or ^{3}H-uridine (Amersham, both with specific activity 5 Ci/mM), 2.5 μCi/ml each, were added to treated or untreated (control) cells for 60 min after 17 h of incubation. For protein labeling a ^{3}H-labelled amino-acid mixture, 1 μCi/ml each of l-leucine, l-valine and l-phenylalanine (UVVVI, Prage, Czech Republic, specific activity 150 mCi/mM), was applied after the same scheme. The acid-insoluble products of the DNA, RNA or protein synthesis were retained on nitrocellulose membrane filters (Synpore RuFS, Czech Republic) and washed with 5% trichloracetic acid. The radioactivity was measured in a non-polar scintillation solution via an Intertechnique counter (Comef, France).

CPE inhibition assay procedure

Monolayer cell cultures grown in Flow 96-well plastic microplates were inoculated with serial 10-fold virus dilutions (1 - 1000 $CCID_{50}$), 0.01 ml per well, by 60 min adsorption at room temperature. Then, the compounds tested at 0.5 $\log_{10}$ increasing consecutive concentrations were added to the maintenance medium (0.2 ml per well of 2% v/v 1M HEPES buffer in Eagle's MEM Flow medium containing 3 μg/ml trypsin, penicillin 100 U/ml, and streptomycin 100μg/ml). The plates were incubated at 37°C for 4 days and viral CPE was followed every day by inverted light microscope at 125 x magnification. Four wells per test sample were used. CPE was scored on a 0 - 4 basis with 4 representing total cell destruction. These data were used to obtain dose-response curves at 10 - 100 $CCID_{50}$ viral doses. From these graphs the minimal inhibitory concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC_{50} value) was determined.

Plaque-inhibition test

It was carried out according to technique of Herrmann(1961)-Siminoff (1961). Monolayer CEF cultures in 70 mm diam. Petri dished (Anumbra, Czech Republic) were inoculated with 100-130 PFU of FPV per dish by 60 min adsorption at room temperature. The compounds tested were incorporated in the agar overlay (1% Bactoagar Difco in Eagle's MEM Difco medium with 10% heated calf serum, 1.65 mg/ml sodium bicarbonate, penicillin 100 U/ml, and streptomycin 100μg/ml). The mean plaque number (3 dished per test sample) and the PFU percentage to the control, respectively, were checked after 72 h of incubation at 37°C.

Kinetic (timing of addition) studies

The one-step cycle design was followed. Monolayer cultures of CK cells were inoculated with influenza virus B/Lee/40 at multiplicity of infection (m.o.i.) 8-10. Mopyridone at MTC was added to the maintenance medium (3% calf serum in Eagle's MEM Difco medium) immediately after virus inoculation (0 h) or at the 2nd, 4th, 6th, 8th, 10th and 12th h. The infectious virus yields were recorded at the 6th, 8th, 10th, 12th and 15th h post virus inoculation (incubation at 36°C) in EID_{50}/ml (in an assay procedure of 72 h incubation at 33°C).

Toxicity testing in embryonated eggs

Substances tested at different doses (mopyridone 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 12 or 24 mg per embryo; rimantadine 0.094, 0.187, 0.375, 0.75, 1.5, 3, 6 or 9 mg per embryo) as serial dilutions in Dulbecco's PBS were injected by allantoic route (in 0.2 ml volume) in 10-11-day-old embryos. The TD_{50} (50% toxic dose) and MTD (maximum tolerated dose) for embryonated eggs were determined by checking-up both the viability and the hatching rate.

Antiviral action testing in embryonated eggs

Virus infection was performed in the allantoic sac (0.1 ml inoculum volum) and the substance tested was introduced (0.2 ml) also by allantoic route 60 min before virus inoculation. Virus and compound dilutions were done in Dulbecco's PBS. The experimental setup used represents parallel viral titrations in the presence or absence of the substance tested. The antiviral effect was measured on the basis of $\Delta\log_{10}$ EID_{50} evaluation.

Compound toxicity determination in mice

Acute (single-dose) toxicity of mopyridone for white mice was determined after the routine procedure (a 7-day observation period, 10 animals per test group), oral and subcutaneous LD_{50} been calculated using the Pershin method (cf. Kudrin and Ponomareva, 1967).

Short-term toxicity was assessed by twice daily oral or subcutaneous dosing for 8 days on groups of 10 mice per each dose. Changes in general condition, behavior and body weight of animals were followed for 15 days after treatment onset and the maximum tolerated dose (MTD) was determined.

Experimental influenza virus infections in white mice and criteria for assessment of the compound effect

White mice were infected intranasally with appropriate inoculation dose (LD_{95} or higher) of influenza virus strains A/Puerto Rico/8/34, A/Aichi/2/68, A/Victoria/35/72 or B/Lee/40 (12-20 animals per test group). Compound and placebo treated animals were observed for 14-16 days post infection and the compound effect was determined on the basis of the following indices: (a) mortality rate with a calculation of the protection index (PI) = [(PC-1) / PC] x 100, where PC (protection coefficient) is the ratio between mortality percentage in placebo and mopyridone treated test groups at the end of the observation period; (b) mean survival time (MST). The maximum error (Δ_p) of the mortality rate relative portion (p) was evaluated by the Fisher's φ-method (the Van der Warden's method being used in cases of p = 0 or 100 %). The standard error of the quadratic deviation of the MST (σ_x) was calculated as described previously (Karparov *et al.*, 1985).

Results

In vitro cytotoxicity studies

Studies in three types of cell cultures (Table 1) found that (a) mopyidone toxicity is lowest in CEF cells; (b) mopyridone is less toxic than rimantadine. The mopyridone $CGIC_{50}$ value is higher than that of rimantadine, 4 times in CEF, 3 times in MDCK cells and approximately 2 times in CK cells, respectively.

The effects of the compound on the cellular DNA, RNA and protein synthesis were studied by using radioisotope methods. It was found that 18-h treatment of confluent CEF monolayers with mopyridone in the concentration range of 0 - 100 μg/ml does not decrease the ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-amino acids incorporation rates as compared to the untreated controls (data not shown).

Susceptibility of influenza viruses to mopyridone

Mopyridone showed high activity against influenza virus A, subtypes H3N2 and H2N2, and influenza virus B by the CPE inhibition assay procedure (Table 2). A clearly higher (ten times and more) of the compound effect was revealed in CK cells as compared to MDCK cells when tested versus A(H3N2) strains. It was worth noting that mopyridone potency against influenza virus A (H2N2, H3N2) strains was similar to that of rimantadine. In the case of A(H3N2) strains in MDCK cells the rimantadine MIC_{50} value was 2 - 10 times lower than that of mopyridone, but the selectivity index ($SI = CGIC_{50}/MIC_{50}$) values were comparable, and in CK cells the mopyridone SI index values were higher as compared to the rimantadine ones.

Influenza virus A/Puerto Rico/8/34 (H1N1) could be qualified as slightly sensitive or resistant (in analogy to rimantadine) to mopyridone (Table 2). A similar low susceptibility to mopyridone was observed when tested other influenza virus A(H1N1) strains: A/England/333/80 (SI = 35.1), A/Chile/1/83 (SI = 8.8) and Taiwan/1/86 (SI = 35.1).

A substantially lower activity mopyridone manifested also against influenza virus A(H7N7). The mopyridone effect on FPV/Weybridge replication in CEF cultures was one thousand fold less than that of rimantadine (Table 2). This was also demonstrated by the plaque-inhibition test in the

Table 1. Effect of mopyridone and rimantadine on the MDCK, CEF and CK cell growth and monolayer state

Cell culture	Mopiridone		Rimantadine	
	$CGIC_{50}$[a] μg/ml	MTC[b] μg/ml	$CGIC_{50}$[a] μg/ml	MTC[b] μg/ml
MDCK	54.8 (49.7,57.0,57.7)	60.0 (60.0,60.0)	18.0 (13.0, 22.6,18.5)	25.0 (20.0,30.0)
CEF	59.6 (57.5, 61.7)	60.0 (50.0, 50.0)	15.1 (16.7, 13.5)	10.0 (10.0, 10.0)
CK	40.2 (42.7, 37.7)	50.0 (50.0, 50.0)	23.7 (23.7, 23.7)	25.0 (25.0, 25.0)

[a]$CGIC_{50}$ value is the mean from 2-3 experiments (in each experiments: 3 culture samples per drug concentration were recorded on the 24, 48 and 72 h after the cell seeding).

[b]MTC value is the mean from 2 experiments (4 culture samples per drug concentration). The results of individual experiments are listed in brackets.

Table 2. Antiviral activity of mopyridone and rimantadine against influenza viruses A and B strains in cell cultures (CPE inhibition assay)

Influenza virus strain	Cell culture	Mopyridone		Rimantadin	
		MIC_{50}[a], μg/ml	SI[b]	MIC_{50}, μg/ml	SI
A/PR/8/34	MDCK	11.8	4.6	12.6	1.4
A/Krasnodar/101/59	MDCK	≤0.1	≤548.0	≤0.1	≤180.0
A/Hong Kong/1/68	MDCK	0.32	171.2	0.05	360.0
A/Aichi/2/68	MDCK	0.27	203.0	0.13	138.5
	CK	≤0.03	≤1340.0	≤0.03	≤790.0
A/Victoria/35/72	MDCK	0.45	121.8	0.04	450.0
	CK	≤0.03	≤1340.0	0.06	395.0
FPV/Weybridge	CEC	14.5	4.1	≤0.01	≤1510.0
B/Lee/40	MDCK	0.05	1096.0	>20.0	<0.9
B/Beijing/1/87	MDCK	0.12	456.7	>10.0	<1.8

[a]Determined in 3-5 experiments.
[b]CGIC50/MIC50.

same cell culture, in which the 50% plaque inhibitory concentration of mopyridone was 4507-fold higher than that of rimantadine (32.9 and 0.0073 μg/ml, respectively).

Timing-of-addition study

In order to determined the mopyridone-susceptible period in influenza virus (B/Lee/40) replication cycle the one-step growth cycle setup in CK cells was performed. The compound was applied at MTC (Fig. 2). A marked inhibition of infectious virus production ($\Delta logs_{10}$ EID_{50} = 2.0) was established when mopyridone was applied to the maintenance medium within the period 0-4th h post virus inoculation. Its addition at the 8th h and later was without effect.

Toxicity study on embryonated eggs

MTD of mopyridone was more than twice higher than that of rimantadine, ≥ 6 mg and 3 mg per embryo, respectively. Obviously, mopyridone TD_{50} lies between 9 and 12 mg per embryo, and rimantadine TD_{50} - between 3 and 6 mg, respectively. These data for rimantadine coinside with Indulen *et al.* (1979) data: MTD and TD_{50} values of 3 and 4 mg per embryo, respectively.

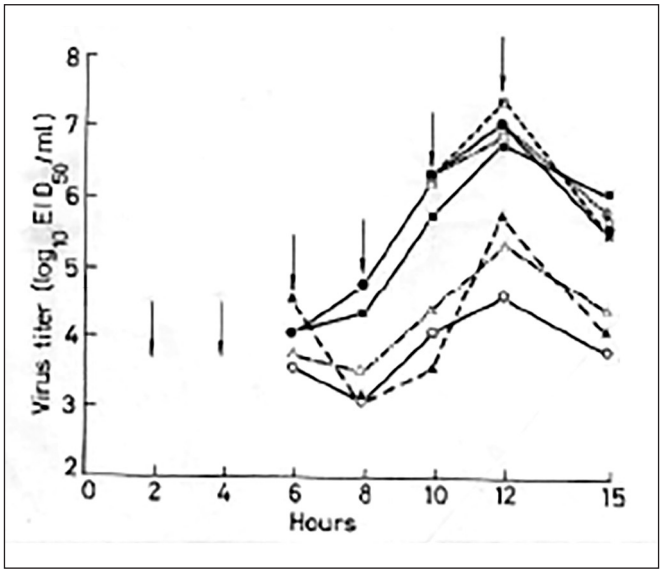

Fig. 2. Effect of mopyridone, added at different time after virus adsorption on influenza virus B/Lee/40 replication in CK cells (one-step growth cycle setup, m.o.i. = 8-10).The arrows indicate time of mopyridone (50 μg/ml) addition: immediately after virus inoculation (○---○), at the 2nd h (▲---▲), at the 4th h (Δ---Δ), at the 6th h (■---■), at the 8th h (□---□); at the 10th h (□---□),at the 12th h (Φ---Φ); control (no mopyridone, ●---●)

Effect of mopyridone on influenza viruses A(H3N2) and B growth in embryonated eggs

Mopyridone activity *in ovo* towards influenza virus A/Hong Kong/1/68 (H3N2) was established to be higher than that of rimantadine on the basis of four criteria: (a) lower minimal inhibitory dose value - <0.75 and 1.5 mg/embryo, respectively; (b) stronger antiviral effect at the optimal effective dose (6 mg/embryo for mopyridone, 1.5 mg for rimantadin) - an inhibition ($\Delta\log_{10}EID_{50}$) of 2.2 and 1.5, respectively; (c) lower toxicity - the 50% toxic dose value between 9 and 12 mg per embryo for mopyridone, and between 3 and 6 mg for rimantadine; (d) higher SI value - >12 and 2-4, respectively. A similar sensitivity to mopyridone *in ovo* was registered for a series of other influenza A(H3N2) strains (A/Philippines/2/82, A/Mississippi/1/85, A/Leningrad/360/860/86) and the Beijing/1/87 strain of influenza B virus, too (not illustrated).

Effect of mopyridone on experimental influenza A and B infections in white mice

Initially, mopyridone was administered subcutaneously in a daily dose of 300 mg/kg - 1/24 of the LD_{50} (single dose toxicity value), 7200 mg/kg (Karparov *et al.*, 1985) - as a 8-days treatment course started on the day of virus inoculation.

Table 3. Effect of mopyridone on influenza A and B virus infections in white mice

Virus strain	Inoculum LD_{50}/mouse	Drug tested	Daily dose, mg/kg	Route	Treatment course, days	Mortality rate[a]	p±Δp	PI, %	MST±σ_x, days
B/Lee/40	40-50	Mopyridone	300.0	s.c.	1-8	2/10	0.024 < 0.2 < 0.488	80.0	13.1±1.4
		Placebo				10/10	0.679 < 0.917 < 1.0		4.5±0.5
	1-3	Mopyridone	150.0	oral	1-8	0/19	0 < 0.047 < 0.175	100.0	>16.0
			75.0			1/19	0.0001 < 0.053 < 0.195	89.4	15.9±0.9
			37.5			2/20	0.011 < 0.1 < 0.265	80.0	15.1±0.8
			18.7			1/19	0.0001 < 0.053 < 0.195	89.4	15.7±0.8
			9.4			5/17	0.108 < 0.294 < 0.525	41.2	14.6±0.9
		Placebo				10/20	0.288 < 0.5 < 0.712		12.5±0.6
	3-5	Mopyridone	300.0	oral	1-8	0/12	0 < 0.071 < 0.283	100.0	>16.0
			150.0			1/12	0.000 < 0.83 < 0.296	84.4	15.7±1.4
			75.0			0/12	0 < 0.071 < 0.283	100.0	>16.0
			37.5			1/12	0.0001 < 0.083 < 0.296	84.4	15.7±1.4
			18.7			3/12	0.057 < 0.25 < 0.521	53.0	13.9±1.2
		Placebo	300.0		p1355Y	8/15	0.287 < 0.533 < 0.771		12.5±0.9
A/PR/8/34 (H1N1)	10-20	Mopyridone	300.0	s.c.	1-8	7/11	0.345 < 0.636 < 0.881	36.6	10.6±1.0
		Placebo				11/11	0.703 < 0.941 < 1.0		6.9±0.7
	10-20	Mopyridone	37.5	oral	1-8	7/13	0.275 < 0.538 < 0.79	46.2	11.6±0.9
		Rimantadine	80.0	oral	1-8	5/15	0.125 < 0.333 < 0.582	66.7	13.0±0.9
			20.0			8/14	0.314 < 0.571 < 0.809	42.9	9.9±0.7
		Placebo				15/15	0.772 < 0.941 < 1.0		8.1±0.5
A/Aichi/2/68 (H3N2)	33	Mopyridone	75.0	oral	1-8	4/15	0.081 < 0.267 < 0.51	73.3	13.3±0.9
			37.5	oral		2/15	0.015 < 0.133 < 0.343	86.7	14.3±1.0
		Placebo				15/15	0.772 < 0.941 < 1.0		5.3±0.4
	33	Mopyridone	37.5	oral	1-8	1/15	0.001 < 0.067 < 0.242	93.3	14.5±1.0
		Rimantadine	20.0	oral	1-8	2/15	0.015 < 0.133 < 0.343	86.7	13.7±0.9
		Placebo				15/15	0.772 < 0.941 < 1.0		5.5±0.4
	≥50	Mopyridone	37.5	oral	1-5	3/14	0.047 < 0.214 < 0.457	78.6	12.6±0.9
			18.7			6/15	0.175 < 0.4 < 0.65	60.0	11.3±0.8
		Rimantadine	20.0	oral	1-5	3/14	0.047 < 0.214 < 0.457	78.6	12.6±0.9
			10.0			6/15	0.175 < 0.4 < 0.65	60.0	10.9±0.7
			5.0			7/15	0.229 < 0.466 < 0.712	53.3	9.9±0.7
		Placebo				15/15	0.772 < 0.941 < 1.0		3.9±0.3
	≥50	Mopyridone	9.4	oral	1-5	15/15	0.772 < 0.941 < 1.0		5.7±0.4
		Rimantadine	20.0	oral	1-5	4/15	0.081 < 0.267 < 0.51	73.3	12.1±0.8
			10.0			7/15	0.229 < 0.467 < 0.712	53.3	10.2±0.7
			5.0			7/15	0.229 < 0.467 < 0.712	53.3	10.2±0.7
		Placebo				15/15	0.772 < 0.941 < 1.0		3.8±0.3
A/Victoria/35/72 (H3N2)	5	Mopyridone	75.0	oral	1-5	4/14	0.089 < 0.286 < 0.541	57.1	13.1±1.0
			37.5			5/15	0.125 < 0.333 < 0.582	50.0	12.3±0.8
			18.7			7/14	0.25 < 0.5 < 0.75	30.1	13.1±1.0
		Rimantadine		oral	1-5	1/15	0.001 < 0.006 < 0.242	90.0	14.5±1.0
		Placebo				10/15	0.457 < 0.667 < 0.9		10.0±0.7

[a]Died/total animal number (p = m/N); [b]P<0.01 at t>2.56; P<0.05 at t>1.96.

In the case of influenza virus B/Lee/40 infection a significant protection was recorded even at the extremely massive virus inoculation dose of 50 LD_{50}, whereas a borderline effect was found in the case of A/Puerto Rico/8/34 (H1N1) infection (Table 3).

Further experiments demonstrated that mopyridone administered orally exerted a marked protective effects in mice infected with influenza virus A/Aichi/2/68 (H3N2) and B/Lee/40. Toxicological studies done beforehand showed a very low LD_{50} value, 8000 mg/kg of mopyridone for this route of administration. In the case of influenza virus B/Lee/40 infection (at a comparatively low virus inoculation dose of 1-5 LD_{50} per mouse) the compound ED_{50} (50% effective dose) varied within the dose range of 9.4-18.7 mg/kg daily in the individual experiments, PI values exceeded 80% within the compound dose range of 37.5 - 300 mg/kg (in a course Days 1 - 9 of infection) and a high SI (LD_{50}/ET_{50}) value of 426 was registered (Table 3).

In influenza virus A/Aichi/2/68 (H3N2) infection, mopyridone administered via a 5 - 8 days course with a 37.5 mg/kg daily dose manifested a high protective effect (PI > 80% at SI value of 426) even at massive virus inocula (more than 30 virus ID_{50} per mouse). This effectivity was comparable to that of rimantadine applied at a daily dose of 20 mg/kg (the optimal effective dose). The ED_{50} values of mopyridone and rimantadine were 18.7 mg/kg and 5-10 mg/kg, respectively.

Influenza virus infection in adult mice (20 g body weight) with another A(H3N2) strain, A/Victoria/35/72, was also susceptible to mopyridone treatment, but in this case the rimantadine activity was found to be superior (Table 3).

In the case of influenza virus A/Puerto Rico/8/34 (H1N1) infection, mopyridone administered orally at a dose of 37.5-75 mg/kg daily (in analogy to rimantadine, 20-40 mg/kg daily) showed a weak efficiency expressed by a lengthening of the mean survival time and a insignificant decrease of the mortality rate.

Discussion

The anti-influenza effect of mopyridone is apparently similar to that of rimantadine. These substances manifested a strong activity against subtype A(H3N2) strains which was of the same range both in *in vitro* and *in vivo* experiments. Furthermore, the activity was reached at almost equal compound concentrations or daily doses. The two compounds showed a weak protective effect in influenza virus A/Puerto Rico/8/34 (H1N1) infected mice in contrast to their inefficiency towards subtype A(H1N1) strains in cell culture experiments. In the case of mopyridone this inconsistency could be explained with an immunomodulatory effect, i.e. a slight stimulation of alveolar macrophages and thymocyte proliferation, and also by a small augmentation of the antigen binding cells (Neychev *et al.,* personal communication). As for the rimantadine, the compound capability to interfere with the development of capillarotoxicity, one of the leading mechanisms in pathogenesis of influenza infection (Ilyenko *et al.,* 1982), could be taken in consideration.

At the same time some striking differences between the effects of the two substances were observed: (i) mopyridone was active against influenza B virus, unsusceptible to rimantadine; (ii) subtype A(H7N7) was significantly more sensitive to rimantadine as compared to mopyridone; (iii) mopyridone manifested a higher activity than rimantadine towards influenza virus A/Hong Kong/1/68 (H3N2) when tested in embryonated eggs; (iv) rimantadine is more toxic than mopyridone (*in vitro, in ovo, in vivo*); (v) the mopyridone effect was distinguished by its considerably higher selectivity *in vivo* (the oral SI values were 107-426 and 14-113 for mopyrione and rimantadine, respectively) (Galabov *et al.,* 1991).

These characteristics presume different mechanisms of action of the two influenza virus replication inhibitors. Actually, experiemnatl evidence is available for two different targets, namely, the M2 protein for the close amantadine hydrochloride (Hay *et al.,* 1985; Wharton *et al.,* 1990), and the M1 protein for mopyridone (Tverdislov *et al.,* 1988; Galabov *et al.,* 1990, 1994; Wassilewa *et al.,* 1995). An initial study on the mechanism of anti-influenza virus action of mopyridone by using flat bilayer lipid membranes and purified influenza A virus structural proteins showed that this compound interacts directly with M1 protein, thus interfering with its adsorption and insertion into the bilayer (Tverdislov *et al.,* 1988). Significant changes were found in the antigenic structures (sites 1A, 2 and 3) of M1 protein of the mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) developed from the wild mopyridone-sensitive strain (Galabov *et al.,* 1990, 1994) as well as in the content of some polar amino acids in this protein. As a consequence, the virions of mopyridone-resistant progenies manifested a series of major deviations in their physico-chemical properties and M1 protein-lipid inter-

actions (Wassilewa *et al.,* 1995).

Literary data in the wide field of experimental chemotherapy shows that mopyridone is the first anti-influenza virus antiviral which targets M1 protein. Thus, the results of the timing-of-addition study merit special attention for understanding the M1 role in the influenza virus replication cycle.

In full agreement with these findings demonstrating different mode of anti-influenza virus action of mopyridone and rimantadine are the data showing a synergistic character of their combined effect (Galabov *et al.,* 1991), as well as an efficiency of mopyridone against *in vitro* replication of rimantadine-resistant influenza A(H3N2) virus mutants (V. Kalnina, personal communication).

All these studies characterize mopyridone as an effective antiviral against influenza viruses A (subtypes H3N2 and H2N2) and B.

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