ALTERATION IN THE ANTIGENIC STRUCTURE OF M1 PROTEIN OF INFLUENZA A VIRUS MUTANT RESISTANT TO A NEW ANTIVIRAL COMPOUND MOPYRIDONE

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Summary. – Using 14 monoclonal antibodies (MoAbs) in solid-phase ELISA it was found that influenza virus A/Hong Kong/1/68 (H3N2) mutants resistant to the antiviral compound mopyridone as compared to the mopyridone-sensitive mutant manifested significant changes in the antigenic structure (sites 1A, 2 and 3) of M1 protein. No differences in M1 were found between rimantadine-resistant and rimantadine-sensitive mutants of influenza virus A(H3N2).

Key words: influenza A(H3N2) virus; antiviral; mopyridone; M1 protein; drug-resistant mutant

Introduction

Mopyridone (1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone) is recently described original substance showing a marked antiviral activity towards influenza A and B viruses as well as against togaviruses both *in vitro* and in experimental infections in white mice (Galabov *et al.*, 1984, 1994; Karparov *et al.*, 1985). Mopyridone in analogy to rimantadine is more efficient *in vitro* against influenza virus A(H3N2) strains as compared to those belonging to A(H1N1) subtype. However, mopyridone manifested a distinct effect towards influenza B virus (resistant to rimantadine) and a borderline one against influenza virus A(H7N7) (strongly sensitive to rimantadine). This efficiency was confirmed *in vivo*, the selectivity ratio value of mopyridone being higher than that of rimantadine (Galabov *et al.*, 1994).

An initial study on the mechanism of anti-influenza virus action of mopyridone by using model flat bilayer lipid membranes and purified influenza A virus structural proteins (surface glycoproteins, M1 protein) showed that this compound interacts directly with M1 protein, thus interfering with its adsorption and insertion into the bilayer. At the same time, mopyridone does not influence the HA-NA complex binding to the lipid membrane (Tverdislov *et al.*, 1988).

This finding directed us to study the M1 protein as a potential target of the antiviral action of mopyridone. Here we report data on the structure of M1 protein isolated from a mopyridone-sensitive mutant and on the development of mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) cultivated in embryonated eggs. Substantial changes in the M1 antigenic structure of the resistant mutants were revealed by use of MoAbs.

Materials and Methods

Compounds. Mopyridone (MCU, M. W. 199.25, white fine crystals, very well soluble in water) was synthesized by D. Sidzhakova (Sidzhakova *et al.*, 1982). Rimantadine hydrochloride was kindly supplied by G. A. Galegov, Institute of Virology, Russian Academy of Medical Sciences, Moscow..

Chick embryos. 10–11 days-old embryonated eggs of Leghorn hens were used.

Virus. Influenza virus A/Hong Kong/1/68 (H3N2) with infectious titer $10^{6.7}$–$10^{7.0}$ EID_{50}/ml was supplied by The A. Kirchenstein Institute of Microbiology, Latvian Academy of Sciences,

Riga (strain 1/89), and the National Influenza Center, Sofia (strain 2/90). They underwent serial allantoic passages.

The mopyridone-resistant mutants used, A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$ and A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$, were selected through five consecutive allantoic passages of the two original virus strains in the presence of mopyridone as follows. The compound, 3 mg per embryo, was injected 60 mins before virus inoculation at 10^{-4}–10^{-8} dilutions in PBS and the allantoic fluid samples were harvested from the HA positive embryos injected with the end-point dilution after 48 hrs incubation at 37 °C.

The mopyridone-sensitive stock virus was prepared through 5–6 passages of the initial virus in parallel with the development of the resistant mutants.

Another influenza virus A(H3N2) strain, A/Caen/1/84, rimantadine-sensitive, and its rimantadine-resistant mutant, A/Caen/1/84$^{Rim\text{-}res}$ (kindly supplied by V.–I. Kalnina, A. Kirchenstein Institute of Microbiology, Latvian Academy of Sciences, Riga) were employed in some experiments.

Infectious virus assay was carried out in 10–12 days-old embryonated eggs by the end-point dilution procedure and EID_{50} evaluation in a standard way.

Virus mopyridone-sensitivity testing. Embryonated eggs infected with virus dose 3.3–33.3 EID_{50} per embryo were treated with mopyridone, 3 mg per embryo, and the infectious and haemagglutinating titers of virus yields were recorded after 48 hrs and compared to the untreated controls.

Virus purification. Allantoic fluids containing the initial mopyridone-sensitive and of the mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) were purified by differential centrifugation and subsequent linear sucrose density gradient centrifugation (10–60 % w/w with 60 % sucrose cushion) according to Johansson *et al.* (1989). The same procedure was employed also for rimantadine mutants of influenza virus A/Caen/1/84 (H3N2).

Proteins were determined after Lowry *et al. (1951).*

Preparation of antiserum to influenza virus M1 protein. M1 protein of influenza virus A/Hong Kong/1/68 was isolated by preparative polyacrylamide gel electrophoresis. The antiserum was obtained by immunization of rabbits by the procedure of Zagidullin *et al.* (1985).

Preparation and characterization of MoAbs to influenza virus M1 protein. MoAbs to M1 protein of the recombinant influenza A virus (X-53a), containing M protein gene of A/PR/8/34, were obtained and characterized according to Bucher *et al.* (1989). A panel of 14 MoAbs against antigenic sites 1A (2BB10-G9, 1G8-A11, 3G12-C12, 9E8-B2, 821-B8-A8), 1B (2E5-C1, 961-G8-H3, 963-DE-G10, 6B9-B8), 2 (1G11-D11, 951-C4-G2, 823-D8-B11) and 3 (611-G10-D3, 951-D10-B3) was used (Bucher *et al.*, 1989).

Antigenic analysis of M1 protein by solid-phase ELISA was carried out as described by Schefer *et al.* (1990). The purified virus, 200 µg/ml, was disintegrated by 1 % lauryl sarcosinate for 30 mins at 37 °C in order to reach maximum release of M1 protein. The virus suspension was then diluted in PBS to a concentration of 2 µg/ml and adsorbed on microtiter plate (Inotech ELISA, England), 100 µl per well, overnight at 4 °C. After coating with bovine serum albumin (0.5 % in PBS containing 0.05 % Tween 20) the plates with added serial two-fold MoAbs dilutions were kept for 1 hr at 37 °C. Antigen-antibody complex was manifested by horseradish peroxidase rabbit anti-mouse conjugate, using o-phenylenediamine (Sigma) as substrate. The reaction was recorded by measuring A_{490}. Two criteria for antibody recognition were followed: (1) the antigen-MoAb interaction curve of the consecutive 2-fold MoAb dilutions; (2) the MoAb titer, the end-point MoAb 2-fold dilution assay giving an A_{490} value 2–3 times higher than that found in the ELISA control samples.

Polyclonal rabbit anti-M1 protein serum, 1000-fold diluted, was used to monitor M1 protein amount adsorbed to the plate.

Results

Table 1 illustrates the sharp differences in susceptibility to mopyridone between the initial influenza virus A/Hong Kong/1/68 strain and two mopyridone-resistant chick embryo isolates of this strain developed independently from two different sources of the parental strain.

Table 1. Effect of mopyridone on influenza virus A/Hong Kong/1/68 (H3N2) mutants growth in embryonated eggs

Virus mutants	Virus inoculum (EID_{50}/embryo)	Antiviral effect of mopyridone (Δ log EID_{50})
A/Hong Kong/1/68 (1/89)[a]	3–5	8.3
A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$	3.3	0.2
	10	0.3
	33	0
A/Hong Kong/1/68 (2/90)[b]	1	> 6.0
	3	≥ 6.8
	10	≥ 2.0
A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$	1	0
	3	0.3
	10	0

[a] From A. Kirchenstein Institute of Microbiology, Riga.
[b] From National Influenza Center, Sofia.

The degree of internal proteins manifestation in various influenza virus strains during direct adsorption in solid-phase ELISA is different, as shown by us previously (Schefer *et al.*, 1990). This should be kept in mind when the antigenic properties of these proteins are comparatively studied. This is why we applied a preliminary virion destruction by the detergent and monitored the M1 protein amount adsorbed by polyclonal antiserum in order to realize maximum appearance of M1 protein of both mopyridone-sensitive and mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68.

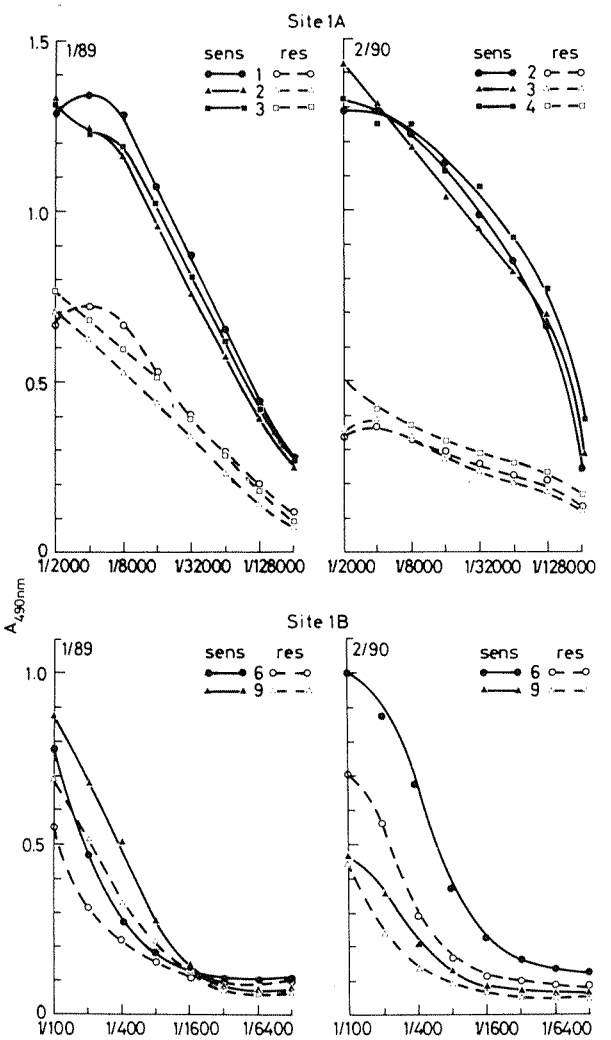

Fig. 1
Antigenic analysis of M1 protein of mopyridone-sensitive and mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) by MoAbs in ELISA
Virus mutants: A/Hong Kong/1/68, mopyridone-sensitive (1/89 and 2/90), and A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$ and A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$, mopyridone- resistant, developed from 1/89 and 2/90, respectively. MoAbs to M1 protein: 2BB10-G9 (1), 1G8-A11 (2), 3G12-C12 (3), 9E8-B2 (4), 2E5-Cl (6), 6B9-B8 (9).
Abscissa: MoAbs dilution.

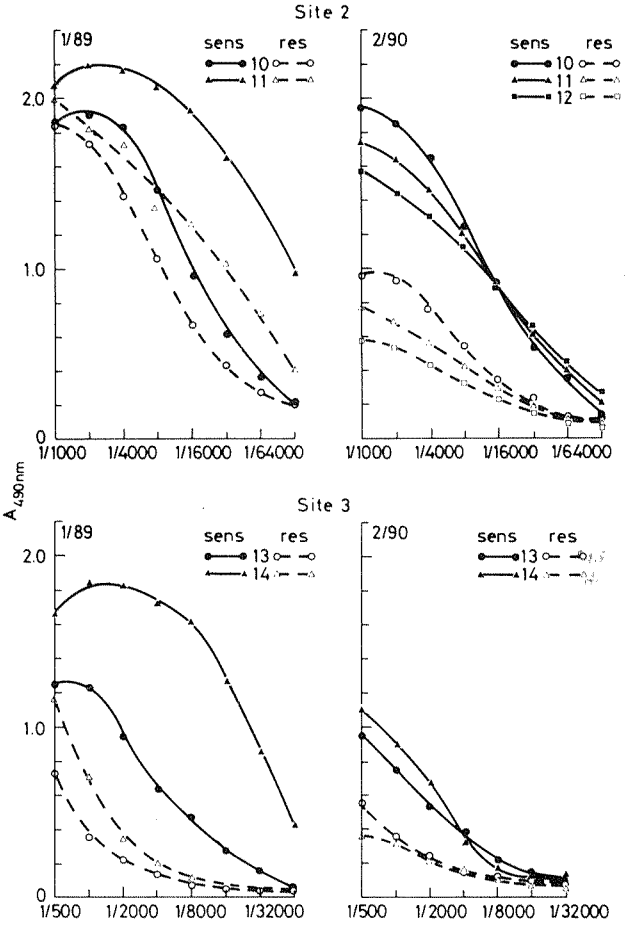

Fig. 2
Antigenic analysis of M1 protein in mopyridone-sensitive and mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) by MoAbs in ELISA
Virus mutants: the same as in Fig. 1. MoAbs to M1 protein: 1G11-D11 (10), 951-C4-G2 (11), 823-D8-B11 (12), 611-G10-D3 (13), 951-D10-B3 (14).
Abscissa: MoAbs dilution.

Firstly, it should be mentioned that the binding of M1 protein with polyclonal antiserum was identical for both sensitive and resistant mutants. The A_{490} values recorded at the antibody dilutions of 1:500 and 1:1000 were as follows: 1.98 and 1.5 with M1 protein of the mopyridone-sensitive strain 1/89, 2.1 and 1.5 with M1 of the respective mopyridone-resistant mutant; 1.09 and 0.78 with M1 of the mopyridone-sensitive strain 2/90, 1.079 and 0.795 with M1 protein of the respective mopyridone-resistant mutant.

Results of a parallel study on the antigenic properties of M1 protein of influenza A virus mopyridone mutants by use of MoAbs are shown in Figs 1 and 2 and Table 2. It is obvious that three out of the four antigenic sites underwent changes during the process of development of mopyridone-resistance

Table 2. ELISA titers of MoAbs to M1 protein of mopyridone-sensitive and mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2)

M1 site	MoAbs		MoAb titer[a] to mopyridone mutants			
			1/89		1/90	
			sensitive	resistant[b]	sensitive	resistant[c]
1A	2BB10-G9	(1)	512 000	256 000	512 000	256 000
	1G8-A11	(2)	256 000	128 000	256 000	256 000
	3G12-C12	(3)	256 000	128 000	256 000	256 000
	9E8-B2	(4)	512 000	256 000	512 000	256 000
	821-B8-A8	(5)	512 000	256 000	512 000	256 000
1B	2E5-C1	(6)	1 600	1 600	6 400	1 600
	961-G8-H3	(7)	800	800	800	800
	963-DE-G10	(8)	800	800	800	400
	6B9-B8	(9)	1 600	1 600	400	400
2	1G11-D11	(10)	64 000	64 000	64 000	16 000
	951-C4-G2	(11)	512 000	128 000	128 000	16 000
	823-D8-B11	(12)	1 024 000	256 000	128 000	16 000
3	611-G10-D3	(13)	32 000	4 000	16 000	4 000
	951-D10-B3	(14)	128 000	4 000	16 000	4 000

[a]Reciprocal value of the end-point MoAb dilution resulting in $A_{490} > 0.12$–0.18. A_{490} of 0.06 was found in the ELISA control sample.
[b]A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$
[c]A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$

Table 3. ELISA titers of MoAbs to M1 protein of rimantadine-sensitive and rimantadine-resistant mutants of influenza virus A/Caen/1/84 (H3N2)

M1 site	MoAbs		MoAb titer[a] to rimantadine mutants	
			sensitive	resistant
1A	2BB10-G9	(1)	1 024 000	1 024 000
	1G8-A11	(2)	1 024 000	1 024 000
	3G12-C12	(3)	1 024 000	1 024 000
1B	2E5-C1	(6)	128 000	128 000
	961-G8-H3	(7)	8 000	4 000
2	1G11-D11	(10)	64 000	64 000
	951-C4-G2	(11)	128 000	64 000
	823-D8-B11	(12)	64 000	64 000
3	611-G10-D3	(13)	32 000	32 000
	951-D10-B3	(14)	512 000	512 000

[a]Reciprocal value of the end-point MoAb dilution resulting in $A_{490} > 0.12$–0.18. A_{490} of 0.06 was found in the ELISA control sample.

in the studied influenza A virus. In fact, only the site 1 remained without changes. This could be due to a generally wea interaction between the MoAbs to site 1B and M1 protein subtype H3N2 influenza A virus strains (Fig. 1, Table 2).

The most characteristic changes, identical f A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$ and A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$ isolate involved the site 1A. It is interesting to note that the ELIS A_{490} values for the site 1A-specific MoAbs with M1 prote of the mopyridone-sensitive mutant were twice or mo times higher than those with M1 protein of the resista ones. This was shorply demonstrated in the MoAb-M protein interaction curves at MoAb dilutions below 1:256 0 (Fig. 1), but no significant differences in the titers we recorded (Table 2).

What concerns the M1 protein antigenic site 2, (1) MoA to this site manifested various behaviour: (1) MoAbs 951-C -G2 and 823-D8-B11 reacted to significantly higher dil tions with Ml of the mopyridone-sensitive mutant; (MoAb 1G11-D11 showed equal titers and similar intera tion curves with M1 protein of the sensitive and resista mutants of 1/89, whereas both criteria were different in th case of 2/90 mopyridone mutants. Evidently, the alter

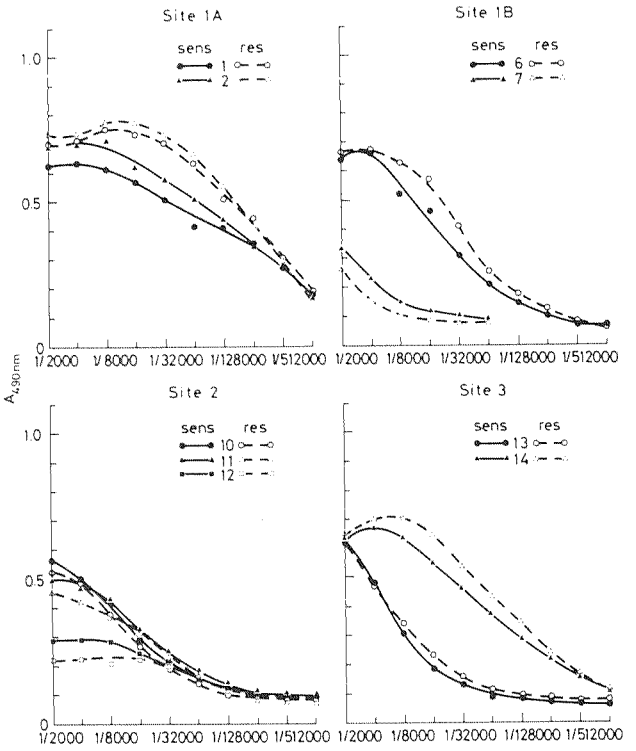

Fig. 3
Antigenic analysis of M1 protein of rimantadine-sensitive and rimantadine-resistant mutants of influenza virus A/Caen/1/84 (H3N2) by MoAbs in ELISA
MoAbs to M1 protein: see legends to Figs 1 and 2.
Abscissa: MoAbs dilution.

tions observed in this antigenic site differ to some extent in the two resistant mutants, A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)1/89}$ and A/HK/1/68 $^{MCU\text{-}res}_{3mg(CE)2/90}$, developed independently (Fig. 2).

MoAbs to the M1 antigenic site 3 reacted much more strongly with M1 of the mopyridone-sensitive mutant as compared to the mopyridone-resistant mutants 1/89 and 2/90 (Fig. 2, Table 2).

In order to test possible direct effect of mopyridone on the M1 protein-MoAbs binding, the mopyridone-sensitive mutant treated by 0.4 % lauryl sarcosinate (30 min at 37 °C) was diluted to 2 μg protein per ml (w/w ratios adequate to those in embryonated eggs test system). ELISA was then carried out by the technique described in *"Materials and Methods"*.

The results obtained (identical curves in presence or absence of the inhibitor) showed that mopyridone does not influence the interaction of MoAbs tested with M1 protein.

An analogical analysis of the antigenic structure of influenza virus A(H3N2) M1 protein was carried out also on the rimantadine mutants of another strain of this influenza A virus subtype, A/Caen/1/84. Fig. 3 and Table 3 demonstrate no differences in all four M1 protein antigenic sites between the rimantadine-sensitive and rimantadine-resistant mutants. These data argue for the specificity of changes observed in the M1 protein of influenza virus A(H3N2) mopyridone-resistant mutants.

Discussion

The presented results can be readily interpreted on the basis of findings by Bucher *et al.* (1989) that the sites 2 and 3 are closely spaced in the M1 protein molecule. The fact that the MoAbs to the sites 2 and 3 react with a synthetic peptide representing the amino acids 83 to 100 implies that this region may be shared by both antigenic sites 2 and 3. Thus, the acquired resistance of influenza virus A/Hong Kong/1/68 to mopyridone could possibly be based on changes within the region of amino acids 83 to 100. This suggestion could be confirmed by direct comparison of the gene 7 nucleotide sequence in the mopyridone-sensitive and -resistant influenza virus mutants.

The changes in the interaction of M1 protein with MoAbs concern antigenic sites 1A, 2 and 3 as revealed the reactions of the panel of MoAbs tested. The lowered reactivity of mopyridone-resistant strains is most probably caused by amino acid change(s) directly on M1 protein, which is reflected in conformational differences of the antigen.

The significant differences found in M1 protein antigenic structure between mopyridone-sensitive and -resistant mutants of influenza virus A(H3N2) support the hypothesis of M1 protein as a target of mopyridone inhibitory action on influenza virus replication. Such differences were not found between the rimantadine-resistant and -sensitive mutants of this influenza A virus subtype. This fact is in line with the recent finding that the minor structural protein M2 is the primary target of the antiviral effect of adamantane derivative amantadine (Hay *et al.*, 1985; Wharton *et al.*, 1990). The synergism of the combined anti-influenza virus effects of mopyridone and rimantadine hydrochloride (Galabov *et al.*, 1991) is obviously based on different viral targets of these two inhibitors.

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