Supporting informations

New Pyrazolobenzothiazine Derivatives As Hepatitis C Virus NS5B Polymerase Palm Site I Inhibitors

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Contents:

Biochemical and biological assays

NS5B polymerase assay. Recombinant NS5B 1b (HC-J4) carrying an N-terminal histidine-tag and 21-amino acid truncation at its C-terminus was purified by Ni-NTA chromatography as previously described.[1] The compounds were dissolved in DMSO as 10 mM stocks and serially diluted in DMSO immediately prior to the assay. Anti-HCV NS5B activity of the compounds was investigated by the standard primer dependent elongation assay as previously described.[2] Preliminary screening was conducted at 25 μM compound concentration to identify candidate NS5B inhibitors. The assays were carried out at 30°C for 1 h and reactions stopped with chilled 5% (v/v) trichloroacetic acid (TCA) containing 0.5 mM pyrophosphate. Nascent RNA products were precipitated on GF-B filters and quantified by liquid scintillation counting. NS5B activity in the presence of DMSO control was set at 100% and that in the presence of the compounds was determined relative to this control. Only compounds exhibiting ≥50% inhibition at 25 μM concentration were further investigated for their IC_{50} values; for the remaining compounds, the term "NA, not active" was reported in Table 1. Dose-response curves of 8-12 concentrations of the compounds in duplicate in two independent experiments were plotted using nonlinear regression analysis and IC_{50} values were determined using CalcuSyn V2 software.

Cells and viruses. The Huh 5-2 and Huh 9-13 HCV subgenomic replicon-containing cells were provided by Prof R Bartenschlager (University of Heidelberg, Heidelberg, Germany).

Antiviral assays. Huh 5.2 cells, containing the hepatitis C virus genotype 1b I389luc-ubi-neo/NS3-3'/5.1 replicon[3] were sub-cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin and 2% Geneticin at a ratio of 1:3 to 1:4, and grown for 3-4 days in 75 cm² tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6 500 cells/well (100 μL/well) in 96-well tissue culture

microtiter plates for evaluation of anti-metabolic effect and CulturPlate (Perkin Elmer) for evaluation of the antiviral effect. The microtiter plates were incubated overnight (37 °C, 5% CO_2, 95-99% relative humidity), yielding a non-confluent cell monolayer.

The evaluation of the anti-metabolic as well as antiviral effect of each compound was performed in parallel. Four-step, 1-to-5 compound dilution series were prepared for the first screen, to collect data for a more detailed dose-response curve, an eight-step, 1-to-2 dilution series was used. Following assay setup, the microtiter plates were incubated for 72 hours (37 °C, 5% CO_2, 95-99% relative humidity). For the evaluation of anti-metabolic effects, the assay medium was aspirated, replaced with 75 µL of a 5% MTS solution in phenol red-free medium and incubated for 1.5 hours (37 °C, 5% CO_2, 95-99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire², Tecan), and optical densities (OD values) were converted to percentage of untreated controls. For the evaluation of antiviral effects, assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 µL of Glo Lysis Buffer (Promega) was added allowing for cell lysis to proceed for 5 min at room temperature. Subsequently, 50 µL of Luciferase Assay System (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire², Tecan). Relative luminescence units were converted into percentage of untreated controls.

The EC_{50} and EC_{90} (values calculated from the dose-response curve) represent the concentrations at which 50% and 90% inhibition, respectively, of viral replication is achieved. For all compounds was possible to calculate EC_{50} values. Compounds **2f**, **2i**, **2j**, **2n**-**p**, **2s**, **3** and **5** did not reach 90% inhibition, thus EC_{90} values could not be determined (ND, not determined in Table 1).

The CC_{50} (value calculated from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50 % as compared to untreated cells.

A concentration of compound is considered to elicit a genuine antiviral effect in the HCV replicon system when the anti-replicon effect is well above the 70% threshold at concentrations where no significant anti-metabolic activity is observed.

Compounds that reproducibly matched the above-outlined selection criteria were evaluated for selective antiviral activity in the Huh 9-13 replicon system. A similar assay setup was used as described above; the antiviral and anti-metabolic effect of the compounds was evaluated in parallel. The anti-metabolic activity of the compounds was quantified as outlined above. For the evaluation of the antiviral effect, assay medium was aspirated and the plates with dry monolayer were stored at -80°C awaiting extraction. Following thawing of the plates at room temperature, the cell monolayer was lysed with 100 µL of cell-to-cDNA lysis buffer (Invitrogen). Lysis of the cells was allowed to proceed for 10 min at room temperature after which all liquid was transferred to a PCR plate (Axygen). The PCR plate was incubated for 15min at 75 °C (T3, Biometra). The lysate was diluted 1:2 with RNase/DNase-free water, after which 5 µL was transferred to a real-time PCR plate (Applied Biosystems). Replicon RNA content was quantified using a real-time quantitative one-step RT-PCR method (RT-qPCR). Per sample, 20 µL master mix was added containing 12.5 µL 2x RT-qPCR mix (Low Rox One-Step RT-qPCR master mix, Abgene), 0.125 µL of a 60 µM forward primer solution (5'-CCA GAT CAT CCT GAT CGA CCA G-3', final [ ] of 300 nM), 0.125 µL of a 60 µM reversed primer solution (5'-CCG GCT ACC TGC CCA TTC-3', final [ ] of 300 nM), 0.3µL of a 5 µM probe solution (5'-ACA TCG CAT CGA GCG AGC ACG TAC-3', final [ ] of 60 nM) and 6.825 µL of DNase/RNase-free water (ACROS). The samples were analyzed using a SDS7500F (Applied Biosystems, standard thermocycling profile: 30 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 sec at 95 °C and 1 min at 60 °C). Replicon RNA quantities were converted to percentage of untreated controls, allowing the calculation of EC_{50} and EC_{90} values. Similar to the Huh 5-2 assay, a compound is only considered to be a selective inhibitor of HCV replication when clear inhibition of virus replication is observed at concentrations that do not elicit a significant anti-metabolic effect on the host cells.

Histological analysis from vital Stains studies for compounds 2a and 2b

When comparing the Huh 9-13 stained with nile red and treated with different inhibitors it is clear that both compounds **2a** and **2b** at 100 μM (panels B and E, respectively) cause a slight loss in cell number as compared to the mock treated control (panel F) (Figure S1). Unlike the HCV NS3 protease inhibitor VX-950 (panel C) that causes pronounced loss of cell numbers at 100 μM, compounds **2a** and **2b** at 100 μM (Panels B and E, respectively) do not impact the general cellular morphology. For compound **2a** an overall increase in neutral lipids is observed with the neutral lipid signal mainly confined to slightly larger lipid droplets that match more the pattern induced by cyclosporin A and not the pattern induced by Entomoxir. For compound **2b** a more pronounced red staining is observed that is indicative of an increased content of charged lipids. The neutral lipid content of the **2b** treated cells is, when compared to the mock treated control, lower and the signal is mainly confined to a few lipid droplets that resemble more the lipid droplets observed in Huh 9-13 cells. In conclusion comparison of nile red stained Huh 9-13 cells showed that, apart from a general reduction in cell number for both compounds, compound **2b** has a greater effect on the charged lipid content of treated cells while compound **2a** influences the neutral lipid content of cells.

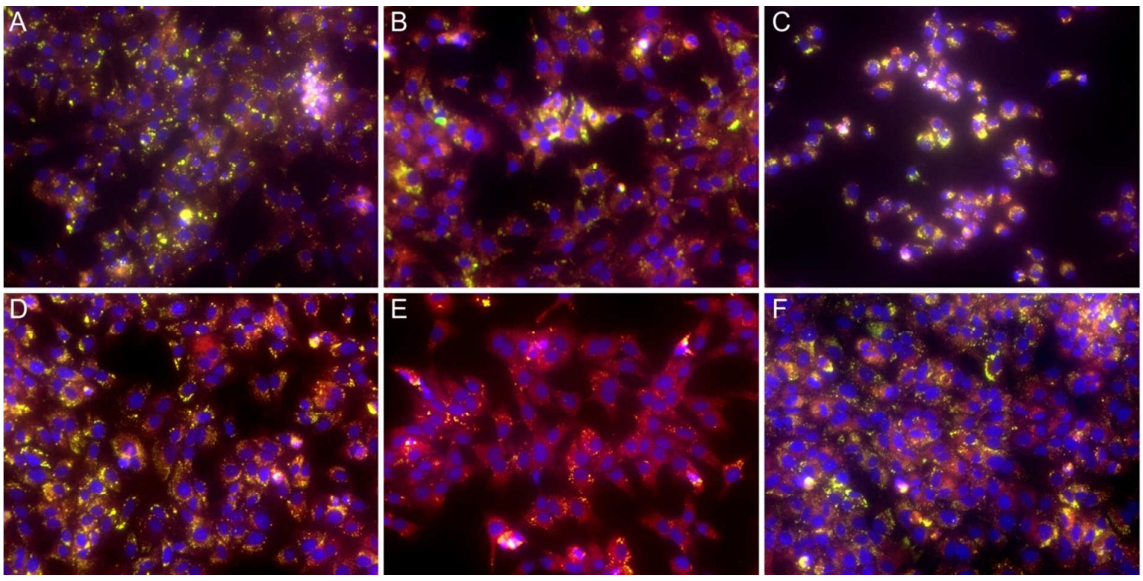

Figure 1S. Huh 9-13 cells were fixed and stained with nile red after 24 hr treatment with either (A) a known inducer of steatosis i.e. cyclosporine A (at 100 μM), (B) compound **2a** (at 100 μM), (C) the HCV NS3 protease inhibitor VX-950 (at 100 μM), (D) a known inhibitor of beta-oxidation i.e. Entomoxir (at 100 μM), (E) compound **2b** (at 100 μM) or (F) mock treatment (DMSO). Nile Red stains both neutral lipids (yellow/gold color) and phospholipids (orange/red color). Nuclei are stained with Hoechst 33342 (blue). This figure was prepared with ScientiFig.[4]

For Huh 9-13 cells stained with lipiTOX red similar observations can be made for the effect of compound **2a** and **2b** on cell numbers (Figure 2S). The effect of compounds **2a** and **2b** on neutral lipid content is however more difficult to discern. In both panels B and C a few cells can be observed that have a phenotype that resembles more the phenotype after cyclosporine A treatment (panel A). The overall impression of both panels B and E is more in conjunction with the mock treated control (panel F). In conclusion comparison of lipiTOX stained Huh 9-13 cells showed a similar reduction in cell number for both compounds as observed with nile red, however the effect of compound **2a** on the neutral lipid content of the cells was less pronounced as compared to the effect observed with nile red.

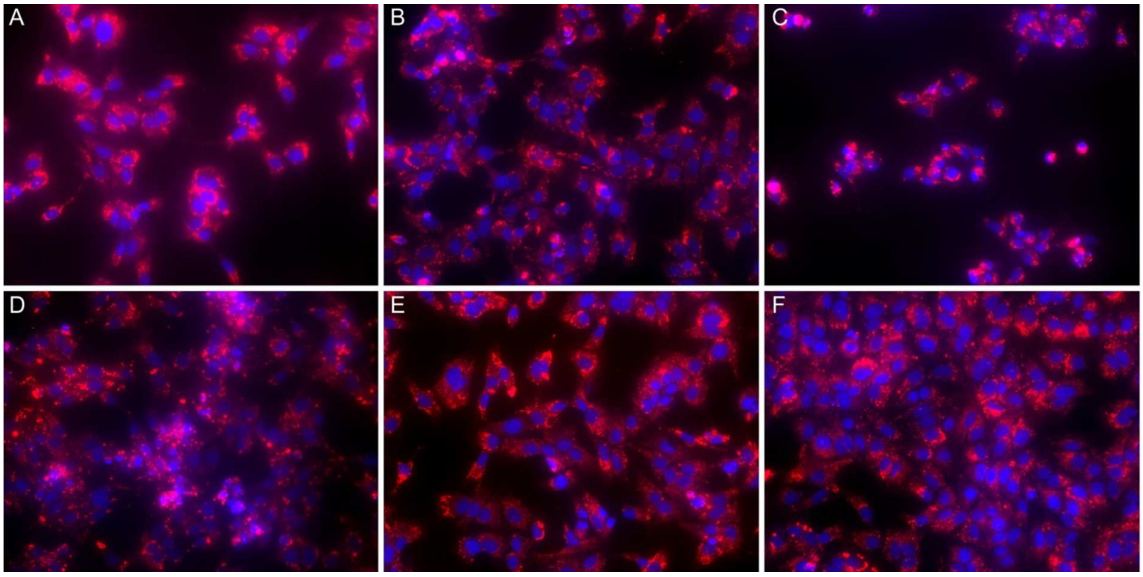

Figure 2S. Huh 9-13 cells were fixed and stained with LipidTOX red after 24 hr treatment with either A. a known inducer of steatosis i.e. cyclosporine A (at 100 μM), B. compound **2a** (at 100 μM), C. the HCV NS3 protease inhibitor VX-950 (at 100 μM), D. a known inhibitor of beta-oxidation i.e. Entomoxir (at 100 μM), E. compound **2b** (at 100 μM) or D. mock treatment (DMSO). LipidTOX red stains neutral lipids (rec). Nuclei are stained with Hoechst 33342 (blue). This figure was prepared with ScientiFig.[4]

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