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Development of resistance to disoxaril in Coxsackie B1 virus-infected newborn mice

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Abstract

Treatment with disoxaril (a WIN compound binding to the hydrophobic pocket within the enterovirus VP1 capsid protein) in newborn mice infected with Coxsackie B1 virus, for 10 days post virus inoculation at a daily subcutaneous dose of 25 mg/kg decreased the virus titer in the mouse brain till day 7. Thereafter (on days 8 and 9) drug-resistant virus progeny in brain samples isolates was noted with disoxaril IC_{50} values of >40 μM as compared to 0.59–1.37 μM in placebo. Study on phenotypic characteristics of the disoxaril-resistant mutants (as obtained in vivo or in cell culture experiments in parallel with the wild disoxaril-sensitive virus) showed: (i) larger size of virus plaques under agar overlay; (ii) more irregular shape of virus plaques; (iii) markedly less thermostability at 50 °C; (iv) slightly increased pathogenicity for newborn mice. The results obtained provide convincing evidence for the development of drug-resistant progeny of Coxsackie B1 virus in the brain of mice following treatment with disoxaril.

Keywords: Coxsackie B1 virus; Disoxaril; Mice; Resistance; Phenotype

1. Introduction

Development of resistance is considered as the main reason for the failure to introduce effective anti-picornavirus chemotherapy in clinical practice. Viral mutants resistant to picornavirus replication inhibitors have been described with almost each drug since the very beginning of antiviral drug investigations, as pointed out by Loddo (1980). The first description of enteroviral drug resistance (poliovirus 1 mutant resistant to guanidine) was reported by Melnick et al. (1961). Later, development of drug-dependent mutants has been established (Loddo et al., 1962). Random genetic mutation can occur at a very high frequency in a number of RNA viruses and especially in picornaviruses (Sierra et al., 2000; Crotty et al., 2001). Therefore, in natural conditions the picornaviral progeny is quite heterogenous. The unlimited number of mutants in the picornaviral population is a result of point mutations occurring randomly during viral replication. Drug-resistant picornaviral progeny will develop quite easily as a result of selection of pre-existing mutants. Establishment of drug resistance could serve as an indicator for a specific antiviral activity (Herrmann and Herrmann, 1977).

Antiviral compounds that bind to the hydrophobic pocket within VP1 capsid protein of enteroviruses and rhinoviruses, and thus stabilize virion and block uncoating, have a leading position among the inhibitors of picornavirus replication. The most effective among them are the WIN- (Diana et al., 1989; Smith et al., 1986; Rossmann, 1989; Pevear, 2001) and Ro-compounds (Andries et al., 1990).

Nowadays, enterovirus infections could be considered as a first range indication for antiviral chemotherapy. That is because enteroviruses, and Coxsackie B viruses in particular, are clinically important pathogens causing a wide variety of human diseases, including acute myocarditis, pericarditis, dilated cardiomyopathy, aseptic meningitis and acquired diabetes.

Use of effective (synergistic) combinations of antivirals may be considered as an effective approach for overcoming the drug resistance in chemotherapy. In the course of a large-scale study for establishing combination effects of anti-enteroviral compounds with various mode of action, we found disoxaril effective both in experiments in vitro (Nikolaeva and Galabov, 1995, 1999) and in vivo, in Coxsackie B1 virus infection in newborn mice (Nikolaeva and Galabov, 2000). Disoxaril (5-[7-[4(4,5-dihydro-2-oxa-

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zolyl)phenoxy]heptyl]-3-methyl-isoxazole, WIN 51711) belongs to the first generation of the highly efficient WIN compounds, which later led to WIN 54954 and WIN 63843 (pleconaril) (Pevear et al., 1999; Pevear, 2001).

As there are no literature data on the viral progeny characteristics in target organs (brain) during the course of enteroviral infection in experimental animals treated with the WIN compounds, we aimed at further clarifying this issue using of Coxsackie B1 virus infection in newborn mice treated with disoxaril.

2. Materials and methods

2.1. Virus

Coxsackie B1 virus stock for in vivo experiments was prepared through five intracerebral passages (0.02 ml/mouse) of the standard laboratory strain (Connecticut 5) in newborn albino mice (ICR line). It represented 10% brain suspension in Dulbecco's phosphate-buffered saline (PBS). Stock virus for in vitro experiments was grown in monolayer FL cells at 37 °C. FL cells are derived from human amnion cells (Fogh and Lund, 1958).

2.2. Cells

FL cells monolayer cultures in Costar 96- or 24-well microplates grown at 37 °C in 5% CO_2 thermostate (Heraeus) or in 20-ml scintillation glass vials were used. Growth medium contained 5% heated calf serum in Dulbecco's MEM (minimal essential medium) (Gibco BRL, Paisley, Scotland, UK). Maintenance solution was 0.5% heated calf serum in Dulbecco's MEM.

2.3. Compound tested

5-[7- [4(4,5-Dihydro- 2-oxazolyl) phenoxy]heptyl]-3-methyl-isoxazole (disoxaril, WIN 51711) was supplied by Sanofi Winthrop, Inc. (Malverne, PA).

2.4. Virus assay

Infectious virus content was determined in parallel by the endpoint dilution method in FL cells in 96-well microplates and by the plaque technique using monolayer cultures in 20-ml scintillation glass vials or 24-well microplates (Costar). The agar overlay (1 or 0.8 ml for scintillation vial and 24-well microplates, respectively) was 1% purified Difco agar in Eagle's MEM Gibco BRL supplemented with 10% heated calf serum, 1.65 μg/ml sodium bicarbonate and antibiotics (penicillin, 100 IU/ml, streptomycin, 100 μg/ml). Following a 48-h incubation at 37 °C, a second overlay (1.5% agar with 0.02% neutral red Gibco in physiological saline) was added, and vials/plates were kept at room temperature. The virus titer in PFU/ml was evaluated.

2.5. Experimental design of in vitro isolation of disoxaril-resistant Coxsackie B1 virus mutant

The standard Coxsackievirus B1(dis-sens/FL), plaque purified, underwent consecutive passages in glass tubes FL cell cultures with maintenance medium containing 30 μM disoxaril. After 48-h cultivation (at a totally confluent CPE) virus yields were harvested following triple freezing, and thawing of infected cell cultures. Drug-resistant progeny, CoxB1(dis-res30 μM/FL), was developed after three passages. Following the plaque purification procedure, the virus stock was checked for virus-drug resistance.

2.6. Experimental design of Coxsackie B1 virus infection in newborn mice treated with disoxaril

Groups of 45–50 newborn mice (ICR line) were inoculated subcutaneously (s.c.) with Coxsackie B1 virus (6 LD_{50}/mouse). Disoxaril treatment, 25 mg/kg s.c. (0.05 ml/mouse) daily, started on the day of infection, and lasted till the 10th day. Virus samples (five brain samples each of the disoxaril treated and the placebo group) were taken every day since day 4 post virus inoculation.

2.7. Determination of virus isolates sensitivity to disoxaril

The plaque-inhibition test was applied on virus progenies that had been previously plaque purified. Monolayer FL cell cultures in 20-ml scintillation vials (diameter (ø) 2.5 mm) or 24-well microplate Costar were inoculated with 50–60 PFU of virus per well, and left for 1 h at room temperature. Then, 1 ml per vial (0.8 ml per well) of the agar overlay (as described above) was laid over the cells. The test compound disoxaril was included in the agar overlay at the following concentrations: 0.32, 1, 3.2, 10, 32, and 100 μM. Following a 48-h incubation period at 37 °C and addition of a second, neutral red containing, agar overlay the percentage of PFU inhibition was evaluated in comparison to the control (without test compound in the agar overlay). The disoxaril dose–response curve was drawn and the 50% minimal inhibitory concentration (MIC_{50}) for each virus sample was evaluated.

2.8. Thermostability test

Virus mutants suspensions of $10^{7.3}$ $CCID_{50}$ each in 1 ml of Dulbecco's MEM containing 0.5% heated calf serum in plastic tubes were placed in a waterbath at 50 °C. Aliquots were removed after various periods of time, and cooled immediately on ice. Virus assays were carried out by the end-point dilution method in monolayer FL cells in 96-well microplates.

2.9. Testing for pathogenicity in mice

Groups of 16–22 newborn (24 h) mice were inoculated s.c. with 0.02 ml of plaque-purified virus mutants

suspensions in PBS containing 10, 1, 0.1 or 0.01 LD_{50}. The infectious titer of stock viruses as assayed in mice was as follows: CoxB1(dis-s/FL) $10^{4.9}$ LD_{50}/0.02 ml, CoxB1(dis-r25 mg/kg/mouse) $10^{4.4}$ LD_{50}/0.02 ml, CoxB1(dis-r30 μM/FL) $10^{4.9}$ LD_{50}/0.02 ml, and CoxB1(dis-s/mouse) $10^{4.7}$ LD_{50}/0.02 ml. Mice were monitored daily for survival over the 14-day post virus inoculation study period, the cumulative mortality rate (percentage) and mean survival time (MST) were evaluated.

2.10. Statistics

Differences between groups were analyzed for significance using the Student's *t*-test or χ^2-test.

3. Results

3.1. Effect of disoxaril on experimental infection with Coxsackievirus B1 in newborn mice

Disoxaril administered s.c. in newborn mice infected with Coxsackie B1 virus (6 LD_{50} s.c.) in a 10-day treatment course, with a single daily dose of 25 mg/kg (0.05 ml/mouse), started immediately after virus inoculation, as selected according to a previous study (Nikolaeva and Galabov, 2000), manifested a marked protective effect. This effect was expressed mainly with a lengthening of the MST by 4.3 days (10.5 and 6.2 days in the treated and placebo groups, respectively) (Table 1). The decrease of the mortality at the end of observation period (14th day) was less than 50%. On day 6, a marked increase of mortality rate was observed.

The protective effect of disoxaril, as shown in Table 1, correlated with a 2.0–2.7 $\log_{10}$ $CCID_{50}$ lower content of infectious virus in the brain samples taken on the first 5 days post-infection from disoxaril-treated animals as compared to the placebo group (Table 2). Later, on days 6–7, viral titer difference diminished (down to 0.8–1.0 $\log_{10}$ $CCID_{50}$) and from day 8, a marked rise of the virus content in treated animals was recorded.

3.2. Sensitivity to disoxaril of virus brain isolates from disoxaril-treated mice

The virus progeny in brain isolates from disoxaril (25 mg/kg) treated mice, infected with Coxsackie B1 virus, was tested for sensitivity to the compound by using the plaque-inhibition test in FL cells. Virus isolates taken after day 7 after infection showed a marked decrease in their sensitivity to disoxaril as compared to the placebo group isolates (Table 3). Disoxaril IC_{50} values attained >40 μM in treated group versus 0.59–1.37 μM in placebo. These data convincingly proved the development of a disoxaril-resistant Coxsackie B1 virus progeny in the infected animals in the course of the 10-day disoxaril administration. This phenomenon can explain the increase of mortality rate in the presence of a continuing disoxaril treatment.

Table 1
Effect of disoxaril (s.c. 25 mg/kg/day)[a] on Coxsackie B1 virus infection in newborn mice

LD_{50}/mouse	Test group	N[b]	Cumulative mortality (%)											MST (days)
			Days after virus inoculation											
			2	3	4	5	6	7	8	9	10	11	12	
6	Placebo	43	0	9.3	14.0	23.3	44.2	53.5	70.0	76.7	83.7	88.4	93.0	6.0
	Disoxaril	60	1.7	5.0	6.7	15.0	25.0	31.7	36.7	40.0	43.3	46.7	50.0***	9.6
15	Placebo	11	0	0	81.8	100								3.8
	Disoxaril	11	0	0	18.2	27.3	36.4	45.5	45.5	63.6	63.6**			8.4

[a] Ten-day treatment course started immediately after virus inoculation.
[b] Number of animals per test group.
** $P < 0.01$ (χ^2-test).
*** $P < 0.001$ (χ^2-test).

Table 2
Effect of disoxaril treatment (s.c. 25 mg/kg/day) course on Coxsackie B1 virus content in the mouse brain

Experiment no.	Test group	Infectious titer ($\log_{10}$ $CCID_{50}$/ml) of the brain samples						
		Days after virus inoculation						
		4	5	6	7	8	9	10
1	Placebo	3.7	4.7	4.3	4.7	3.0	<2.0	<2.0
	Disoxaril	<2.0	<2.0	3.3	4.0	4.8	>4.8	3.7
2	Placebo	3.8	4.0	3.8	4.7	4.7	2.7	2.8
	Disoxaril	<2.0	2.0	3.0	4.3	>4.8	2.0	2.0

Table 3
Sensitivity to disoxaril in the plaque-inhibition test (in FL cells) of virus brain isolates from disoxaril (25 mg/kg/day) treated newborn mice with Coxsackie B1 virus infection

Experiment no.	Test group	Disoxaril IC_{50} values (μM) of viral brain samples taken on day (after virus inoculation)						
		4	5	6	7	8	9	10
1	Placebo	0.64	0.65	0.74	0.64	1.2	0.64	0.59
	Disoxaril	ND	ND	3.3	35.0	>40.0	29.0	32.0
2	Placebo	0.92	0.93	0.8	1.37	1.0	0.8	0.82
	Disoxaril	0.65	0.56	0.72	11.82	>40.0	ND	ND

As a next step, several phenotypic characteristics of the disoxaril-resistant Coxsackie B1 virus brain isolates were characterized, namely: (i) plaque size and shape under agar overlay; (ii) stability at 50 °C; (iii) pathogenicity for newborn mice. In vitro selected disoxaril-resistant mutants, as well as drug-sensitive wild progenies were included in this study for comparison. Four viral progenies were tested: disoxaril-sensitive wild strain, both grown in FL cells (CoxB1(dis-s/FL)) and adapted for mouse brain through a series of intracerebral passages (CoxB1(dis-s/mouse)), and disoxaril-resistant mutants developed in vitro, CoxB1(dis-r30 μM/FL) and CoxB1(dis-r25 mg/kg/mouse), isolated from mouse brains collected on 7 and 8 days after the infection and treatment onset. The mutants used in the following experiments, described in Sections 3.3–3.5, were additionally cloned and tested for disoxaril sensitivity.

3.3. Plaque characteristics of Coxsackie B1 virus disoxaril mutants

The plaques of CoxB1(dis-s/FL), CoxB1(dis-r25 mg/kg/mouse), CoxB1(dis-r30 μM/FL) and CoxB1(dis-s/mouse) were compared by plaque assay. A larger diameter of virus plaques in both in vivo and in vitro selected resistant progenies was observed as compared to the disoxaril-sensitive strains (Table 5). The plaque size of in vivo and in vitro developed progenies was identical: $ø = 1.9 \pm 0.1$ mm of the disoxaril-resistant mutants and 0.9 ± 0.3 mm of the disoxaril-sensitive mutants (the standard deviation was calculated on the base of 20 measurements per each mutant sample). The shape of the two disoxaril-resistant mutants was more irregular than that of the disoxaril-sensitive strains.

3.4. Thermostability (50 °C) of Coxsackie B1 virus disoxaril mutants

Thermal inactivation curves at 50 °C of the four Coxsackie B1 virus mutants were followed by recording the residual infectivity (Fig. 1). The disoxaril-resistant mutants manifested a highly decreased thermostability as compared to the disoxaril-sensitive ones. So, the $ET_{\Delta=3\log}$ value of the CoxB1(dis-r30 μM/FL) was approximately five times shorter than that of the CoxB1(dis-s/FL) one: 6 min 40 s and 31 min 45 s, respectively. Similarly, the drug-resistant mutant isolated from mouse brain (CoxB1(dis-r25 mg/kg/mouse)), was markedly more thermosensitive than the drug-sensitive brain progeny (CoxB1(dis-s/mouse)): $ET_{\Delta=3\log}$ values of 13 min 56 s and 26 min 6 s, respectively.

3.5. Pathogenicity for newborn mice of Coxsackie B1 virus disoxaril mutants

A slightly increased pathogenicity of the disoxaril-resistant Coxsackievirus B1 mutants was found in comparison with the disoxaril-sensitive strains: in CoxB1(dis-r30 μM/FL) with 0.45 $\log_{10}$ LD_{50} and in CoxB1(dis-r25 mg/kg/mouse) with 0.4 $\log_{10}$ LD_{50}.

This phenomenon was very markedly manifested at lower virus inocula (0.1 or 0.01 LD_{50}). As seen in Table 4, an increase of the mortality rate and a shortening of the MST were recorded in mice infected with disoxaril-resistant Coxsackievirus B1 mutants CoxB1(dis-r30 μM/FL) and CoxB1(dis-r25 mg/kg/mouse). No significant differences were found at virus inocula ≥1 LD_{50}.

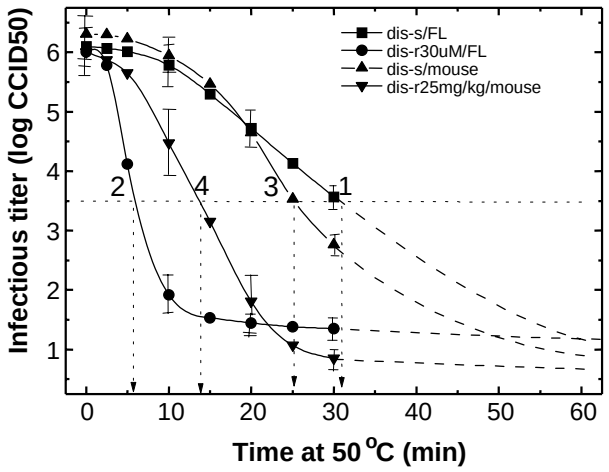

Fig. 1. Curves of thermoinactivation at 50 °C of the Coxsackie B1 virus disoxaril mutants: (1) CoxB1(dis-s/FL); (2) CoxB1(dis-r30 μM/FL); (3) CoxB1(dis-s/mouse); (4) CoxB1(dis-r25 mg/kg/mouse). Data are presented as means ± S.D.

Table 4
Pathogenicity for newborn mice of disoxaril mutants of Coxsackie B1 virus

Cox B1 disoxaril mutants	Virus inoculation dose (LD_{50}/mouse)					
	0.1			0.01		
	N[a]	Mortality (%)	MST (days)	N	Mortality (%)	MST[b] (days)
CoxB1(dis-s/FL)	20	30.0	8.5	16	12.5	9.4
CoxB1(dis-r30 μM/FL)	22	45.4	7.8	16	25.0	8.8
CoxB1(dis-s/mouse)	18	11.1	9.6	20	0	10.0
CoxB1(dis-r25 mg/kg/mouse)	22	45.5***	7.8	22	22.2***	8.7

[a] Number of animals per test group.
[b] Mean survival time.
*** $P < 0.001$ for CoxB1(dis-r25 mg/kg/mouse) vs. CoxB1(dis-s/mouse) (χ^2-test).

Table 5
Phenotypic characteristics of disoxaril mutants of Coxsackie B1 virus

Cox B1 disoxaril mutants	MIC_{50} (μM)	Plaque diameter (mm)	Plaque shape	Stability at 50 °C $ET_{\Delta=3\log}$ (min)	Pathogenicity for mice
CoxB1(dis-s/FL)	0.84	0.9 ± 0.3	Round	32	Normal
CoxB1(dis-r30 μM/FL)	>30.0	1.9 ± 0.1***	Irregular	7	Slightly increased
CoxB1(dis-s/mouse)	0.59–1.37	0.9 ± 0.3	Round	26	Normal
CoxB1(dis-r25 mg/kg/mouse)	>40.0	1.9 ± 0.1***	Irregular	14	Slightly increased

*** $P < 0.001$ for dis-r vs. dis-s CoxB1 mutants (Student's *t*-test).

4. Discussion

Study on the antiviral action of disoxaril against Coxsackie B1 virus showed a sharp discrepancy between the strong inhibitory effect on viral replication in vitro (in cell cultures) and the moderate values of the effect recorded in vivo, in an experimental infection in newborn mice. This is especially well demonstrated when the viral load in the target organ (brain) of infected and disoxaril-treated animals was traced. Following a period of a strong suppression of virus replication (embracing days 1–5), a marked decrease of antiviral effect was recorded despite continuing disoxaril administration.

Our investigations clearly demonstrate that the above phenomenon is due to development of disoxaril-resistant progeny. These data are unique in the literature concerning WIN compounds. The first isolation of a drug-resistant enterovirus mutant from experimental animals has been described by Melnick et al. (1961). In this case, monkeys, infected with poliovirus 1 (Mahoney) were treated with guanidine hydrochloride.

Analysis of the phenotypic characteristics of the disoxaril mutants of Coxsackie B1 virus as summarized in Table 5 demonstrates a correlation of the drug resistance with the size and shape of virus plaques under agar overlay. Moreover, this marker in the resistant mutants is related with a marked increase of thermosensitivity. This finding is in accordance with the data of Groarke and Pevear (1999) concerning the significantly lower thermostability of Coxsackievirus B3 mutants resistant to another WIN compound, pleconaril. Likewise, an increased thermosensitivity of echovirus 9 strains, isolated from patients treated with pleconaril and possessing a lower drug sensitivity was recorded (Pevear et al., 2001). Evidently, integrity of the drug-binding hydrophobic pocket of VP1, which plays a decisive role in virion stability (Rombaut et al., 1991; Moeremans et al., 1992), is seriously compromised in the viral progeny resistant to pocket-binding inhibitors.

Our data on an increased pathogenicity for newborn mice of the disoxaril-resistant Coxsackie B1 virus mutants merit special attention. They are in contrast to the results of Groarke and Pevear (1999) in a study on pleconaril-resistant Coxsackie B3 virus mutants, characterized as attenuated and less virulent for mice than the drug-sensitive wild-type virus. The discrepancy found could be based on the significant differences in the experimental protocols and the use of different Coxsackievirus species and strains with different tropism (neurotropic and viscerotropic, respectively), as well.

The correlation of the phenotypic markers size (ø) of the viral plaques under agar overlay in cell cultures (marker S) and the neurovirulence (marker N or NV) for newborn mice in the case of disoxaril mutants of Coxsackievirus B1 as established in our study are in line with the known correspondence between these two markers in enteroviruses (Voroschilova, 1979).

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