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Pyrazolopyrimidines: Potent Inhibitors Targeting the Capsid of Rhino- and Enteroviruses

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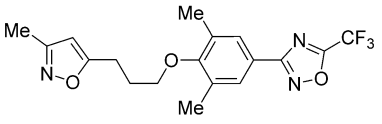
There are currently no drugs available for the treatment of enterovirus (EV)-induced acute and chronic diseases such as the common cold, meningitis, encephalitis, pneumonia, and myocarditis with or without consecutive dilated cardiomyopathy. Here, we report the discovery and characterization of pyrazolopyrimidines, a well-tolerated and potent class of novel EV inhibitors. The compounds inhibit the replication of a broad spectrum of EV in vitro with IC_{50} values between 0.04 and 0.64 μM for viruses resistant to pleconaril, a known capsid-binding inhibitor, without affecting cytochrome P450 enzyme activity. Using virological and genetics methods, the viral capsid was identified as the target of the most promising, orally bioavailable compound 3-(4-trifluoromethylphenyl)amino-6-phenylpyrazolo[3,4-*d*]pyrimidine-4-amine (OBR-5-340). Its prophylactic as well as therapeutic application was proved for coxsackievirus B3-induced chronic myocarditis in mice. The favorable pharmacokinetic, toxicological, and pharmacodynamics profile in mice renders OBR-5-340 a highly promising drug candidate, and the regulatory nonclinical program is ongoing.

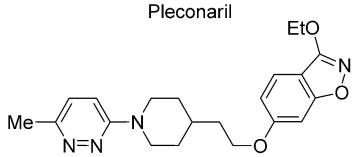
The genus enterovirus (EV) of the family *picornaviridae* includes more than 100 rhinovirus (RV) serotypes and about 70 EV serotypes, each of them classified into species A–D.[1] These viruses are capable of causing a wide range of acute and chronic diseases.[2] Each year, millions of people suffer from the common cold, asthma, chronic obstructive pulmonary disease exacerbation, otitis or pneumonia after infection with rhinoviruses.[2b] EVs such as echoviruses 9, 11, 30 (ECHO-9, -11, and -30) and coxsackieviruses B1-6 (CVB1-6) can cause severe conditions, including aseptic meningitis, encephalitis, and acute and chronic myocarditis with or without consecutive dilated cardiomyopathy.[2a]

Broad-spectrum anti-enteroviral drugs are urgently needed, but despite intensive research no specific drugs have been approved for the treatment of EV infections so far.[3] Drug candidates include the RNA synthesis inhibitor enviroxime and the protease 2C inhibitor AG 7088.[3a,b] Pleconaril, a capsid inhibitor with broad-spectrum activity against EVs,[4] failed approval by the US Food and Drug Administration for the oral treatment of the common cold due to a lack of efficacy and safety concerns related to the induction of certain cytochrome P450 enzymes.[5] Results from a phase II clinical trial (NCT00394914) with an intranasal formulation of pleconaril have yet to be published.[6] 1,2-Benzisoxazoles represent a second class of potential capsid inhibitors, with drug candidate vapendavir currently under development.[7] Its binding mode and activity spectrum closely resemble those of pleconaril,[4a,b,7–8] which may be a factor in the observed cross-resistance with pleconaril.[7b]

High-level resistance to capsid inhibitors resulting from single amino acid substitution of residues forming the hydrophobic inhibitor binding pocket of viral capsid protein 1 (VP1)



Pleconaril



Vapendavir

have been observed.[9] It has been suggested that substituted amino acids block the integration of the inhibitors into the pore. Such single amino acid substitutions in VP1 were also shown to confer high-level pleconaril resistance to EVs,[4a,c,8,10] whereby I1092L substitution, situated in proximity of the central, methylated phenoxy group of pleconaril in VP1, was most commonly observed for CVB3.[4c,8] We demonstrated the crucial role of the central ring of capsid inhibitors in anti-enteroviral

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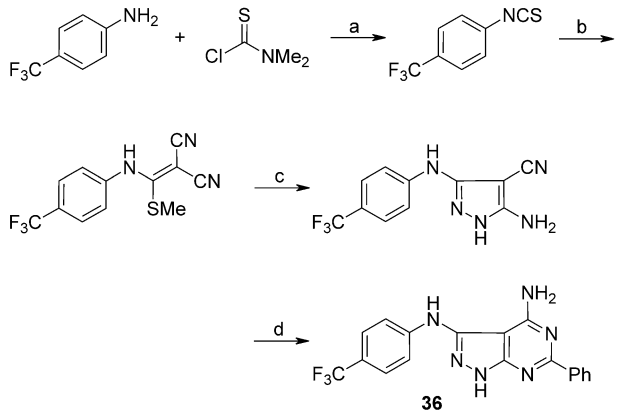

Scheme 1. Synthetic route to compound **36**.[12] *Reagents and conditions*: a) toluene, reflux, 3 h, 95%; b) NaH, $CH_2(CN)_2$, DMF, 20 °C, 5 h, 95%; c) NH_2NH_2 H_2O, EtOH, reflux, 2.5 h, 67%; d) PhC(NH)NH2, NaAc, butanol, melt at 170–180 °C, 25 min, 25%.

activity and showed that selected ring decorations can break drug resistance.[11] These and further considerations led to the exploration of a series of pyrazolo[3,4-*d*]pyrimidines as novel drug candidates for the treatment of EV infections (Scheme 1; see also, Table S1 in the Supporting Information).[12] Herein, we report their synthesis, physicochemical characteristics, structure–activity relationships and unique anti-enteroviral activities.

All synthesized compounds (~80, plus pleconaril as a reference) were initially subjected to cytotoxicity (see the Supporting Information) and antiviral testing (inhibition of the cytopathic effect, CPE) in HeLa cell cultures. Most of them were well tolerated (Table S2 in the Supporting Information). Analogues derived from a 3-phenylamino-6-phenylpyrazolo[3,4-*d*]pyrimidine-4-amine scaffold were particularly promising, exhibiting strong activity against CVB3, RV2, 5, 8, 42, and 48 (RV5, RV42, and RV48 are known[4a] and confirmed by our data as high-level pleconaril-resistant; activity measured as described in Table S2 in the Supporting Information). Amino groups at positions 3 and 4 of the central ring are critical for antiviral activity. Different substitutions of the aniline are possible. Most active compounds comprise at least one halogen or trifluoromethyl group (e.g., compounds **24**, **25**, **32, 36**, and **41**).

Compound **36** (MW: 370.3; log*P* = 2.85; Scheme 1) showed particularly favorable physicochemical, antiviral, and pharmacokinetic properties (Figure 1 and Table 1; see also, Tables S2 and S4 in the Supporting Information). It was synthesized in four steps with high yield,[12b] as summarized in Scheme 1.

Compound **25**, an analogue with a 4-fluoroaniline moiety, was also highly active in vitro but almost inactive in vivo (results not shown). The compound is hydroxylated in vivo to form the 100- to 5000-fold weaker metabolite **15**. This renders **25** unsuitable for oral administration. By introducing an electron-withdrawing trifluoromethyl group into **36**, its pharmacokinetic and metabolic properties were improved (Tables S4 and S5 in the Supporting Information).

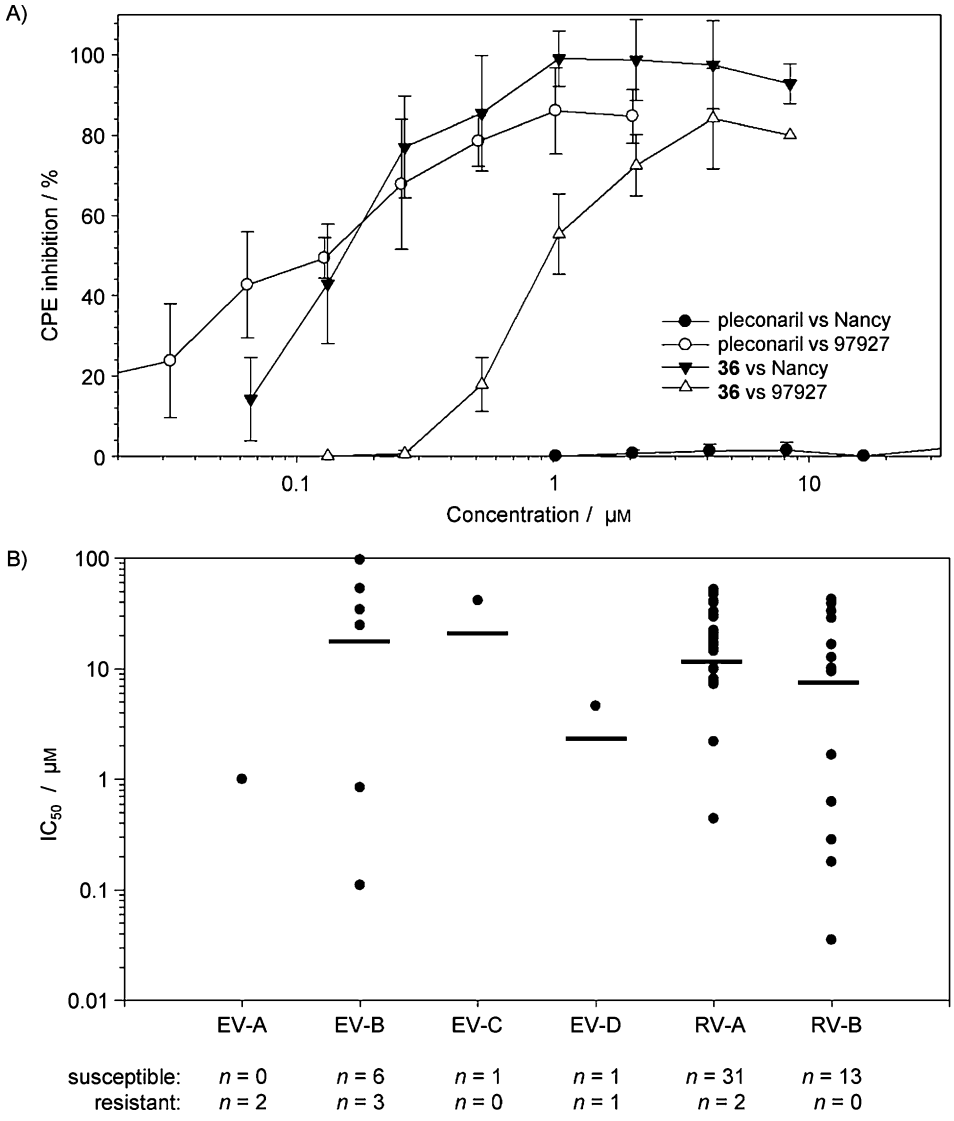

Figure 1. Anti-enteroviral activity of **36**. A) Dose-dependent inhibition of CVB3 Nancy- and CVB3 97927-induced cytopathic effect in HeLa cells by compound **36** in comparison to pleconaril. Data represent the mean ± SD (error bars) of at least three independent determinations. B) Distribution of mean IC_{50} values of compound **36** among the studied enterovirus strains. Mean and/or SD values were calculated from the results of at least three assays.

Table 1. Impact of amino acid substitutions detected in compound **36**-resistant CVB3 Nancy variants on plaque inhibition in HeLa cells.

CVB3 Nancy	Amino acid substitution	Pleconaril[b]	IC_{50} [μM][a] Compd **36**	Compd **25**
wildtype	none	n.a.	0.16 ± 0.03	0.25 ± 0.06
36-resistant	P1094S	n.a.	35.03 ± 0.01	3.43 ± 0.97
36-resistant	S1190T	n.a.	n.a.	n.a.
36-resistant	I1207N	n.a.	n.a.	n.a.
36-resistant	I1207 M	n.a.	n.a.	31.53 ± 7.89
36-resistant	S2209C	n.a.	n.a.	32.47 ± 4.59

[a] Data represent the mean ± SD of at least three independent determinations in plaque reduction assays performed in duplicate; [b] not active (n.a.) due to naturally occurring pleconaril resistance (L1092).

Compound **36** strongly inhibited the cytopathic effect of the pleconaril-resistant CVB3 Nancy (L1092[4c]) as well as pleconaril-susceptible CVB3 97927 (I1092) with IC_{50} values of 0.16 and 1.05 μM, respectively (Figure 1 A; see also the Supporting Information).

We postulate that inhibitor binding to viral capsid proteins leads to the prevention of virus adsorption and might also be responsible for the observed antiviral effect during and after adsorption. The detection of amino acid substitutions in VP1 (2×P1094S, 2×S1190T, 1×I1207N, 1×I1207M) and VP2 (2×S2209C) of high-level **36**-resistant CVB3 Nancy variants supports this (Table 1). In agreement with pleconaril studies,[8] a concentration of 1 μg mL^{-1} (2.7 μM) of **36** was used for their isolation. In ten independently prepared pools of wild-type CVB3 Nancy, the mean frequency of resistance to **36** was ~7×10^5, corresponding well with the data published for pleconaril.[8] Based on these phenotypic and genotypic data, we expect that the lipophilic **36** binds inside the hydrophobic pocket of VP1 in a similar fashion to pleconaril and vapendavir.[7b,15] The potential binding mode of **36** as derived by automated ligand docking with GOLD[16] corroborates this mechanism of action (Figure 2 B,C). The model suggests that the observed mutations causing high-level resistance induce larger conformational rearrangements leading to the disruption of hydrophobic interactions formed by the trifluorophenyl moiety of **36** (which is postulated to correspond to the methylisoxazole moiety in pleconaril).

A panel of 46 RV serotypes comprising viruses from species A (n = 33) and B (n = 13), two EV species A, nine EV species B, one EV species C, and two EV species D were assessed regarding their sensitivity to **36** in CPE inhibition assays in HeLa cells.[13] Compound **36** inhibited 52 of the 60 strains tested (Figure 1 B). Strong antiviral activity was detected against ECHO11 and EV68, which are known to cause outbreaks of aseptic meningitis and acute lower respiratory tract infections, respectively.[14] The highest activities were measured against high-level pleconaril-resistant viruses including RV5, RV42, RV44, RV48, and RV69.[4a,c,10] The IC_{50} values for these specific EVs ranged from 0.04 to 0.64 μM (Table S2 in the Supporting Information).

The effect of 0.5 and 1 μM of **36** on different steps of the viral replication cycle was studied in plaque reduction assays with CVB3 Nancy in HeLa cells (Figure 2 A). Pretreatment of cells with **36** prior to virus inoculation had no effect on virus-induced plaque formation. In contrast, incubation of **36** with cell-free virus resulted in strong loss of virus infectivity as shown by high percentage of plaque reduction in Figure 2 A. Here, high concentrations of CVB3 Nancy were incubated with or without the inhibitor in test medium for one hour at 37 °C. Then the mixture was diluted 1:10^6 to obtain noneffective inhibitor concentrations and added to confluent cell monolayers for virus adsorption.

Acute (>3 g kg^{-1}) and chronic (12.5, 50 and 200 mg kg^{-1} doses, 30 days treatment in male BALB/c mice) toxicological studies in mice showed no indications of adverse anatomical, behavioral or physiological effects (see Table S3 in the Supporting Information). In vitro ADMET profiling of **36** (Table S4 in the Supporting Information) included analysis of plasma protein binding and recovery (96.7% and 100%, respectively), plasma stability (100%), mutagenicity (none), and hERG channel inhibition (low). Compound **36** is neither an inducer nor an inhibitor of relevant cytochrome P450 isoforms. A 100% metabolic stability of **36** in human liver microsomes was observed (see the Supporting Information, Table S4) explaining in vitro its efficacy after oral administration in mice. The in vitro data were confirmed by pharmacokinetics studies in mice during a 100 mg kg^{-1} per os administration. The in vivo results show that **36** has druglike pharmacokinetic properties, as reported in the Supporting Information (Table S5), and in part summarized here: C_{max}: 1.25 μg mL^{-1}; t_{max}: 3 h; AUC_{0-t}: 6418.3 ng mL h^{-1}; $AUC_{0-\infty}$: 9598.53 ng mL h^{-1}; $t_{1/2}$: 3.5 h; C_{max}/AUC_{0-t}: 0.2 h^{-1}; MRT: 3.54 h; Cl: 10.42 mL h kg^{-1}; K_{el}: 0.18 h^{-1}; V_d: 52.72 mL kg^{-1}.

Effective compound concentrations were reached by oral administration of 100 mg kg^{-1} of **36** twice daily, for 7 days. When treatment was started 3 h prior to (prophylactically) or 1 and 3 days p. i. (therapeutically), **36** exerted a strong antiviral effect in a model of CVB3-induced chronic myocarditis in NMRI mice (Figure 3; see also, Tables S6 and S7 in the Supporting Information). Placebo-treated, CVB3-infected mice markedly lost body weight (Figure 3 A) and became ill (Figure 3 B) on day 3 p. i. Prophylactic and therapeutic treatment significantly improved both study parameters (Figure 3). As a result of viral replication, pronounced inflammation as well as necrosis and fibrosis of myocardial tissue were observed in hematoxylin-eosin and sirius red-stained heart specimens, respectively, in placebo-treated animals on day 28 p. i. (Figure 3 C). CVB3 also completely destroyed the exocrine pancreas tissue (Figure 3 C). Compound **36** significantly decreased tissue damage as well as inflammation in the heart and pancreas when treatment was started 3 h before, or 1 or 3 days p. i. No therapeutic effect was observed when treatment started 7 days p. i. (results not shown).

In conclusion, pyrazolopyrimidines represent a novel class of compounds targeting the capsid of clinical important RVs and EVs. Notably, lead compound **36** is orally available, inhibits a broad-spectrum of EVs and RVs, helps to overcome the resistance of known capsid inhibitors, and does not exert an effect on relevant cytochrome isoforms. The favorable pharma-

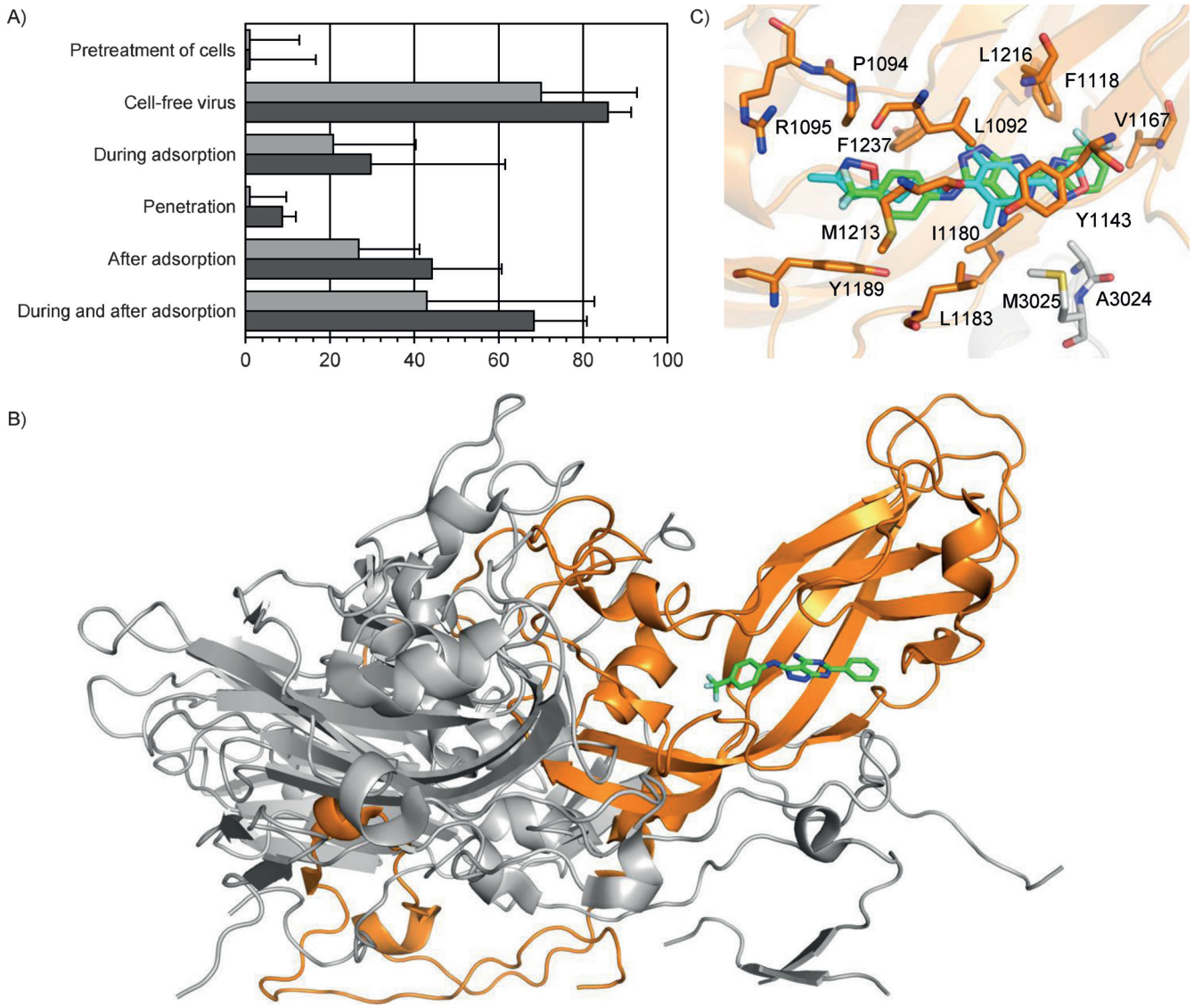

Figure 2. Mechanism of action of compound **36**. A) 0.5 μM (■) or 1.0 μM (■) of compound **36** were added to cells or only to cell-free virus before infection and at different time points during infection in a plaque reduction assay with CVB3 Nancy in HeLa cells. B) Viral capsid protein (VP1: orange; VP2-4: grey) with the highest-ranked docking pose obtained for **36** (carbon atoms in green; ChemPLP score[17] = 90.6). C) Detail of this docking pose. Carbon atoms of pleconaril are turquoise, and that of **36** are green. Nitrogen, oxygen, sulfur and fluorine atoms are colored blue, red, yellow and light blue, respectively. The molecular shapes of **36** and pleconaril as positioned within the hydrophobic pocket show similarities. Compound **36** shows excellent shape complementarity with the hydrophobic pocket.

cokinetic, toxicological, and pharmacodynamics profile in mice renders **36** a highly promising drug candidate.

Experimental Section

Chemistry: The synthesis of compound **36** as shown in Scheme 1 is described in detail in the Supporting Information together with synthetic protocols for access to control compounds (guanidine hydrochloride and pleconaril).

Computational methods: Information on the homology model used to predict the binding mode and ligand docking methods is also given in the Supporting Information.

In vitro assays: Also presented in the Supporting Information are details of the in vitro studies performed, including analysis of cytotoxicity and antiviral activity (CPE inhibition assay) of pyrazolopyrimidines in HeLa cells, mechanism of action studies (modified plaque-reduction assay), isolation[8] and phenotypic (plaque-reduction assay), as well as genetic characterization of **36**-resistant variants. Information on the cells, virus strains, growth conditions, and virus titer determination are also described.

In vivo models: The in vivo studies on the prophylactic and therapeutic antiviral effect, the pharmacokinetic, and the toxicity of **36** were performed in mice. The experimental design was reviewed and approved by local government (ThüringerLandesamt für Lebensmittelsicherheit und Verbraucherschutz); the registration number is 02-001/07 (antiviral studies), and by the State Scientific Center for Antibiotics, Moscow; the registration number is 267/2010 (pharmacokinetic and toxicity). For details, see the Supporting Information.

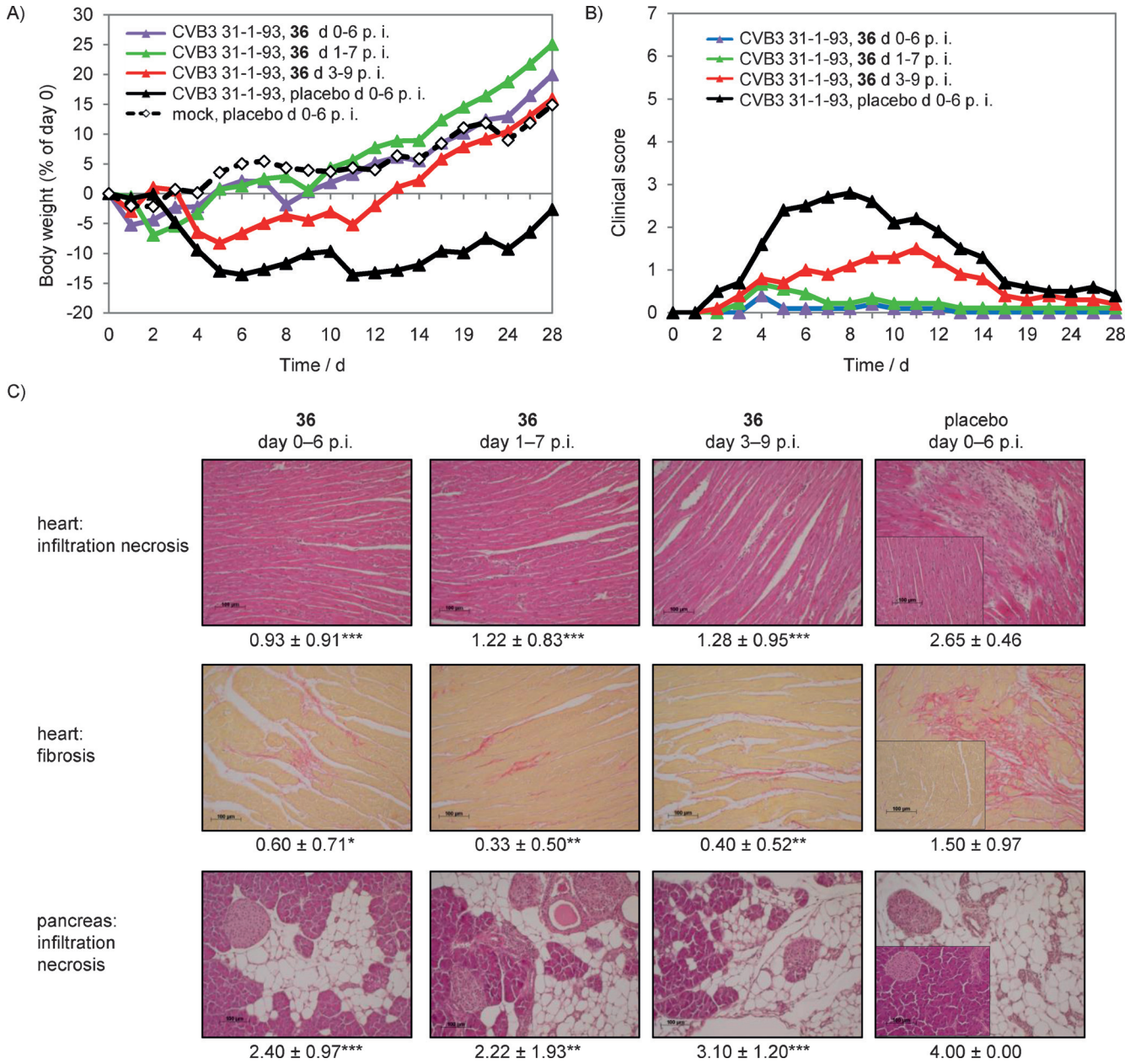

Figure 3. Prevention of CVB3-induced chronic myocarditis in NMRI mice. A) Influence of treatment with **36** on body weight, B) on symptoms shown as clinical score, and C) on histopathological changes in heart and pancreas tissue. Statistical analysis of histopathological and clinical score was performed using the Mann-Whitney U test to compare **36**-treated, and placebo-treated infected mice. After confirming normal distribution of data with the Kolmogorov-Smirnov test, a t-test was applied for statistical analysis of body weight change of untreated, **36**-treated mice in comparison to untreated, placebo-treated infected mice. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

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Keywords: antivirus agents · drug discovery · structure–property relationships · synthetic drugs · virology

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