Attenuated Virulence of Pleconaril-Resistant Coxsackievirus B3 Variants

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Pleconaril (VP 63843) is a novel orally bioavailable small molecule with broad antipicornavirus (enterovirus and rhinovirus) activity. Ten independently derived pleconaril-resistant variants of coxsackievirus B3 were isolated from cell culture. The molecular basis of drug resistance and the biologic properties of the drug-resistant viruses were investigated. RNA sequence analysis revealed amino acid changes in the drug-binding pocket of the resistant variants. Thermal stability studies showed the drug-resistant viruses to be significantly less stable than wild type virus. When evaluated in a murine model in which wild type virus infection is 100% lethal, the drug-resistant viruses showed attenuated virulence with both reduced mortality and delayed time to death. Virus titers in heart and spleen were dramatically lower in drug-resistant virus-infected mice than in wild type virus-infected animals. The study results indicate that pleconaril-resistant virus variants are attenuated and significantly less virulent than drug-sensitive wild type virus.

The present study was designed to investigate the molecular basis of pleconaril-resistant variants isolated in cell culture and to describe their biologic phenotype, with particular interest in assessing the virulence of drug-resistant viruses in an animal model. CB3 was chosen for these studies for several reasons. CB3 is a ubiquitous enterovirus capable of causing a wide range of human disease, including myocarditis, myositis, pancreatitis, hepatitis, respiratory syndromes, and central nervous system disorders [4]. Pleconaril inhibits the replication of CB3 in cell culture and is effective in treating mice infected with CB3 (unpublished data). The murine model of CB3 infection is well established and has been used for several decades to study the pathogenesis of enterovirus disease [4]. In infected BALB/c mice, CB3 can be isolated from brain, heart, spleen, pancreas, and liver. Mortality in mice is due to the direct viral destruction of these target organs [4].

Methods

Cells and virus. LLC-MK2 cells (American Type Culture Collection, Bethesda, MD) were grown at 37°C in MEM Earles medium containing 5% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS). HeLa (Wis) cells (University of Wisconsin, Madison) were grown at 37°C in MEM-Hanks’ medium supplemented with 5% heat-inactivated FBS. Confluent cell cultures were infected with a myocarditic strain of CB3 (CB3m) C. Gauntt, University of Texas, San Antonio) at an MOI of ~0.1. CB3 was purified by a modification of the procedure of Mapoles et al. [5].

Isolation of drug-resistant mutants. Ten independently prepared pools of wild type CB3 (10^6 pfu/pool) were individually incubated with 1 μg/mL pleconaril in M199 medium. After 1 h, the material was plated on LLC-MK2 cells and overlaid with agar containing pleconaril (1 μg/mL). One clearly isolated plaque from each pool was picked and subjected to two rounds of plaque purification in the presence of 1 μg/mL pleconaril.

Viral RNA sequencing. The region in VP1 that encodes the drug-binding pocket was sequenced directly from the RNA as described [6]. Four primers were used based on the published nucle-
otide (nt) sequence [7]: 5'-TCTGTGTGGTGAGGCT-3' (nt 2824–2841); 5'-GCCGCCGGGCTTCCC-3' (nt 2962–2980); 5'-GCCGTAATTCCGCTCTCT-3' (nt 3051–3068); and 5'-GTA-GATTCTAATGGTGC-3' (nt 3141–3158).

Mouse infection. Groups of 10 male BALB/c mice (17–20 g; Taconic Farms, Hudson, NY) were infected intraperitoneally with either 600 or 6000 pfu of virus in PBS. Control groups were inoculated with PBS alone. Mice were monitored daily for survival over the 18-day study period. Viral titers in heart and spleen were determined. Organs were excised from mice sacrificed 5 days after infection and homogenized as 20% (wt/vol) suspensions in M199 medium with a Duanl tissue grinder (Kontes, Vineland, NJ). The homogenate was twice freeze-thawed and then centrifuged at 181g to remove debris. The supernatant was collected and stored at −80°C until plaque assay.

Results

Isolation of pleconaril-resistant CB3. Pleconaril inhibits CB3 plaque formation in a dose-dependent manner with an IC₅₀ of 0.005 μg/mL (data not shown). To determine a suitable concentration of pleconaril for use in the selection of resistant virus variants, the number of plaques formed on cells infected with 10⁶ pfu of virus in the presence of varying concentrations of pleconaril was determined. Figure 1 shows that there was a concentration-dependent decrease in plaque formation that continued until an apparent plateau was reached at a drug concentration of ~0.5 μg/mL. At this concentration, plaque formation had been reduced by ~6 log₁₀. Based on this curve, a concentration of 1 μg/mL was used for isolation of resistant variants. Under these selection conditions, the frequency of resistance to pleconaril in the wild type virus population was ~5 × 10⁻³. Ten independently prepared pools of wild type CB3 were used to isolate individual plaques of pleconaril-resistant variants. One pleconaril-resistant plaque from each pool was purified and used for subsequent study. Single-step growth curves of the resultant pleconaril-resistant viruses (CB3-R1–R10) were similar to that of wild type CB3, with virus rate of replication and titers similar in both the presence or absence of drug in the culture medium. Further, the drug-resistant phenotype appeared stable in both cell culture and after virus passage in mice (data not shown).

Molecular basis of drug resistance. The RNA sequence in the region encoding the drug-binding pocket in VP1 was determined for the wild type and for 10 pleconaril-resistant viruses. Relative to the wild type virus, 9 of the 10 resistant isolates (CB3-R1–R8 and CB3-R10) had nt changes that resulted in a single amino acid change at residue 92 of VP1 (Ile to Met). For CB3-R9, in addition to a change at residue 92 (Ile to Leu), a second change occurred at VP1 residue 207 (Leu to Val). It is not known whether both changes in CB3-R9 are required for expression of the resistant phenotype. However, a mouse-adapted strain of CB3 (strain Nancy), which is resistant to pleconaril, also has Leu at residue 92 in VP1 [8].

Thermal stability of drug-resistant viruses. Capsid inhibitors can stabilize picornaviruses against heat inactivation, suggesting that the drug-binding pocket plays a role in virion stability [9, 10]. Therefore, it was suspected that the changes in amino acid residues within the drug-binding pocket of VP1 observed in the pleconaril-resistant viruses might affect virion stability. To investigate this possibility, wild type CB3 and three of the pleconaril-resistant variants were subjected to heat treatment by incubation in culture medium at 46°C for 30 min prior to being titered. Under these conditions, wild type virus was completely stable after the 30-min heat treatment. In contrast, CB3-R9 lost 90% of its infectivity, and CB3-R1 and CB3-R3 lost >99% of their infectivity under the same conditions. These data indicate that the changes necessary to confer pleconaril-resistance dramatically decreased the thermal stability of the viruses.

Mouse virulence. Infection of BALB/c mice with CB3 is lethal [4]. To determine if the mouse virulence of the pleconaril-resistant viruses was altered, wild type CB3 and pleconaril-resistant viruses CB3-R1, CB3-R3, and CB3-R9 were evaluated for their ability to cause lethal infection in mice. Mice were infected with either 600 or 6000 pfu of each virus and were followed for 18 days. For wild type CB3-infected mice, deaths began at day 6 and 100% mortality occurred by day 9 (600 pfu) or day 11 (6000 pfu). In the case of all three pleconaril-resistant viruses tested, mortality was significantly reduced at both virus challenge doses. At the 600 pfu inoculum, CB3-R1, CB3-R3, and CB3-R9 proved lethal in 30%, 60%, and 50% of animals, respectively (table 1). The final mortality at the 600

Figure 1. Dose-response curve of pleconaril inhibition of Coxsackievirus B3 (CB3). LLC-MK₂ cells were infected with 10⁶ pfu of wild type CB3 in presence of varying concentrations of pleconaril. Proportion of surviving virus, determined by plaque assay, is plotted against concentration of pleconaril. Dotted line indicates theoretical continuation of curve assuming that 100% of virus is susceptible to inhibition by pleconaril.
pfu dose was 50%, 30%, and 70% for CB3-R1, CB3-R3, and CB3-R9, respectively. In addition to an overall reduced ability of the drug-resistant viruses to cause lethal infection in mice, the time to onset of death was delayed 2–3 days in animals infected with the pleconaril-resistant variants (table 1).

To investigate further the reduced ability of the pleconaril-resistant viruses to cause lethality in mice, heart and spleen tissue from mice infected with wild type, CB3-R9, or CB3-R1 viruses were examined for virus titer. Tissues were taken at day 5, 1 day prior to the onset of animal deaths in the wild type–infected mice, and virus titers were determined by plaque assay. Levels of virus in tissues obtained from CB3-R1– and CB3-R9–infected mice were 2–3 orders of magnitude lower than those in the tissues of wild type virus–infected animals (table 1).

**Discussion**

Picornaviruses, particularly the enteroviruses and rhinoviruses, are ubiquitous human pathogens and cause immeasurable human morbidity. Enteroviruses are associated with over 20 clinically recognized syndromes, including viral meningitis, encephalitis, viral respiratory infection, neonatal enteroviral disease, myocarditis, conjunctivitis, gastroenteritis, hepatitis, and chronic infections in immunocompromised persons. Rhinoviruses are the most frequently recognized cause of acute upper respiratory infections [11]. It is clearly desirable to have a pharmaceutical means to manage and control diseases caused by enteroviruses and rhinoviruses.

Pleconaril (VP 63843) is an orally bioavailable small molecule inhibitor of enterovirus and rhinovirus capsid function. The drug acts by specifically integrating into the viral capsid at a unique hydrophobic site in the VP1 protein. When the drug occupies this binding pocket, virus attachment to cells and virus uncoating are blocked, which in turn prevents the release of viral RNA and RNA replication. Pleconaril inhibits the replication of >96% of the most commonly isolated serotypes of enterovirus and >90% of the more than 100 rhinoviruses tested at drug levels that can be readily achieved in humans (unpublished data). This broad-spectrum antipicornavirus activity, coupled with its admirable safety profile, makes pleconaril an exciting drug candidate for the treatment of enterovirus and rhinovirus diseases.

Pleconaril is currently undergoing clinical evaluation for treatment of viral meningitis and viral respiratory infection. In a clinical study in adult enteroviral meningitis, treatment with pleconaril resulted in a 53% \((P=.008)\) reduction in disease duration [12]. Schiff et al. [13] reported on the oral efficacy of pleconaril in coxsackievirus A21–induced respiratory disease in infected volunteers. Finally, in agammaglobulinemic children with chronic enterovirus central nervous system infections, a single short course of therapy with pleconaril resulted in clinical improvement and the elimination of virus [14].

These initial clinical results are encouraging. However, as for any pharmaceutical product targeting an infectious agent, it is important to understand the potential for the emergence of drug-resistant variants. The present studies were designed to investigate this issue directly. Pleconaril-resistant CB3 variants were isolated in cell culture. Of 10 independently isolated high-level resistant viruses, all possessed an amino acid substitution (and in 1 case, two amino acid changes) within the drug-binding site. Resistant variants were isolated from coxsackievirus A21–infected volunteers. Finally, in agammaglobulinemic children with chronic enterovirus central nervous system infections, a single short course of therapy with pleconaril resulted in clinical improvement and the elimination of virus [14].

These initial clinical results are encouraging. However, as for any pharmaceutical product targeting an infectious agent, it is important to understand the potential for the emergence of drug-resistant variants. The present studies were designed to investigate this issue directly. Pleconaril-resistant CB3 variants were isolated in cell culture. Of 10 independently isolated high-level resistant viruses, all possessed an amino acid substitution (and in 1 case, two amino acid changes) within the drug-binding pocket of VP1. In fact, all 10 resistant viruses studied here exhibited a change at residue 92, suggesting that this site is critical to the development of pleconaril resistance. The biologic characterization of the pleconaril-resistant viruses both in cell culture and in animals revealed them to be significantly less robust than the parental wild type virus. They showed reduced thermal stability and a reduced capacity to replicate in mice and cause lethal infection. The characteristics of three of the pleconaril-resistant CB3 variants are summarized in table 1.

These results have important implications for the use of pleconaril in the treatment of picornavirus diseases. It is anticipated that the patient treatment period with pleconaril will be brief, consequently reducing the likelihood that resistant virus variants will emerge. However, should pleconaril-resistant variants arise, the results of the present study suggest that they are likely to exhibit a weakened and attenuated phenotype, similar to that of an attenuated vaccine strain. It is noteworthy that in another study of virus resistance to a picornavirus capsid
inhibitor, resistant rhinovirus variants isolated from infected persons treated with the uncoating inhibitor R61837 possessed a diminished capacity to cause clinical illness [15]. The findings with rhinovirus and the enterovirus results of the present study with pleconaril suggest that capsid inhibitor-resistant virus variants may be of little clinical consequence.

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References