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The Enterovirus 3C Protease Inhibitor SG85 Efficiently Blocks Rhinovirus Replication and Is Not Cross-Resistant with Rupintrivir

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The novel enterovirus protease inhibitor (PI) SG85 effectively inhibits the *in vitro* replication of 14 rhinoviruses representative of species A and B (median 50% effective concentration, 0.04 μM). A low-level SG85-resistant variant was selected that carried amino acid substitutions S127G and T143A in the 3C protease. Both substitutions are required for low-level resistance to SG85, as demonstrated by reverse genetics. Interestingly, there is no cross-resistance to SG85 and rupintrivir (another PI); a structural explanation is provided for this observation.

Rhinoviruses (RV) are nonenveloped, positive-sense, single-stranded RNA viruses that belong to the family of *Picornaviridae*, genus *Enterovirus* (1). Currently, more than 150 RV have been identified, which genotypically group into RV-A, RV-B, and RV-C (2). RV infections not only cause common colds but may also trigger exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (3–5). Because of the high (and still rising) number of variants, development of a vaccine will be hard to achieve. Therefore, treatment with antivirals is a more realistic strategy to reduce the burden of these infections. An RV inhibitor, in particular, is needed for the prophylaxis and treatment of RV-induced exacerbations of asthma and COPD (6).

The RV 3C protease ($3C^{pro}$) is a promising target for drug development efforts because of the high level of conservation in its substrate-binding site, its role as an indispensable enzyme for virus replication, and its unique cleavage specificity (after Gln), which has not been observed in any other known host cell protease (7–11). Peptidomimetics with Michael acceptor warheads permanently disable the protease by covalent binding to its catalytic site (12, 13). The peptidomimetic rupintrivir (Pfizer AG7088; Fig. 1) effectively inhibits RV and enterovirus replication *in vitro* but largely failed to fulfill its promise in clinical trials (14–17).

Comparison of the known crystal structures of enterovirus $3C^{pro}$s revealed that the enterovirus 68 (EV68) $3C^{pro}$ can be considered an intermediate between the proteases of RV02 and poliovirus (18). Therefore, it was selected for the design of broad-spectrum enterovirus $3C^{pro}$ inhibitors, yielding SG85, a peptidic α,β-unsaturated ethyl ester with Michael acceptor properties, as the most promising candidate. SG85 is an efficient inhibitor of EV68 $3C^{pro}$ and inhibits the replication of enteroviruses in cell-based assays (18, 19).

We demonstrate here that SG85 effectively inhibits the replication (in multicycle virus-cell-based cytopathic effect [CPE] reduction assays [20]) of 14 RV serotypes that are representative of RV-A and -B (Table 1). Median 50% effective concentrations (EC_{50}s) of 0.04 ± 0.02 μM and 0.02 ± 0.01 μM were obtained against RV-A and RV-B, respectively. The $3C^{pro}$ inhibitor rupintrivir (Axon Medchem, The Netherlands) was, on average, 4-fold more active (Table 1), but this varied with the type (e.g., equipotent activity against RV63 and 14-fold more potent against RV02). Akin to rupintrivir, SG85 also strongly inhibited the replication of enterovirus 71 (EV71) and, to a lesser extent, protected cells against coxsackievirus B3 (CVB3), echovirus 11 (ECHO11), and poliovirus 1 (PV1) replication (18) (Table 1).

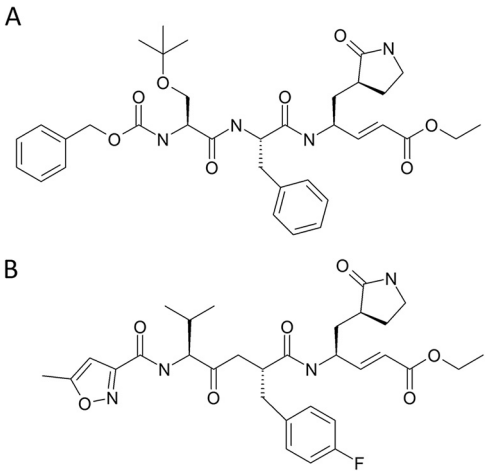

FIG 1 Structural formulae of SG85 (A) and rupintrivir (B).

Serial passaging with increasing concentrations of SG85 for extended periods of time did not result in the isolation of SG85-resistant RV14 virus variants (data not shown). Instead, a clonal selection procedure was used. To this end, several hundred infected cultures (in 96-well plates) were treated with a fixed concentration of the compound studied equal to the EC_{99} or a higher concentration. Supernatants collected from those few cultures where a CPE developed under drug pressure were selected for

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TABLE 1 Antiviral activities of SG85 and rupintrivir against 14 RV and 4 enteroviruses in virus-cell-based assays

Virus strain	Median EC_{50} (μM) ± MAD[a]	
	SG85	Rupintrivir
RV-A		
RV02	0.14 ± 0.03[a]	0.010 ± 0.006[a]
RV09	0.032 ± 0.008[a]	0.011 ± 0.001[a]
RV15	0.031 ± 0.004[a]	0.013 ± 0.001[a]
RV29	0.04 ± 0.03[a]	0.008 ± 0.002[a]
RV41	0.038 ± 0.007[a]	0.0036 ± 0.0008[a]
RV59	0.04 ± 0.02[a]	0.0102 ± 0.0001[a]
RV63	0.01 ± 0.01[a]	0.0106 ± 0.0009[a]
RV85	0.15 ± 0.02[a]	0.015 ± 0.008[a]
RV89	0.05 ± 0.06[a]	0.0040 ± 0.0002[a]
RV-B		
RV14	0.055 ± 0.008[a]	0.018 ± 0.002[a]
RV42	0.02 ± 0.02[a]	0.0026 ± 0.0008[a]
RV70	0.05 ± 0.05[a]	0.0111 ± 0.0001[a]
RV72	0.008 ± 0.005[a]	0.00356 ± 0.0001[a]
RV86	0.02 ± 0.02[a]	0.0096 ± 0.0008[a]
EV-A EV71	0.10 ± 0.04[a]	0.004 ± 0.002[a]
EV-B CVB3	0.5 ± 0.1[a]	0.25 ± 0.04[a]
EV-B ECHO11	33 ± 7[a]	2.8 ± 0.7[a]
EV-C PV1	39 ± 18	8.2 ± 2.7[a]

[a] Antiviral activity was determined in a CPE reduction assay with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium readout. The results shown are from dose-response curves set up from four or more experiments of which at least two were independent. MAD, median absolute deviation.
[b] One hundred percent inhibition of a virus-induced CPE can be achieved with this compound (as determined by microscopic inspection).

further expansion of the virus (20). Using this method, we obtained resistant variants in 1 out of 20 cultures in the case of pleconaril (data not shown), whereas for SG85, such variants were observed in only 1 of 273 cultures. This suggests that the virus quasispecies of the inoculum contained very few variants with some natural level of resistance to the compound and/or that it is very difficult for the virus to acquire these substitutions. The variant that was obtained proved to be >4-fold less sensitive than the wild-type virus to the antiviral effect of SG85 (data not shown). Such a low level of drug resistance may not be clinically relevant. However, other factors, such as the pharmacokinetic profile of the compounds in humans, will contribute as well. Genotyping revealed double mutations in the $3C^{pro}$-encoding gene (S127G and T143A). To confirm the causal link between the mutant genotype and the resistant phenotype, single and double mutants were generated by using an infectious clone of RV14 (D. Blaas, Vienna, Austria; referred to as $RV14_{IC}$ here). The two single mutants, i.e., those that carried either S127G or T143A, did not show decreased susceptibility to the antiviral effect of SG85 (Table 2). However, the double mutant ($RV14_{IC}$ S127G T143A) has low-level resistance and proved 3-fold less susceptible than wild-type $RV14_{IC}$ to the antiviral effect of SG85. Of note, the sensitivity of any of the mutants to rupintrivir was not altered (Table 2). The reverse-engineered double mutant had a level of resistance (3-fold) comparable to that of the phenotypically selected variant. It has been reported that *in vitro*-selected rupintrivir-resistant RV14 (7- to 16-fold resistant) carries four substitutions (i.e., T129A, T131A, Y139H, T143P) in 3C (21). Even though the quadruple mutant also carries a substitution at position 143 (21), we did not observe cross-resistance between SG85 (which selects for the substitutions S127G and T143A) and rupintrivir (Table 2). In the same study, reverse-engineered variants with a single substitution, including one at position T143, showed no reduced susceptibility to the antiviral effect of rupintrivir (21). This is in line with our observations that besides the substitution T143A, the substitution S127G in $3C^{pro}$ is also required for resistance to the Michael acceptor-based peptidomimetic SG85.

SG85 and rupintrivir are less active against enterovirus species B and C (Table 1). For a better understanding, a sequence alignment was made of $3C^{pro}$ of the RV strains that were used in this study, along with the 3C sequences of an RV-C15 isolate (W1, GU 219984.1), CVB4, PV1, and EV68 (Fig. 2). The serine residue at position 127 was conserved throughout the RV strains. At the corresponding position (residue 128), EV68 and PV1 carry a glycine, which corresponds to the substitution that was detected in the low-level SG85-resistant RV14 variant. A crystal structure of RV02 $3C^{pro}$ in complex with a peptidic Michael acceptor (compound III) revealed the presence of a hydrogen bond between the backbone amide of the P2 residue of the inhibitor and the side chain oxygen of S128 (22). It may be assumed that the same interaction will exist in the SG85 complex of the RV14 protease and will thus be lost with the S127G substitution, presumably resulting in a loss of free energy of binding that decreases the efficiency of the inhibitor. Rupintrivir, however, lacks a P2 backbone amide and such a hydrogen-bonding interaction, and therefore, the S127G substitution does not accumulate as part of a drug resistance mechanism. It should be noted that we made multiple attempts to express both the wild-type and mutant proteases. However, be-

TABLE 2 Antiviral activities of SG85, rupintrivir, and pleconaril on the replication of reverse-engineered $RV14_{IC}$ strains that contain mutations in the $3C^{pro}$-encoding gene

$RV14_{IC}$ strain	EC_{50} (μM) ± MAD (RR)[a]		
	SG85	Rupintrivir	Pleconaril
Wild type	0.018 ± 0.001	0.010 ± 0.001	0.327 ± 0.004
S127G mutant	0.019 ± 0.001 (1)	0.0094 ± 0.0002 (1)	0.31 ± 0.01 (1)
T143A mutant	0.018 ± 0.001 (1)	0.008 ± 0.001 (1)	0.108 ± 0.004[b] (0.3)
S127G T143A mutant	0.047 ± 0.001[b] (3)	0.012 ± 0.001 (1)	0.13 ± 0.02[c] (0.4)

[a] Antiviral activity was determined in a CPE reduction assay with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium readout. Data are in duplicate from three independent assays. The early-stage RV inhibitor pleconaril (kindly provided by V. Makarov, RAS Institute of Biochemistry, Russia) was included in this assay as a reference. MAD, median absolute deviation. RR, relative resistance (EC_{50} of mutated strain/EC_{50} of wild type).
[b] $P < 0.0001$ (unpaired *t* test).
[c] $P < 0.001$ (unpaired *t* test).

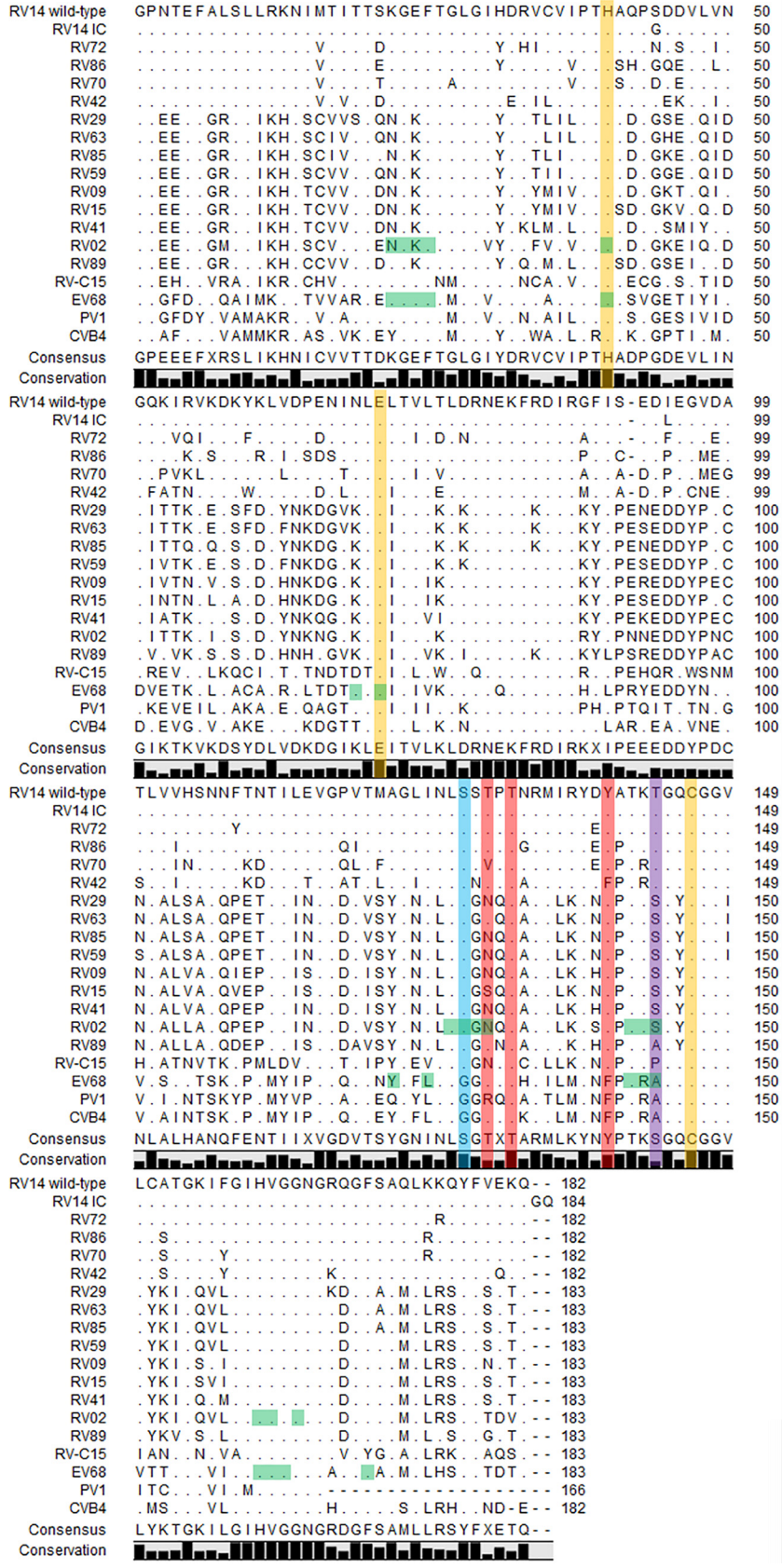

FIG 2 Alignment of the 3C^{pro} amino acid sequences of 15 RV (RV-A and -B) genotypes, EV68 (EV-D), and PV1 (EV-C). A yellow background indicates residues of the catalytic triad, a purple background indicates a residue that is mutated in both the SG85- and rupintrivir-resistant RV14 variants, a blue background indicates residues that are mutated in the SG85-resistant RV14 variant, a red background indicates residues that are mutated only in rupintrivir-resistant RV14 variants, and a green background indicates residues in the structure of the RV02 3C^{pro} that interact with compounds I and III (22) or residues in EV68 3C^{pro} that interact with SG85 (18). This sequence alignment was created in CLC sequence viewer 7 (Qiagen).

cause of the limited solubility of these proteins, we were not able to biochemically characterize these enzymes.

The threonine at position 143 is conserved in the RV-B strains, whereas the majority of RV-A strains carry a serine (at corresponding position 144) and the RV-C strain included in this analysis carries a proline. It remains to be determined whether RV-C ($3C^{pro}$) is sensitive to SG85 (and/or rupintrivir). For RV89, EV68, and PV1, an alanine residue is present at this position, which was also detected in the low-level SG85-resistant RV14 variant. The crystal structures of EV68 $3C^{pro}$ in complex with SG85 (18) and of RV $3C^{pro}$ in complex with compound III or rupintrivir (22) reveal that whereas the main chain of residue 144 is part of the S1 pocket of the protease, its side chain, whether Ala or Ser/Thr, is oriented away from the inhibitor; therefore, it is not immediately clear how the S/T143A substitution would affect the binding of the compound. In agreement with these observations, both the $RV14_{IC}$ S127G T143A double mutant and PV1 show decreased susceptibility to the antiviral effect of SG85, whereas RV89 (which is akin to the $RV14_{IC}$ single mutant, carries only A143) is inhibited as well as wild-type $RV14_{IC}$.

Protease inhibitors (PI) are being successfully used for the treatment of infections with HIV and hepatitis C virus. The development of novel RV and enterovirus 3C inhibitors should be considered further. Such inhibitors have the potential to exert broad-spectrum antiviral activity and (unlike with most HIV and HCV PI) high-level drug-resistant variants do not, or at least do not readily, develop. Moreover, we demonstrate here that PI of enterovirus and RV 3C with nonoverlapping drug resistance profiles can be developed.

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