Supporting Information

2,4-Diaminoquinazolines as Dual Toll Like Receptor 7/8 Modulators for the Treatment of Hepatitis B virus

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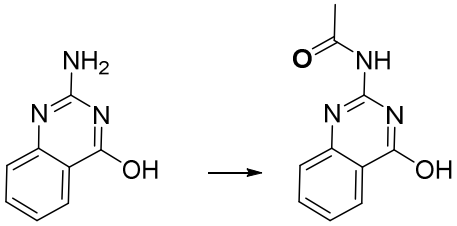
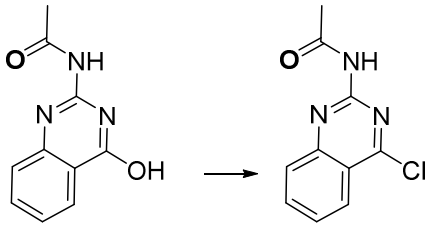
Contents of Supporting Information

Example alternative synthesis

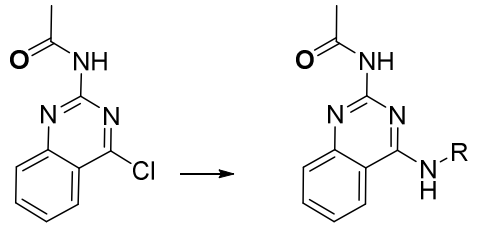
(S)-2-((2-aminoquinazolin-4-yl)amino)butan-1-ol (**5**) and *(S)-2-((2-aminoquinazolin-4-yl)amino)-4-methylpentan-1-ol* (**8**).



N-(4-hydroxyquinazolin-2-yl)acetamide. Into a 1L round bottom flask equipped with a magnetic stir bar was placed 2-amino-4-hydroxyquinazoline (8.8 g, 46.03 mmol) and acetic anhydride (150 mL). The flask was equipped with a reflux condenser and the mixture was heated to reflux with stirring for 15 h. The precipitate was isolated by filtration and washed with diisopropylether then dried in vacuo to afford a white solid.

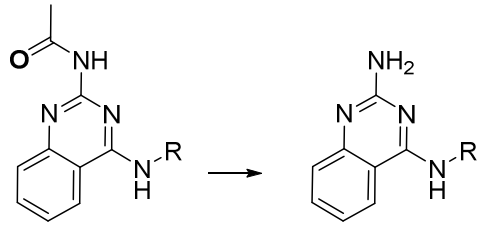


N-(4-chloroquinazolin-2-yl)acetamide. Into a 250 mL round bottom flask equipped with a magnetic stir bar was added *N*-(4-hydroxyquinazolin-2-yl)acetamide (4.5g, 19.3 mmol), and CH_3CN (100 mL). $POCl_3$ (5.56 mL, 5 59.8 mmol) was added dropwise over 30 minutes, followed by the addition of DIPEA (10.3 mL, 59.8 mmol). The reaction mixture became a brown solution and stirring was continued for 2 h at room temperature. The reaction mixture was poured into 1M NaOH (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over $MgSO_4$, the solids were removed via filtration and the filtrate was used as such in the next step.



(S)-N-(4-((1-hydroxybutan-2-yl)amino)quinazolin-2-yl)acetamide. The filtrate from the previous step, containing *N*-(4-chloroquinazolin-2-yl)acetamide in CH_3CN, was treated with DIPEA (9.2 mL, 53.6 mmol) and amine (35.8 mmol). The reaction mixture was stirred for 16 h at rt. The solvent was removed under reduced pressure and the crude was reconstituted in dichloromethane and washed with water. The organic layer was dried ($MgSO_4$), the solids were removed by filtration, and the solvents of the filtrate were evaporated to dryness to obtain the desired compound.

(S)-N-(4-((1-hydroxy-4-methylpentan-2-yl)amino)quinazolin-2-yl)acetamide. The method to prepare *(S)-N-(4-((1-hydroxy-4-methylpentan-2-yl)amino)quinazolin-2-yl)acetamide* was the same as described for *(S)-N-(4-((1-hydroxybutan-2-yl)amino)quinazolin-2-yl)acetamide* .



(S)-2-((2-aminoquinazolin-4-yl)amino)butan-1-ol (**5**). Into a 25 mL glass tube, a solution of (*S*)-*N*-(4-((1-hydroxybutan-2-yl)amino)quinazolin-2-yl)acetamide in CH_3OH (10 mL) was treated with 30% $NaOCH_3$ in CH_3OH. The mixture was heated at 75°C for 16h. After concentration, the residue was purified by prep. HPLC (RP Vydac Denali C18 – 10 μm, 200 g, 5 cm). Mobile phase (0.25% NH_4HCO_3 aq., CH_3CN).

(S)-2-((2-aminoquinazolin-4-yl)amino)-4-methylpentan-1-ol (**8**). The method to prepare **8** was the same as described for **5**.

Description of Biological Assays

Assessment of TLR7 and TLR8 activity. The ability of compounds to activate human TLR7 and/or TLR8 was assessed in a cellular reporter assay using HEK293 cells transiently transfected with a TLR7 or TLR8 expression vector and NFκB-luc reporter construct. In one instance, the TLR expression construct expresses the respective wild type sequence or a mutant sequence comprising a deletion in the second leucine-rich repeat (dlRR2) of the TLR. Such mutant TLR proteins have previously been shown to be more susceptible to agonist activation (US 7498409).

Briefly, HEK293 cells were grown in culture medium (DMEM supplemented with 10% FCS and 2 mM Glutamine). For transfection of cells in 10 cm dishes, cells were detached with Trypsin-EDTA, transfected with a mix of CMV-TLR7 or TLR8 plasmid (750 ng), NFκB-luc plasmid (375 ng) and a transfection reagent and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. Transfected cells were then detached with Trypsin-EDTA, washed in PBS and resuspended in medium to a density of 1.67 x 10^5 cells/mL. Thirty microliters of cells were then dispensed into each well in 384-well plates, where 10 µL of compound in 4% DMSO was already present. Following 6 hours incubation at 37°C, 5% CO_2, the luciferase activity was determined by adding 15 µL of Steady Lite Plus substrate to each well and readout performed on a ViewLux ultraHTS microplate imager. Dose response curves were generated from measurements performed in quadruplicates. Lowest effective concentrations (LEC) values, defined as the concentration that induces an effect which is at least twofold above the standard deviation of the assay, were determined for each compound. The Z'-factor of the assay is 0.51 and the %CV of the LEC is between 14 and 50% depending on the compound.

Compound toxicity was determined in parallel using a similar dilution series of compound

with 30 µL per well of cells transfected with the CMV-TLR7 construct alone (1.67×10^5 cells/mL), in 384-well plates. Cell viability was measured after 6 hours incubation at 37°C, 5% CO_2 by adding 15 µL of ATP lite per well and reading on a ViewLux ultraHTS microplate imager. Data was reported as CC_{50}.

Suppression of HCV replicon replication. Activation of human TLR7 results in robust production of interferon by plasmacytoid dendritic cells present in human blood. The potential of compounds to induce interferon was evaluated by looking at the antiviral activity in the HCV replicon system upon incubation with conditioned media from peripheral blood mononuclear cells (PBMC). The HCV replicon assay is based on a bicistronic expression construct, as described by Lohmann *et al.* (Science (1999) 285: 110-113; Journal of Virology (2003) 77: 3007-15 3019) with modifications described by Krieger *et al.* (Journal of Virology (2001) 75: 4614-4624). The assay utilized the stably transfected cell line Huh-7 luc/neo harboring an RNA encoding a bicistronic expression construct comprising the wild type NS3-NS5B regions of HCV type 1b translated from an Internal Ribosome Entry Site (IRES) from encephalomyocarditis virus (EMCV), preceded by a reporter gene (Firefly-luciferase) and a selectable marker gene (neoR, neomycine phosphotransferase). The construct is flanked by 5' and 3' NTRs (non-translated regions) from HCV type 1b. Continued culture of the replicon cells in the presence of G418 (neoR) is dependent on the replication of the HCV RNA. The stably transfected replicon cells that replicate HCV RNA autonomously and to high levels, encoding *inter alia* luciferase, were used for profiling of the conditioned cell culture media.

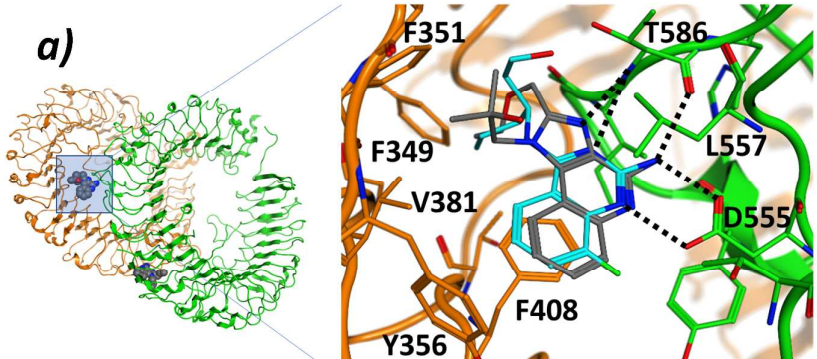
Briefly, PBMCs were prepared from buffy coats of at least two donors using a standard Ficoll centrifugation protocol. Isolated PBMCs were resuspended in RPMI medium supplemented with 10% human AB serum and 2×10^5 cells/well were dispensed into 384-well plates containing compounds (70 µL total volume). After overnight incubation, 10 µL of supernatant was transferred to 384-well plates containing 2.2×10^3 replicon cells/well in 30 µL (plated the day before). Following 24 hours of incubation, replication

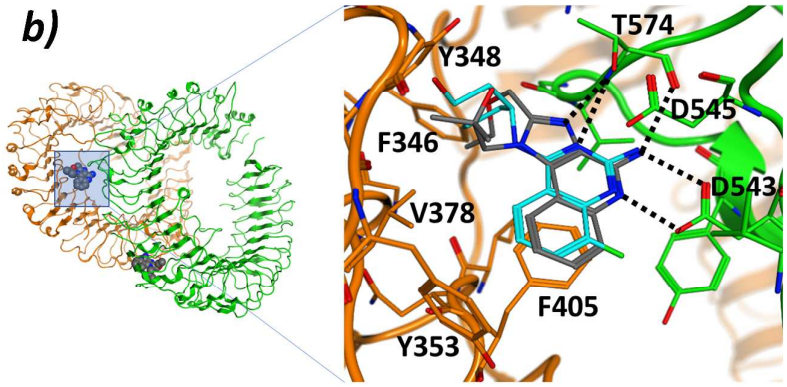
was measured by assaying luciferase activity using 40 µL/well Steady Lite Plus substrate and measured with ViewLux ultraHTS microplate imager. The inhibitory activity of each compound on the Huh7-luc/neo cells were reported as EC_{50} values, defined as the compound concentration applied to the PBMCs resulting in a 50% reduction of luciferase activity which in turn indicates the degree of replication of the replicon RNA on transfer of a defined amount of PBMC culture medium. Recombinant interferon α-2a (Roferon-A) was used as a standard control compound. All compounds showed CC_{50} of >24 µM in the HEK 293 TOX assay described above.

Mouse *in vivo* study. IFNα production was measured with an ELISA kit from PBL Assay Science, able to detect the subtypes of murine IFNα. Levels of interferon in plasma and in liver homogenates were assessed. All in vivo studies were performed in AAALAC-accredited sites, and ethical approval by the corresponding ethical committee was obtained.

Molecular Modeling

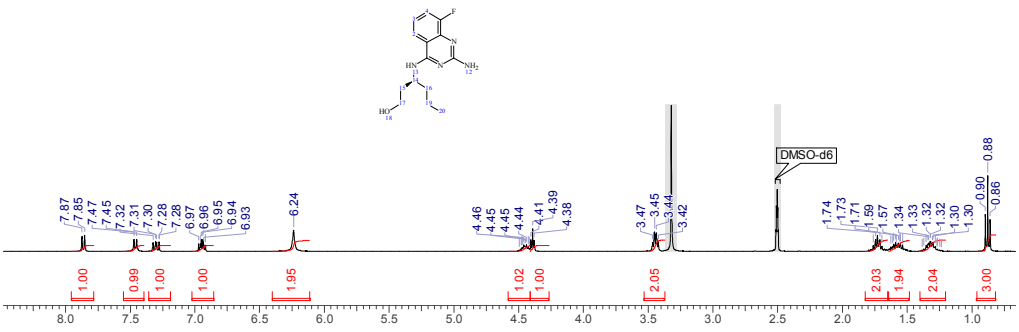
Figure S1: Compound **31** docked to Resiquimod binding pocket on (a) monkey TLR7 dimer interface (PDB ID:5GMH) and (b) human TLR8 dimer interface (PDB ID: 3W3N). The two monomers are colored orange and green, respectively (ribbon representation of the co-crystal structure on left with Resiquimod displayed as spheres with gray carbon atoms & details of key interactions on the right while depicting overlay of the ligand docking pose with Resiquimod). The carbon atoms of **31** are colored in cyan whereas the receptor residues are colored in orange and green (different monomers).





^{1}H and ^{13}C NMR Spectrum

^{1}H NMR (DMSO-d_6, 400 MHz)



^{13}C NMR (DMSO-d_6, 91 MHz)

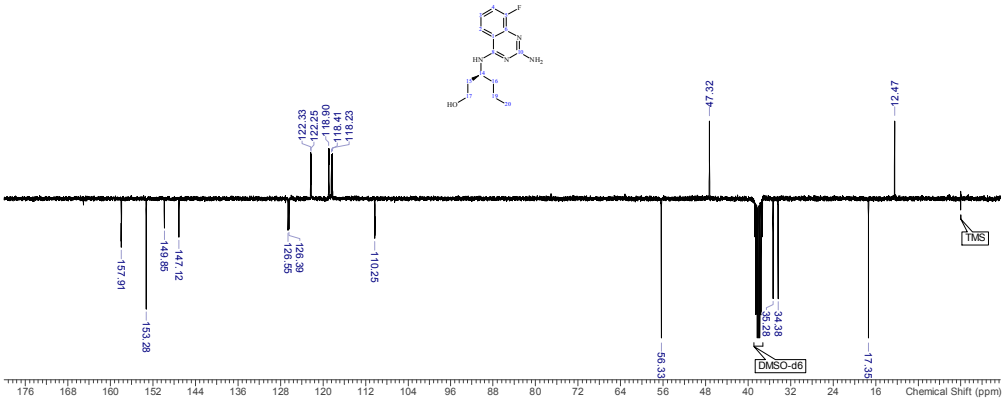


Table S2. Metabolic Stability Compounds 7-14[a]

Compound	Human (%)	Mice (%)
7	17	66
8	12	72
9	6	27
10	23	68
11	5	45
12	23	72
13	45	82
14	17	78

[a]Percent metabolized measured after 15 minutes at 1μM in liver microsomes.