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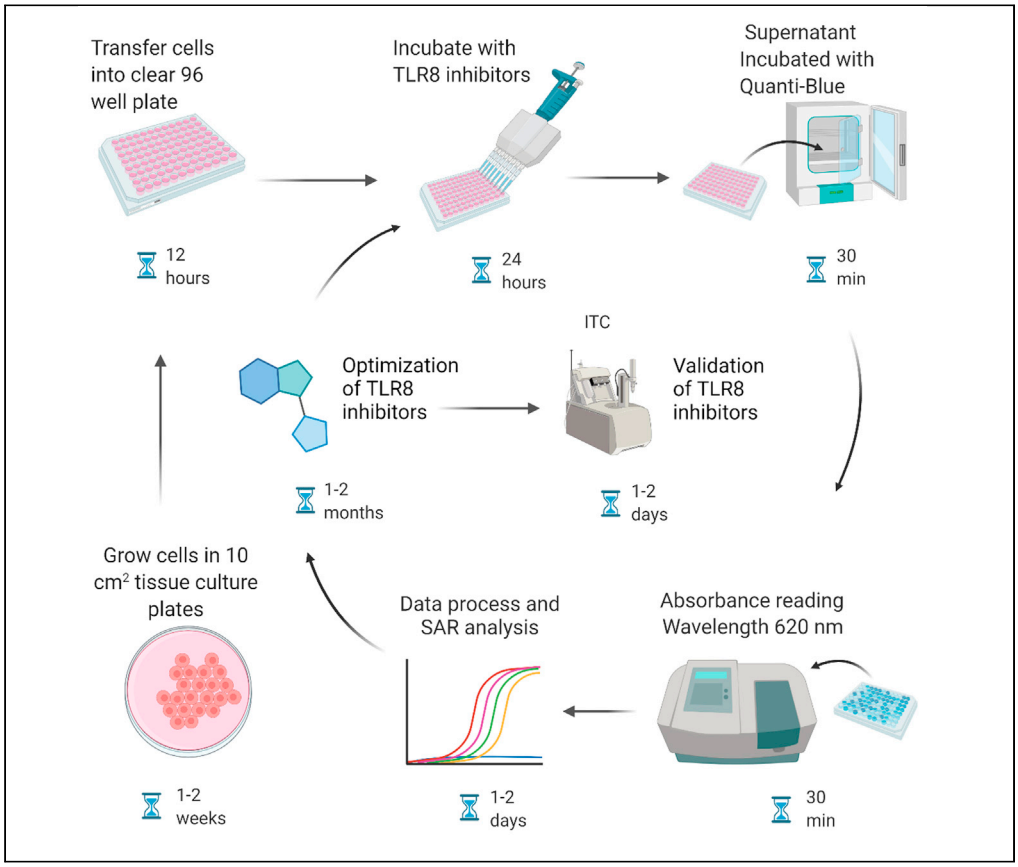
Protocol for evaluation and validation of TLR8 antagonists in HEK-Blue cells via secreted embryonic alkaline phosphatase assay



Toll-like receptor 8 (TLR8) is a pattern recognition receptor that senses RNA degradation products and initiates immune responses. TLR8 overactivation is associated with autoimmune diseases. Herein, we describe the evaluation and validation of TLR8 antagonists in HEK-Blue cells via secreted embryonic alkaline phosphatase (SEAP) assay, WST assay, ITC, and immunoblotting. These assays can facilitate the development of TLR8 antagonists; this protocol can also be adapted to analyze agonists and antagonists for other TLRs.

Zhenyi Hu, Tuan Zhang, Shuangshuang Jiang, Hang Yin

zhenyi.hu@yale.edu (Z.H.)
yin_hang@tsinghua.edu.cn (H.Y.)

Highlights

A protocol for secreted embryonic alkaline phosphatase assay for TLR8 inhibition

Validation of TLR8 antagonist specificity

Validation of TLR8 antagonist induced downstream signaling inhibition

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Protocol for evaluation and validation of TLR8 antagonists in HEK-Blue cells via secreted embryonic alkaline phosphatase assay

Zhenyi Hu,[1,4,5,*] Tuan Zhang,[1,2,3] Shuangshuang Jiang,[1,2,3] and Hang Yin[1,2,3,6,*]

[1]School of Pharmaceutical Sciences, Tsinghua University, 100084 Beijing, China

[2]Tsinghua-Peking Center for Life Sciences, Tsinghua University, 100084 Beijing, China

[3]Beijing Advanced Innovation Center for Structural Biology, Tsinghua University, 100084 Beijing, China

[4]Present address: Department of Molecular, Cellular and Developmental Biology, Yale University, CT 06511, USA

[5]Technical contact

[6]Lead contact

*Correspondence: zhenyi.hu@yale.edu (Z.H.), yin_hang@tsinghua.edu.cn (H.Y.)
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SUMMARY

Toll-like receptor 8 (TLR8) is a pattern recognition receptor that senses RNA degradation products and initiates immune responses. TLR8 overactivation is associated with autoimmune diseases. Herein, we describe the evaluation and validation of TLR8 antagonists in HEK-Blue cells via secreted embryonic alkaline phosphatase (SEAP) assay, WST assay, ITC and immunoblotting. These assays can facilitate the development of TLR8 antagonists; this protocol can also be adapted to analyze agonists and antagonists for other TLRs.

For complete details on the use and execution of this protocol, please refer to Hu et al. (2018).

BEFORE YOU BEGIN

This protocol includes methods for evaluation and validation of TLR8 antagonist. Before performing the experiments, all reagents and TLR small molecular modulators (TLR8 agonist, antagonist, etc.) (Wang et al., 2020) should be obtained from commercial sources or synthesized in house and subsequently dissolved in DMSO as stock solution. All cell lines should be purchased and prepared in a ready-to-use mode with careful considerations.

Cell lines preparation

Timing: 1–2 months

1. HEK-Blue cells are human embryonic kidney (HEK) cells transfected with SEAP (secreted embryonic alkaline phosphatase) gene. The SEAP reporter gene is under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding site, thus, the activation of NF-κB will lead to expression of SEAP. The activity of SEAP can be easily measured with the alkaline phosphatase detection reagent - QUANTI-Blue.(Zhang et al., 2018) The HEK-Blue TLR cells are HEK 293 cells co-transfected with SEAP and various TLRs. In our research, the HEK-Blue Null1, HEK-Blue cells with overexpression of TLR2, TLR4, TLR7, and TLR9 were purchased from Invivogen (California, USA).(Cheng et al., 2011; Yin and Flynn, 2016) HEK-Blue cells overexpressing TLR3, TLR5 and TLR8 were generated from HEK-Blue Null1 cells by lentiviral infection with TLRs, respectively. THP-1 cells were purchased from ATCC (Virginia, USA).

HEK-blue cells culture medium preparation

Timing: 1–2 h

2. DMEM media was supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin (PenStrep), 100 mg/mL Normocin, 5 μg/mL blasticidin and 2 mM L-glutamine.

HEK-Blue cells test medium preparation

Timing: 1–2 h

3. FBS was deactivated in water bath at 56°C for 30 min.
4. Inactivated FBS was then used to supplement DMEM media at concentration of 10%. 50 U/mL penicillin and 50 μg/mL streptomycin (PenStrep), 100 mg/mL Normocin, 5 μg/mL blasticidin and 2 mM L-glutamine were also used to supplement DMEM.

Note: Heat inactivation is critical for SEAP reporter assay as heat can deactivate serum alkaline phosphatase, this serves to minimize SEAP assay background and enhance assay sensitivity.

THP-1 cell medium preparation

Timing: 1–2 h

5. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (PenStrep), 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol.

Thawing Cryopreserved HEK-Blue cells

Timing: 1–2 weeks

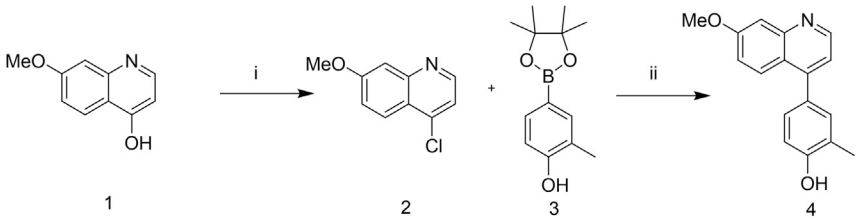
6. The cryovials of HEK-Blue cells were taken from liquid nitrogen and quickly thawed in the 37°C warm water until little ice is left.
7. 2×10^6 HEK-Blue cells were transfer into a 50 mL falcon tube filled with 40 mL warmed HEK-Blue cells culture medium and mixed well.
8. Spin cells 300×*g* for 5 min.
9. The supernatant was discarded into bleach and the cell pellet resuspended with HEK-Blue cells culture medium without PenStrep in a 3.5 cm dish.
10. Culture the cells up to 3 passages until no less than 2×10^6 cells to transfer to a 10 cm dish, full medium will then be used.
11. Culture until 80% confluent of cells, and it's ready for SEAP assay.

Cell culture

Timing: 1–2 weeks

12. HEK-Blue cells were grown in humidified incubators containing 5% CO_2 at 37°C. HEK-Blue TLR cells were cultured in DMEM medium as described above in step 2.
13. THP-1 cells were grown in humidified incubators containing 5% CO_2 at 37°C. THP-1 cells were cultured in RPMI 1640 medium as described above in step 5.

Note: We recommend that these cell lines to be routinely tested for mycoplasma infection.



Scheme 1. Representative synthesis route of TLR8 inhibitors

Reagents and conditions: (i) $POCl_3$, reflux 12 h; (ii) K_2CO_3, $PdCl_2dppf{\cdot}CH_2Cl_2$, 1,4-dioxane, H_2O, 100°C, 12 h.

Small molecule design and synthesis

Timing: 1–2 months

Synthetic route of TLR modulators may vary based on their structural features, here we use our TLR8 inhibitor as one example to show its synthesis and structural confirmation.

14. 7-Methoxy-4-quinolinol (compound **1** in Scheme 1, 175 mg, 1.0 mmol) was stirred in $POCl_3$ (5 mL) at 90°C for 16 h.
15. The reaction was then quenched with saturated sodium carbonate solution and extracted with ethyl acetate (50 mL × 3). The combined organic layers were dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (eluent: dichloromethane/ethyl acetate, 10%–100%) to give the title compound (compound **2**, Scheme 1) as a white solid (163 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (d, *J* = 4.8 Hz, 1H), 8.12 (d, *J* = 9.2 Hz, 1H), 7.43 (d, *J* = 2.5 Hz, 1H), 7.35 (d, *J* = 4.8 Hz, 1H), 7.29 (dd, *J* = 9.2, 2.6 Hz, 1H), 3.97 (s, 3H).
16. A mixture of 4-chloro-7-methoxy-quinolone (compound **2**, , 80 mg, 0.42 mmol, Scheme 1), 2-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-phenol (compound **3**, 97 mg, 0.42 mmol, Scheme 1), K_2CO_3 (158 mg, 1.1 mmol) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium (II), complex with dichloromethane (18 mg, 0.02 mmol) in dioxane (5 mL) and H_2O (1 mL) was heated at 100°C 12 h in an atmosphere of nitrogen.
17. The reaction was then quenched with water and extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (eluent: dichloromethane/methanol, 0%–10%) to give the title compound 2-Methyl-4-(7-methoxyl-4-quinolinyl)-phenol as white solid (compound **4**, 77 mg, 85%, Scheme 1). ESI–MS m/z: 266.1181$[M+H]^+$; Purity: 99.0% (measured by ^{1}H NMR) . ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.69 (s, 1H), 8.79 (d, *J* = 4.5 Hz, 1H), 7.85 (d, *J* = 9.3 Hz, 1H), 7.44 (d, *J* = 2.6 Hz, 1H), 7.26–7.20 (m, 3H), 7.20–7.16 (m, 1H), 6.95 (d, *J* = 8.2 Hz, 1H), 3.93 (s, 3H), 2.21 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 159.84, 155.98, 150.27, 150.14, 147.57, 131.66, 127.96, 127.90, 126.93, 124.32, 121.13, 119.20, 119.12, 114.71, 107.77, 55.46, 16.03.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-NF-κB p65 (1:1000 dilution)	Cell Signaling Technology	Cat#82422
Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (1:1000 dilution)	Jackson ImmunoResearch	Cat#111-035-144
Lamin A/C Antibody (1:1000 dilution)	Cell Signaling Technology	Cat#2032

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Penicillin-Streptomycin	Gibco	Cat#15070063
Normocin	InvivoGen	Cat#ant-nr-1
Blasticidin	InvivoGen	Cat#ant-bl-05
L-glutamine	Gibco	Cat#25030081
Pam3CSK4	InvivoGen	Cat#tlrl-pms
Pam2CSK4	InvivoGen	Cat#tlrl-pm2s-1
Poly(I:C)	InvivoGen	Cat#tlrl-pic
Flagellin	InvivoGen	Cat#tlrl-bsfla
R848	InvivoGen	Cat#tlrl-r848
LPS	InvivoGen	Cat#tlrl-b5lps
ODN 2006	InvivoGen	Cat#tlrl-2006
Critical commercial assays		
SEAP reporter assay	InvivoGen	Cat#rep-qb1
WST-1 cell proliferation assay	Roche	Cat#5015944001
Cell culture microplate, 96 well, F-bottom	Greiner Bio-One	Cat#655160
RPMI 1640 Medium	Gibco	Cat#12633012
DMEM	Gibco	Cat#10569010
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#23225
NE-PER™ Nuclear and Cytoplasmic Extraction Reagents	Thermo Fisher Scientific	Cat#78833
Deposited data		
TLR8/CU-CPT9a structure	Protein Data Bank	PDB ID: 5Z14
TLR8/CU-CPT9a structure	Protein Data Bank	PDB ID: 5Z15
Experimental models: Cell lines		
Human: HEK-Blue-hTLR2 cells	InvivoGen	Cat#hkb-htlr2
Human: HEK-Blue-hTLR3 cells	lentiviral infection with TLR3	N/A
Human: HEK-Blue-hTLR4 cells	InvivoGen	Cat#hkb-htlr4
Human: HEK-Blue-hTLR5 cells	lentiviral infection with TLR5	N/A
Human: HEK-Blue-hTLR7 cells	InvivoGen	Cat#hkb-htlr7
Human: HEK-Blue-hTLR8 cells	lentiviral infection with TLR8	N/A
Human: HEK-Blue-hTLR9 cells	InvivoGen	Cat#hkb-htlr9
Human: THP-1	ATCC	Cat#TIB-202
Software and algorithms		
GraphPad Prism	GraphPad Software Inc	https://www.graphpad.com/scientific-software/prism/
OriginPro	OriginLab Corporation	https://www.originlab.com/index.aspx?go=Products/Origin
Others		
DTX 880 Multimode Detector	Beckman Coulter	Cat# 20056
MicroCal iTC200	GE Healthcare	N/A
ChemiDoc xrs	Bio-Rad Laboratories	N/A

MATERIALS AND EQUIPMENT

Preparation of CU-CPT9a stock solution		
Compound	Final concentration	Amount
CU-CPT9a	10 mM	2.65 mg
DMSO	0.1%	1 mL
Total	n/a	1 mL stock solution

Note: 10 mM CU-CPT9a stock solution was obtained by dissolving 2.65 mg of CU-CPT9a powder in 1 mL DMSO. Stock solution can be stored at −20°C for months. Multiple freeze-thaw circles should be avoided.

Antibody dilution buffer		
Compound	Final concentration	Amount
Bovine serum albumin (BSA)	5%	0.5 mL
1× PBS	n/a	10 mL
Total	n/a	10 mL

Note: Antibody dilution buffer was prepared immediately before use. This buffer can be stored on ice or at 4°C for several hours.

Preparation of ITC buffer		
Compound	Final concentration	Amount
MES pH 5.5 (1 M)	25 mM	250 μL
NaCl (5 M)	200 mM	400 μL
DMSO	2.5%	250 μL
ddH_2O	n/a	9.1 mL
Total	n/a	10 mL

Note: ITC buffer was prepared immediately before use. This buffer can be stored at 4°C for several days.

STEP-BY-STEP METHOD DETAILS

SEAP reporter assay

Timing: 2 days

The SEAP reporter gene is under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding site, thus, the activation of NF-κB will lead to expression of SEAP. In this way, activation of NF-κB can be measured easily by activation of SEAP. The method was used to monitor the inhibition effect of TLR8 antagonists in HEK-Blue TLR8 cells which were activated by TLR8 agonist (R848).

1. Cultured HEK-Blue TLR8 cells in 10 cm dishes were washed with 5 mL PBS 3 times gently.
2. 10 mL of DMEM medium was added to dish, then cells were harvested from dishes by aspirating. To separate cells, the medium was passed through 10 mL serological pipette for 10 times.
3. Cultured HEK-Blue TLR8 cells were spined at 300×*g* for 5 min at 4°C. Supernatant was discarded into bleach and the cell pellet was resuspended with DMEM medium.
4. HEK-Blue TLR8 cells were seeded in tissue culture treated 96-well flat-bottom clear plates with a density of 7.5×10^4 cells/well in DMEM test media, see HEK-Blue cells test medium preparation for details.
5. R848 was used to activate TLR8 in HEK-Blue TLR8 cells. DMSO (0.1%) treated cells were used as negative control (three replicates) and R848 (1 μg/mL) treated cells were used as positive control (three replicate). To test TLR8 antagonists, cells were treated with both R848 (1 μg/mL) and various concentration of appropriate TLR8 antagonists. For example, cells were treated with TLR8 antagonist (compound 4 in scheme 1) starting at concentration of 10 μM and serial dilution of 1:4. See Table 1 and Figure 1 for an example of a plate map. SEAP assays for each sample were conducted with three biological replicates, each in triplicate.
6. Plates were then incubated in humidified incubators containing 5% CO_2 at 37°C for 24 h.

Table 1. Example setup of 96-well plate for SEAP assay

Wells	Treatment
B2, C2, D2, E2, F2, G2	DMSO (0.1%)
B3, C3, D3	R848 (1 μg/mL)
E3. F3, G3	R848 (0.5 μg/mL)
B4, C4, D4, E4, F4, G4	Compound 4 (10 μM) + R848 (1 μg/mL)
B5, C5, D5, E5, F5, G5	Compound 4 (2.5 μM) + R848 (1 μg/mL)
B6, C6, D6, E6, F6, G6	Compound 4 (0.625 μM) + R848 (1 μg/mL)
B7, C7, D7, E7, F7, G7	Compound 4 (0.156 μM) + R848 (1 μg/mL)
B8, C8, D8, E8, F8, G8	Compound 4 (0.039 μM) + R848 (1 μg/mL)
B9, C9, D9, E9, F9, G9	Compound 4 (0.0097 μM) + R848 (1 μg/mL)
B10, C10, D10, E10, F10, G10	Compound 4 (0.0024 μM) + R848 (1 μg/mL)
B11, C11, D11, E11, F11, G11	R848 (1 μg/mL)

⚠ CRITICAL: after 24 h, 30 μL of culture media was transfer to a 96-well flat-bottom clear plate and 100 μL of Quanti-Blue (Invivogen, California, USA) was added, the plate was then incubated at 37°C until color changed to dark blue (30 min–1 h). See an example of SEAP results in Figure 1.

7. Readout of absorbance at 620 nm was quantified using Beckman-Coulter DTX 880 Multimode Detector. Readout of only R848 treated cells were normalized as 100% activation, and untreated cells as 0% activation. See an example of SEAP results analysis of several representative compounds (CU-CPT9a-c) in Figure 2.

Note: Wash HEK-Blue TLR8 cell with PBS very gently due to their weak adhesion to cell culture dishes. We recommend adding PBS slowly to the bottom of tilted cell culture dishes, then swirl the dishes carefully to wash cells. To avoid edge effect, we recommend that wells around edges of 96-well plates should not be used.

WST-1 cell proliferation assay

Timing: 2 days

The WST-1 cell proliferation assay provides an easy and sensitive way to measure the viability of cells. In this assay, the tetrazolium salt is cleaved to formazan by cellular mitochondrial dehydrogenase, thus, the number of living cells can be monitored by the amount of dye generated in WST-1 assay. The WST-1 reagent is more soluble and stable compared to others such as MTT and MTS, so it was chosen in our method to assess the toxicity of TLR8 antagonists.

8. Cultured HEK-Blue TLR8 cells in 10 cm dishes were washed with 5 mL PBS 3 times gently.
9. 10 mL of DMEM medium was added to dish, then cells were harvested from dishes by aspirating. To separate cells, the medium was passed through 10 mL serological pipette for 10 times.
10. Cells were centrifuged at 300×*g* for 5 min at 4°C. Discard the supernatant into bleach and re-suspend the cell pellet with DMEM medium.
11. HEK-Blue TLR8 cells were seeded in tissue culture treated 96-well flat-bottom clear plates with a density of 7.5×10^4 cells/well in DMEM test media, see HEK-Blue cells test medium preparation for details.
12. To test cytotoxicity of TLR8 antagonists, cells were treated with both R848 (1 μg/mL) and various concentration of appropriate TLR8 antagonists. For examples, cells were treated with TLR8 antagonist starting at concentration of 10 μM and serial dilution of 1:4. See Table 1 and Figure 3 for an example of a plate map. Each test was conducted with three biological replicates, each in triplicate.
13. Plates were then incubated in humidified incubators containing 5% CO_2 at 37°C for 24 h.

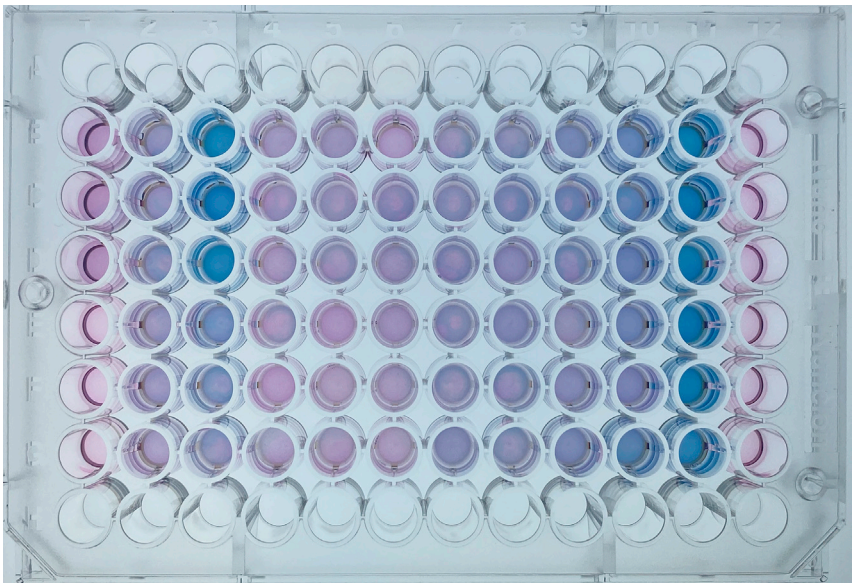


Figure 1. Picture of SEAP assay plate

Secreted embryonic alkaline phosphatase reacts with Quanti-Blue and produce optical density at 620–655 nm. Blue color indicated activation of NF-κB, TLR8 antagonists downregulated NF-κB activation and stayed as pink color. To avoid edge effect, wells around edges of 96-well plates were not used. Compound 4 (Scheme 1) was used as an example.

14. After incubation of 24 h, supernatant was removed and discarded, and 100 μL of 1:10 diluted WST-1 (Roche) in DMEM test medium was then added to each well as indicated by the manufacturer's protocol. The plate was then incubated at 37°C until color changes (30 min-1 h).
15. Readout of absorbance at 450 nm was quantified using Beckman-Coulter DTX 880 Multimode Detector. Readouts of untreated cells were normalized as 100% survival. See Figure 4 for an example of cytotoxicity test result of TLR8 antagonists (CU-CPT9a-c).

Validation specificity of TLR8 antagonist

TLR selectivity assay

Timing: 1–2 weeks

Due to the high similarities among TLRs (Kanzler et al., 2007), it's important to achieve selectivity towards TLR8 in our development of TLR8 antagonist, in this assay, we used SEAP assay to measure inhibition of TLR8 antagonists in HEK-Blue cells overexpressing TLRs other than TLR8.

16. Cultured HEK-Blue cells overexpressing TLR1-9 in 10 cm dishes were washed with 5 mL PBS 3 times gently.
17. 10 mL of DMEM medium was added to dish, then cells were harvested from dishes by aspirating. To separate cells, the medium was passed through 10 mL serological pipette for 10 times.
18. Cells were centrifuged at 300×*g* for 5 min at 4°C. Discard the supernatant into bleach and resuspend the cell pellet with DMEM medium.
19. HEK-Blue TLR cells were seeded in tissue culture treated 96-well flat-bottom clear plates with a density of 7.5×10^4 cells/well in DMEM test media, see HEK-Blue cells test medium preparation for details.
20. To test the specificity of TLR8 antagonists, HEK-Blue cells overexpressing TLRs other than TLR8 were treated with both TLR8 antagonists and corresponding antagonist. For HEK-Blue hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, and hTLR9 cells, Pam3CSK4 (100 ng/mL), poly(I:C) (5 μg/mL),

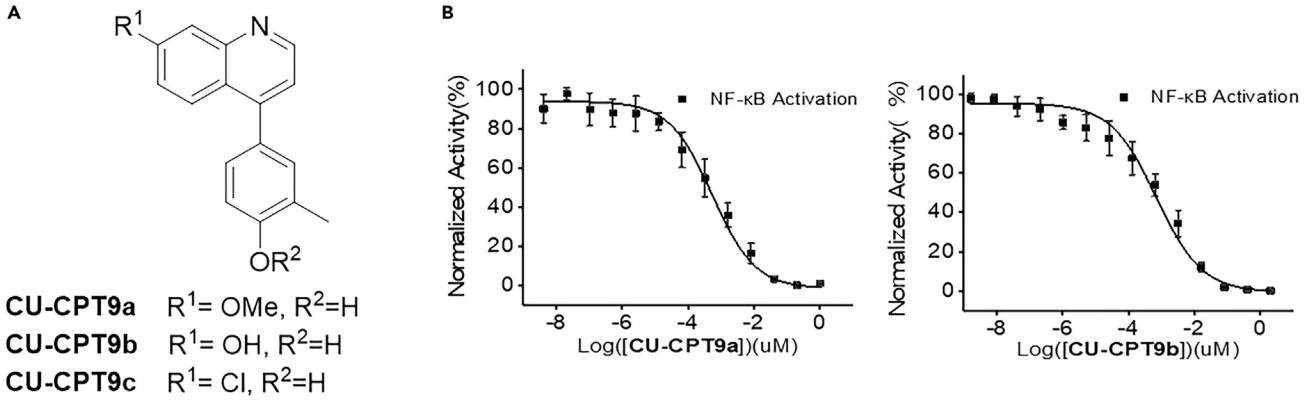


Figure 2. Measure TLR8 inhibition with SEAP assay

(A) Structures of representative TLR8 antagonists: CU-CPT9a-c.

(B) SEAP assay showing dose-dependent inhibitory effects of TLR8 signaling by CU-CPT9a and CU-CPT9b in hTLR8 HEK-Blue cell using R848 (1 μg/mL) as agonist (data are mean ± SD; n = 3 independent experiments). Figure was reproduced from previous research article (Hu et al., 2018) with permission.

LPS (lipopolysaccharide) (20 ng/mL), flagellin (50 ng/mL), R848 (1 μg/mL), ODN2006 (0.15 μM) were used as agonists respectively.

21. Plates were then incubated in humidified incubators containing 5% CO_2 at 37°C for 24 h.
22. After about 24 h, 30 μL of culture media was transferred to a new 96 well flat bottom clear plate and 100 μL of Quanti-Blue (Invivogen, California, USA) was added, the plate was then incubated at 37°C until color changes (30 min-1 h).
23. Readout of absorbance at 620 nm was quantified using Beckman-Coulter DTX 880 Multimode Detector. Readout of only appropriate agonists treated cells were normalized as 100% activation, and untreated cells as 0% activation. SEAP assays for each sample were conducted with three biological replicates, each in triplicate. Please see Figure 5 for an example of specificity assay, CU-CPT9a was tested in HEK-Blue cell lines overexpressing various TLRs, and it only showed inhibition toward TLR8.

Isothermal titration calorimetry (ITC)

Timing: 1–2 weeks

ITC can directly measure the heat that is released during binding of TLR8 antagonists to TLR8. ITC was used in this protocol to confirm and measure the direct binding between TLR8 and antagonists.

24. Prepare buffer composed of 25 mM MES pH 5.5, 0.20 M NaCl, and 2.5% DMSO at 25°C using a MicroCal iTC200 (GE Healthcare, Illinois, USA).
25. TLR8 inhibitors were dissolved in buffer to a final concentration of 100 μM, TLR8 protein was dissolved in buffer to a final concentration of 10 μM.
26. The titration conditions were as follows: Fill the calorimeter syringe with ligand solution according to the instructions in the instrument manual. 100 μM inhibitors were injected into 10 μM hTLR8 as instructed in the instrument manual. The titration sequence included a single 0.4 μL injection followed by 18 injections, 2 μL each, with a spacing of 120 s between the injections.
27. OriginLab software (GE Healthcare, Illinois, USA) was used to analyze the raw ITC data. Data points were fitted in OriginLab as instructed in the instrument manual. Example ITC result of CU-CPT9a (Figure 6) demonstrated that the antagonist was bound to TLR8 with high affinity (K_d=21 nM).

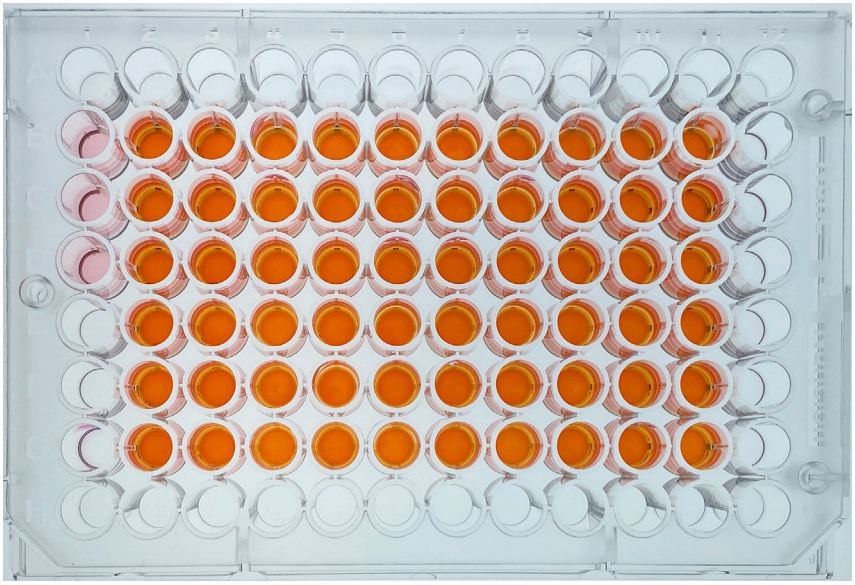


Figure 3. Picture of WST-1 assay plate

WST-1 was cleaved by cellular mitochondrial dehydrogenase and produce optical density at 450 nm. To avoid edge effect, wells around edges of 96-well plates were not used. Compound 4 (Scheme 1) was used as an example.

Validation of downstream signaling inhibition by TLR8 antagonist

Activation of TLR8 leads to upregulation of several downstream signaling proteins such as IRAK4 and TRAF6 (O'Neill et al., 2013), which eventually leads to activation and translocation of NF-κB p65 subunit into nuclear to initial immune responses. To validate the inhibitory effect of TLR8 antagonists on NF-κB, we used immunoblotting to monitor downregulation of nucleus p65.

Immunoblotting

Timing: 1–2 weeks

28. THP-1 cells were seeded in 6-well plates with a density of 2 × 10^6 cells/well in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Pen-Strep), 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol. THP-1 cells were treated with phorbol-12-myristate-13-acetate (PMA) (100 ng/mL) to stimulate THP-1 cells' transfer to macrophage-like phenotypes, which enable us to test TLR8 antagonists in a physiology relevant setting.
29. The cells were incubated in humidified incubators containing 5% CO_2 at 37°C for 24 h.
30. After differentiation, the supernatant was removed and replaced with unsupplemented RPMI, these cells were then treated with R848 (1 μg/mL) along with 0, 5, 50, 500 nM of appropriate TLR8 antagonists (CUCPT9a in Figure 7) and incubated for 2 h.
31. After incubation of 2 h, THP-1 cells were collected, nuclear protein fraction was extracted using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Massachusetts, USA). To cell pellet was added 100 μL ice-cold CER I, and suspension was incubated on ice for 10 min.
32. 5.5 μL ice-cold CER II was then added and incubated on ice for 5 min. The cell suspension was then centrifuged at 16,000×*g* for 5 min. Supernatant was discardeded and pellet was collected and added with 50 μL ice-cold NER.
33. The sample was incubated on ice and vortexed for 15 s every 10 min, for a total of 40 min.
34. The sample was then centrifuged at 16,000×*g* for 10 min at 4°C. The supernatant was collected for the following assay.

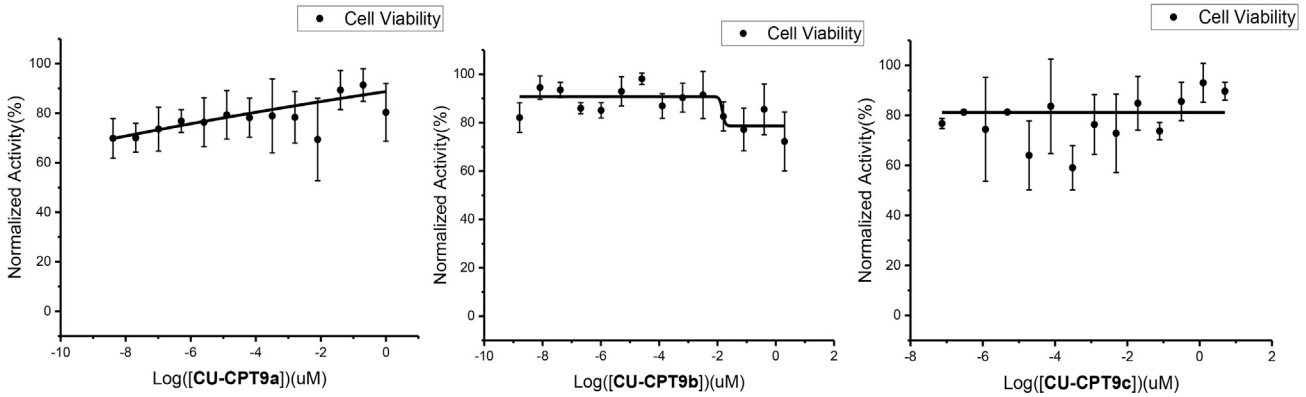


Figure 4. Cytotoxicity measured by WST-1 of 3 representative TLR8 antagonists (CU-CPT9a-c)

WST-1 assay showed no significant dose-dependent cytotoxicity was induced by CU-CPT9a-9c in HEK-Blue TLR8 cells. (mean ± SD; n = 3). Figure was reproduced from previous research article (Hu et al., 2018) with permission.

Pause point: Protein samples collected from step 34 can be stored at −80°C before further assay.

35. Protein levels of samples collected from step 34 were measured with BCA assay according to the manufacturer's manual (Thermo Fisher Scientific, Massachusetts, USA).
36. Protein samples (20 μg protein each lane) were loaded and run in 10% Tris-glycine SDS-PAGE, and then transferred onto a nitro-cellulose membrane (BioRad, California, USA) using electro-blotting.
37. The membrane was blocked with milk in TBST for 1 h at 25°C. NF-κB P65 (CST; 8242) (1:1000) was then used as primary antibody in BSA buffer, it was incubated 12 h at 4°C.
38. Membrane was washed 3 times with TBST, 5 min each.
39. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) antibody (Jackson Immuno Research; 111-035-144) (1:1000 dilution in TBST) as secondary antibody, the membrane was incubated with secondary antibody dissolved in TBST (5% milk) at 25°C for 1 h.
40. Then blots were washed 3 times with TBST, 5 min each, then visualized using Thermo SuperSignal West Pico kit (Thermo Fisher Scientific, Massachusetts, USA). Lamin A/C (CST; 2032), a nuclear protein, were used as internal controls for nuclear fractions. Figure 7 showed that 500 nM CU-CPT9a significantly inhibited nuclear p65.

EXPECTED OUTCOMES

For about 100 TLR8 antagonists we designed and synthesized, all of them were tested at least in triplicates (Figure 1). SEAP assay provided an easy cell-based assay with relatively high-throughput, enabling a quick screening of TLR8 antagonist library. Through various assays listed above, we obtained several highly potent TLR8 antagonists with IC_{50} less than 1 nM. For detailed structure-activity relationships of these antagonists, please see our paper (Hu et al., 2018). WST-1 demonstrated that our selected TLR8 antagonists did not induce significant cytotoxicity (Figures 3 and 4). Specificity was also shown using SEAP assays on HEK-Blue cells overexpressing other TLR receptors (Figure 5). Biophysical assay such as ITC confirmed the direct binding of TLR8 antagonist to TLR8 protein, also, it showed the high affinity of our TLR8 antagonist (Figure 6). To validate the inhibition effect of TLR8 signaling, western blot demonstrated the down regulation of NF-κB (Figure 7). With these developed assays, researchers could adapt them for development of small molecule modulators targeting other TLRs. Using the SEAP assay reported here, TLR modulators are expected to be discovered based on their abilities to regulate NF-κB. Other assays such as ITC or western blot can confirm the on-target mechanism of TLR modulators.

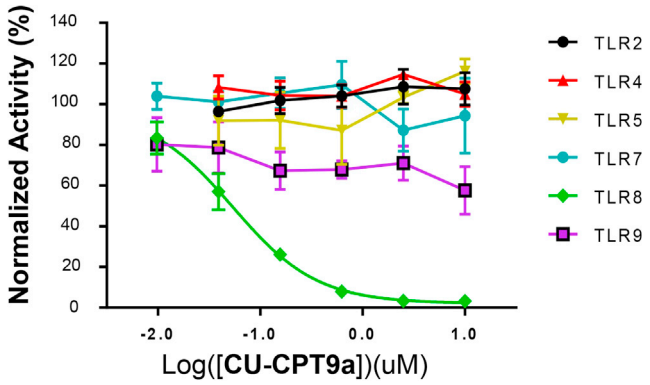


Figure 5. Specificity test of CU-CPT9a in HEK-Blue cells overexpressing individual TLRs

For HEK-Blue hTLR2, hTLR3, hTLR4, hTLR5, hTLR7/8, and hTLR9 cells, Pam3CSK4 (100 ng/mL), poly(I:C) (5 μg/mL), LPS (lipopolysaccharide) (20 ng/mL), flagellin (50 ng/mL), R848 (1 μg/mL), ODN2006 (0.15 μM) were used as TLR-specific agonists, respectively. Experiments were performed in triplicates. (data are mean ± SD; n = 3 independent experiments) Figure was reproduced from previous research article (Hu et al., 2018) with permission.

LIMITATIONS

In the aforementioned assays, specificity of small-molecule TLR8 antagonists were validated in HEK-Blue cells overexpressing other TLR receptors. Moreover, our ITC data clearly showed the binding of our TLR8 antagonist with TLR8 proteins. Western blots showed deregulation of NF-κB, which is a major downstream protein of TLR8 (Kawai and Akira, 2007; Kawasaki and Kawai, 2014). These data suggested on-target effect of CPT9a. However, these HEK-Blue cell lines are specifically engineered cell lines for direct monitoring of TLRs, this could not exclude the possibility that CPT9 serial of compounds could also target other proteins such as other kinases, which also are involved in down-regulating of NF-κB pathway (Lim and Staudt, 2013; Loo and Gale, 2011). Cautions and extra measures might be necessary when adopting our methods for development of other TLR modulators. Other assays such as KINOMEscan or proteomic study can be used to explore other possible protein targets of these small molecules.

Also, our TLR8 antagonists were validated *in vitro* in different cell assays, it has not been validated in animal models. To further development of TLR modulators, *in vivo* assays in animal models could help to provide more validation evidence as well as biological exploration of TLRs' functions (Duffy and O'Reilly, 2016).

TROUBLESHOOTING

Problem 1

Weak SEAP signal.

As in step 7, read out of SEAP assay could be below 0.3, this is considered as weak signal.

Potential solution

In SEAP assay, HEK-Blue cells were seeded at density of cell density of 7.5×10^4 cells/well in DMEM, lower density could lead to weak signal.

Cell supernatant was incubated with Quanti-blue reagent for 0.5–2 h, usually longer incubation will increase signal absorbance. The optimal absorbance should be between 0.8–1.2.

Problem 2

High background absorbance for SEAP assay.

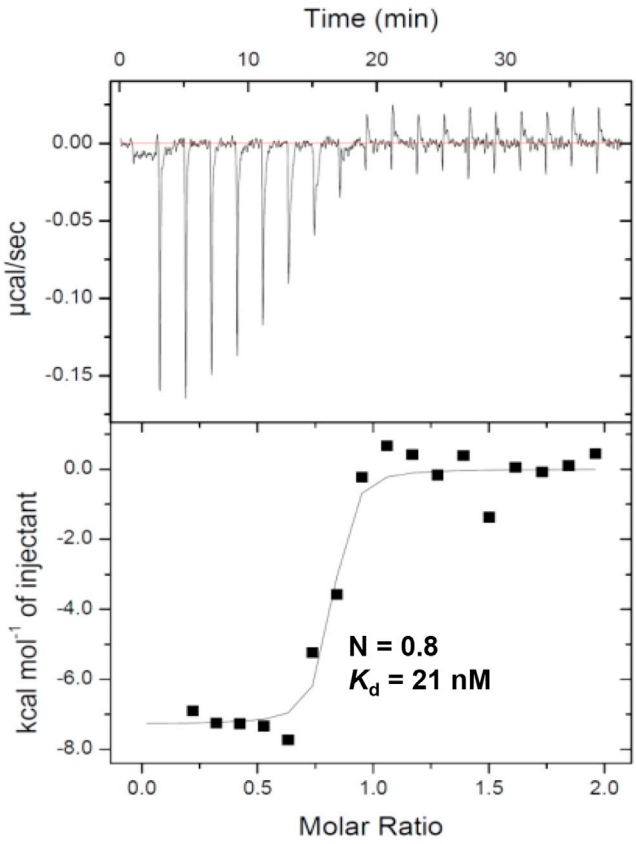


Figure 6. ITC analysis of TLR8 titrated with CU-CPT9a

Heat monitored in ITC correspond to direct binding between TLR8 and its antagonist. Direct binding of TLR8 with CU-CPT9a was confirmed with high affinity (K_d = 21 nM). Figure was reproduced from previous research article (Hu et al., 2018) with permission.

As in step 7 and 23, HEK-Blue cells that were not activated with agonists may show high signal.

Potential solution

Longer incubation of cell supernatant with Quanti-blue reagent will increase signal absorbance, however, it could also lead to high background absorbance.

Quanti-blue reagent is light sensitive, it should be used within two weeks after preparation. It should be stored at 4°C and avoid light.

Problem 3

As in step 7, HEK-Blue cells overexpressing TLRs could show low sensitivity to activation of agonists.

Potential solution

From our experience, HEK-Blue cells exhibited inconsistent response to TLR modulators after 30 passages. So, we recommend thawing new batch of cells when the HEK-Blue cells pass 30 passages.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hang Yin (yin_hang@tsinghua.edu.cn).

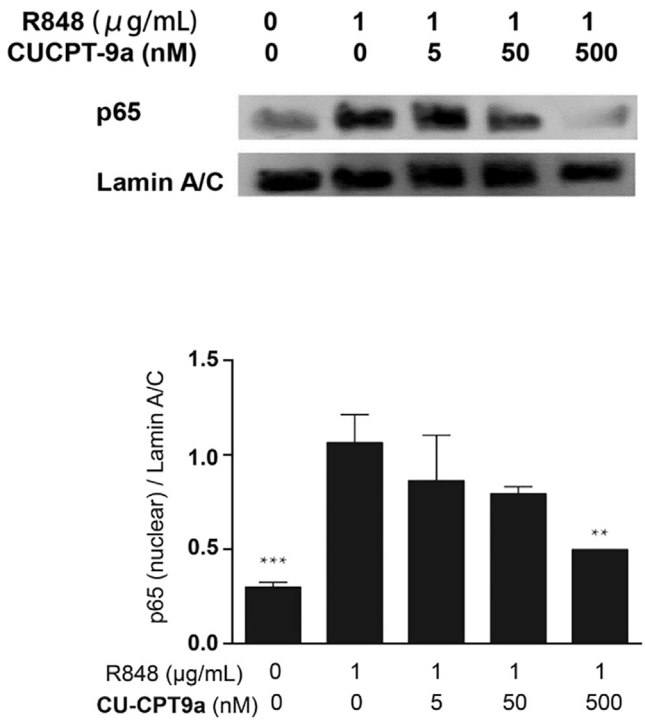


Figure 7. Western-blot confirmed inhibitory effect of CU-CPT9a

Representative images of the Western blotting of nuclear p65 in the presence of R848 and various concentrations of CU-CPT9a (top). Quantitative data analyses of p65/Lamin A/C (bottom). Ratios were calculated using densitometric analysis with image J software. Lamin A/C was used for internal controls (n = 3 independent experiments. *P*-values were determined using one-way ANOVA, *P < 0.05; **P < 0.01; ***P < 0.001). Figure reproduced from previous research article (Hu et al., 2018) with permission.

Materials availability

This study did not generate new reagents.

Data and code availability

The final atomic coordinates and experimental structure factors were deposited in the Protein Data Bank with accession codes 5Z14 and 5Z15 for TLR8/CU-CPT9a complex, and TLR8/CU-CPT9c complex structures, respectively.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

H.Y. designed the project and supervised the project. Z.H. wrote the manuscript, all authors edited the manuscript.

DECLARATION OF INTERESTS

H.Y. and Z.H. have filed a patent application based on the technology in this manuscript.

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