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Rationally designed small-molecule inhibitors targeting an unconventional pocket on the TLR8 protein-protein interface

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ABSTRACT: Rational designs of small-molecule inhibitors targeting protein-protein interfaces have met little success. Herein, we have designed a series of triazole derivatives with a novel scaffold to specifically intervene with the interaction of TLR8 homomerization. In multiple assays, **TH1027** was identified as a highly potent and specific inhibitor of TLR8. Successful solution of the X-ray crystal structure of TLR8 in complex with **TH1027** shed in-depth mechanistic insight of its binding mode, validating that **TH1027** located between two TLR8 monomers and recognized an unconventional pocket thereby preventing TLR8 from activation. Further biological evaluations showed that **TH1027** dose-dependently suppressed the TLR8-mediated inflammation responses in both human monocyte cell lines, peripheral blood mononuclear cells (PBMCs), and rheumatoid arthritis patient specimens, suggesting strong therapeutic potential against autoimmune diseases.

INTRODUCTION

Toll-like receptor (TLR) family are a group of highly conserved transmembrane proteins which form the first barrier of the immune defense in higher animals.[1] Through the recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), TLRs trigger inflammatory and antiviral responses.[2] Ten different TLRs (TLR 1 through 10) have been identified in human to date which respond to a variety of PAMPs, including lipopeptides (TLR1/2 or TLR2/6),[3,4] viral double-stranded RNA (TLR3 and 10),[5,6] lipopolysaccharide (TLR4),[7] bacterial flagellin (TLR5),[8] viral or bacterial single-stranded RNA (TLR7 and TLR8),[9,10] and cytidine–phosphate guanosine (CpG)-rich unmethylated DNA (TLR9).[11] All these TLRs play a key role in the innate and adaptive immune responses during pathogen invasion and the repair of damaged tissues. However, the persistent activation of TLRs, for instance, TLR8 has also been suggested to contribute to the pathogenesis of various autoimmune diseases.[12,13] Therefore, the development of small-molecule inhibitors selectively targeting TLR8 may provide useful tools to further understand the regulation mechanism and provide a new perspective for discovering new therapies for autoimmune diseases. However, due to the flexible interface of the TLR8 dimeric complex and the high similarity of TLR7 and TLR8 in sequence, it is a great challenge to develop small-molecule TLR8-selective modulators. To our knowledge, resiquimod (R848),[14] the synthetic dual hTLR7/8 agonist, has been confirmed effective for the treatment of genital herpes in clinical. A series of TLR8 specific agonists have been discovered, including furo[2,3-c]pyridines,[15] furo[2,3-c]quinolones,[16] thiazolo[4,5-c]quinolones,[17] benzimidazol-2-amines,[18] 2-aminoimidazoles,[19] pyrimidine-2,4-diamines.[20] There are also TLR7 and TLR8 dual agonists including imidazo-quinolines,[21] pyrimidine derivatives,[22] pyrimidine-2,4-diamines.[23] And TLR7/8 dual antagonists under development are mainly 3H imidazo-quinolines[24] and benzanilide scaffolds.[25] Despite that

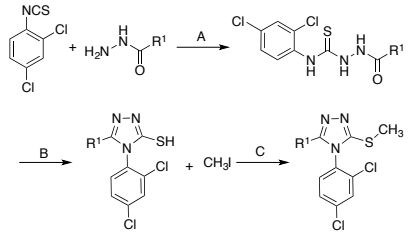
TLR8 was first discovered more than 20 years ago[26] and remarkable clinical potential has been implied,the development of TLR8 specific inhibitors lagged far behind. Most recently, our group has reported the first series of inhibitors of TLR8, designated as **CU-CPT**s,[27,28] bearing a pyrazolo[1,5-a] pyrimidine scaffold and a quinoline scaffold, which stabilized the preformed TLR8 dimer in its resting state (Figure S1). Nonetheless, new chemical scaffolds are still much needed to target TLR8 due to its biological relevance. Herein, we reported a new series of small-molecule inhibitors of TLR8 with a 1,2,4-triazole scaffold targeting the unliganded TLR8 dimeric complex, laying the groundwork for future therapeutic development.

RESULTS and DISCUSSION

Rational design of small-molecule inhibitors of TLR8

In order to identify inhibitors with a novel scaffold that can specifically target the newly discovered binding site on the interface of TLR8 dimer (Figure 1A),[27] we carefully analyze the crystal structure of apo TLR8 dimer (PDB: 3W3G) and come up with following solutions: First of all, aiming to utilize the hydrogen bonding potential of Y348 within the binding pocket, we introduced different heterocycles especially with more hydrogen bond acceptors which may increase the possibility of forming hydrogen bonds and the water solubility. Then we introduced aromatic rings which might have hydrophobic interactions with the surrounding hydrophobic amino acid residues like F405, F261, F494*, F495* and F346 (throughout this paper, asterisks are used to indicate the residues of the second copy of TLR8 monomer). Aiming to these molecular traits, we screened a Maybridge library containing 14,400 drug-like molecules. Using the SEAP reporter assay, we identified 72 compounds as hits, which inhibited TLR8-mediated signaling more than 85% at 4 μM. Within these compounds, 13 hits were selected due to their negligible cytotoxicity (Listed in SMILES file). Eventually, three compounds, **56-G5**, **169-B11** and **169-E2** were selected (Figure S2), as they all shared a 1,2,4-triazole scaffold and met the demanding of the above rationale. Then we chose the most active compound **56-G5** as a starting compound to carry out further studies.

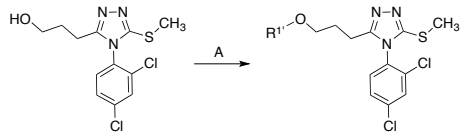
Scheme 1. Synthesis of compounds **56-G5**, **1a-1f**[a].



R^1= -$(CH_2)_3$OH (**56-G5**); -OH (**1a**); -CH_2OH (**1b**); -$(CH_2)_2$OH (**1c**); -$(CH_2)_4$OH (**1d**); -$(CH_2)_5$OH (**1e**); -$(CH_2)_3$ (**1f**).

[a]Reagents and conditions: (A) EtOH, reflux, 10 h; (B) 2M NaOH (aq), reflux, 10 h, then 2M HCl (aq), stirred for 10 mins; (C) CH_3CN, K_2CO_3, rt, 12 h.

Scheme 2. Synthesis of compounds **1g** and **1h**[a].



1a

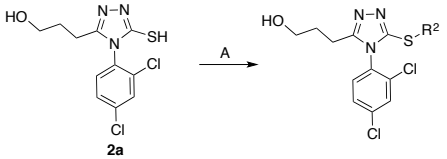
$R^{1'}$= -CH_3 (**1g**); CH_3CO- (**1h**).

[a]Reagents and conditions: (A) NaH, anhydrous THF, CH_3I, rt, 12 h (for **1g**); acetic anhydride, 4-dimethylaminopyridine, anhydrous dichloromethane, rt, 12 h (for **1h**).

Structure-activity relationship studies

With the scaffold identified, we developed a synthetic route (Scheme 1-4) for 1,2,4-triazole derivatives and carried out structure and activity relationship (SAR) studies (Table 1). The SAR results indicated that the length of linker at the R^1 position had no significant influence as long as the chain is not too short (**1a** versus **56-G5**, **1b, 1c**, **1d and 1e**). Comparison of different compounds demonstrated that a three-carbon chain at R^1 position displayed relatively better inhibitory activity. Next, removal of the hydroxyl group at R^1 position (**1f**) rendered a modest effect on its activity, while methylation (**1g**) or acetylation (**1h**) of hydroxyl group led to slight decrease of the potency. These results implied that the proper length of the carbon chain and the hydroxyl group may be both important for the formation of hydrogen bonds with surrounding amino acid residues. Therefore, in the following SAR studies, the hydroxyl group linked with a three-carbon chain was fixed at the R^1 position. Furthermore, when the methyl group was replaced with hydrogen (**2a)** or more hydrophilic groups (**2d, 2e**) at the R^2 position, the inhibitory activity was completely compromised. Introducing the ethyl (**2b**) or isopropyl group (**2c**) at the R^2 position sharply reduced the potency, suggesting a small and hydrophobic substituent was favored here.

Scheme 3. Synthesis of compounds **2a-2e**[a].

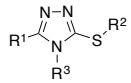


2a

R^1= -CH_2CH_3 (**2b**); -$CH(CH_3)_2$ (**2c**); -$CONH_2$ (**2d**); -CH_2CONH_2(**2e**).

[a]Reagents and conditions: (A) ethyl iodide, K_2CO_3, acetone, rt, 12 h (for **2b**); 2-iodopropane, K_2CO_3, acetone, rt, 12 h (for **2c**); trichloroacetyl chloride, acetonitrile, DMF, rt, 1 h, then 7M ammonia methanol solution, reflux for 3h (for **2d**); 2-iodoacetamide, K_2CO_3 and acetonitrile, rt, 12 h (for **2e**).

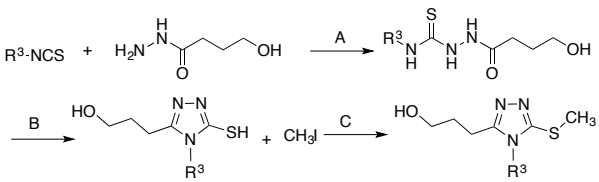
Table 1. Structure-activity relationship analyses of the TLR8 inhibitors in HEK-Blue hTLR8 cells



No.	R^1	R^2	R^3	IC_{50}[μM] [a]
56-G5	-$(CH_2)_3OH$	-CH_3	2,4-di-Cl-C_6H_3-	0.29 ± 0.01
1a	-OH	-CH_3	2,4-di-Cl-C_6H_3-	9.00 ± 0.41
1b	-CH_2OH	-CH_3	2,4-di-Cl-C_6H_3-	0.55 ± 0.07
1c	-$(CH_2)_2OH$	-CH_3	2,4-di-Cl-C_6H_3-	0.59 ± 0.03
1d	-$(CH_2)_4OH$	-CH_3	2,4-di-Cl-C_6H_3-	1.06 ± 0.10
1e	-$(CH_2)_5OH$	-CH_3	2,4-di-Cl-C_6H_3-	0.52 ± 0.05
1f	-$(CH_2)_2CH_3$	-CH_3	2,4-di-Cl-C_6H_3-	0.63 ± 0.02
1g	-$(CH_2)_3OCH_3$	-CH_3	2,4-di-Cl-C_6H_3-	1.18 ± 0.14
1h	-$(CH_2)_3OCOCH_3$	-CH_3	2,4-di-Cl-C_6H_3-	2.08 ± 0.10
2a	-$(CH_2)_3OH$	-H	2,4-di-Cl-C_6H_3-	＞20
2b	-$(CH_2)_3OH$	-CH_2CH_3	2,4-di-Cl-C_6H_3-	0.39 ± 0.04
2c	-$(CH_2)_3OH$	-$CH(CH_3)_2$	2,4-di-Cl-C_6H_3-	10.12±0.81
2d	-$(CH_2)_3OH$	-$CONH_2$	2,4-di-Cl-C_6H_3-	＞20
2e	-$(CH_2)_3OH$	-CH_2CONH_2	2,4-di-Cl-C_6H_3-	＞20
3a	-$(CH_2)_3OH$	-CH_3	4-Cl-C_6H_4-	1.61 ± 0.12
3b	-$(CH_2)_3OH$	-CH_3	4-OCH_3-C_6H_4-	3.86 ± 0.31
3c	-$(CH_2)_3OH$	-CH_3	4-CF_3-C_6H_4-	4.84 ± 0.82
3d	-$(CH_2)_3OH$	-CH_3	2-Cl-C_6H_4-	0.98 ± 0.14
3e	-$(CH_2)_3OH$	-CH_3	3-Cl-C_6H_4-	0.35 ± 0.02
3f	-$(CH_2)_3OH$	-CH_3	3-CF_3-C_6H_4-	0.21 ± 0.02
3g	-$(CH_2)_3OH$	-CH_3	3-CH_3-C_6H_4-	0.29 ± 0.03
3h	-$(CH_2)_3OH$	-CH_3	3,4-di-Cl-C_6H_3-	0.19 ± 0.01
3i	-$(CH_2)_3OH$	-CH_3	2,3-di-Cl-C_6H_3-	0.80 ± 0.07
3j	-$(CH_2)_3OH$	-CH_3	2,4,6-tri-Cl-C_6H_2-	2.81 ± 0.44
3k	-$(CH_2)_3OH$	-CH_3	2,3,4-tri-Cl-C_6H_2-	0.60 ± 0.10
3l	-$(CH_2)_3OH$	-CH_3	3,4,5-tri-Cl-C_6H_2-	0.11 ± 0.01
3m（TH1027）	-$(CH_2)_3OH$	-CH_3	3,4,5-tri-CH_3-C_6H_2-	0.030± 0.002
3n	-$(CH_2)_3OH$	-CH_3	3-pyridyl-	＞20
3o	-$(CH_2)_3OH$	-CH_3	cyclohexyl-	＞20
3p	-$(CH_2)_3OH$	-CH_3	1-naphthyl-	0.39 ± 0.06

[a] SEAP reporter assay was used to measure the inhibitory activities of compounds. IC_{50} values and corresponding standard deviations were determined from at least three biological replicates.

Scheme 4. Synthesis of compounds **3a**-**3p**[a]



Cmpd	R^3
3a	4-Cl-C_6H_4-
3b	4-OCH_3-C_6H_4-
3c	4-CF_3-C_6H_4-
3d	2-Cl-C_6H_4-
3e	3-Cl-C_6H_4-
3f	3-CF_3-C_6H_4-
3g	3-CH_3-C_6H_4-
3h	3,4-di-Cl-C_6H_3-

Cmpd	R^3
3i	2,3-di-Cl-C_6H_3-
3j	2,4,6-tri-Cl-C_6H_2-
3k	2,3,4-tri-Cl-C_6H_2-
3l	3,4,5-tri-Cl-C_6H_2-
3m(TH1027)	3,4,5-tri-CH_3-C_6H_2-
3n	3-pyridyl-
3o	cyclohexyl-
3p	1-naphthyl-

[a]Reagents and conditions: (A) EtOH, reflux, 10 h; (B) 2M NaOH (aq), reflux, 10 h, then 2M HCl (aq), stirred for 10 mins; (C) CH_3CN, K_2CO_3, rt, 12 h.

To further investigate whether different substituents and positions on the benzene ring would influence the activities, we synthesized 4-Cl (**3a**), 4-OCH_3 (**3b**), 4-CF_3 (**3c**), 2-Cl (**3d**), 3-Cl (**3e**), 3-CF_3 (**3f**) and 3-CH_3 (**3g**) substituted compounds. Results indicated that the activity of 3-substituted compounds was significantly higher than those with 4-substitutions or 2-substitutions. All 3–Cl (**3e**), 3-CF_3 (**3f**) and 3-CH_3 (**3g**) substituted compounds showed similar activities which indicated that electronic effects are not obvious here. Next, we explored the multi-substitutions on benzene ring (**3h**-**3l**) and results showed that 3,4,5-tri-chlorophenyl group (**3l**) at R^3 position showed the most potent inhibitory activity, suggesting that this substitutions fitted better to the hydrophobic pocket. Then we tried to replace the 3,4,5-tri-Cl substitution with 3,4,5-trimethyl substitution and the inhibitory activity was increased about 4-fold (**3l** versus **TH1027**), suggesting **TH1027** may match better to the target hydrophobic pocket. Also, cyclohexyl (**3o**) and 3-pyridyl (**3n**) substitution at the R^3 position showed no activities which may indicate the existence of a hydrophobic pocket around R^3 position. In summary, with extensive and systematic SAR exploration, we have identified **TH1027** as the lead compound. Both synthetic small molecule R848 and single stranded RNA are previously established as nonselective TLR8 activators.[10,14] In the cellular assay, **TH1027** showed dose-dependent inhibitory effects in blocking R848- or ssRNA06-induced TLR8 activation with an IC_{50} of 30.0 ± 2.0 nM (Figure 1B). Importantly, **TH1027** was found to have no significant cytotoxicity at concentrations up to 100 μM in HEK-Blue TLR8 cells (Figure S3).

K_d determination and on-target validation in cells

Next, isothermal titration calorimetry (ITC) confirmed that **TH1027** directly bound to the ectodomain of human TLR8 proteins with the dissociation constant (K_d) of 225 ± 38 nM

(Figure 1C). The heat change induced by its synthetic ligand R848 was markedly suppressed in the presence of **TH1027**, indicating that **TH1027** prevented R848 from binding to TLR8 (Figure S4).

A major challenge of developing TLR inhibitors is to achieve high specificity. There are at least 13 homologous TLRs present in murine macrophages and 10 homologous TLRs in human, all sharing common structural motifs including leucine-rich repeats.[2] To evaluate its specificity, we tested **TH1027** against a panel of homologous TLRs, including TLR1/2, TLR2/6, TLR3, TLR4, TLR5, TLR7 and TLR9, using TLR-specific ligands to selectively activate a particular TLR signaling pathway. Result showed that **TH1027** inhibited TLR8-mediated signaling without affecting other TLRs, proving its highly selectivity in a complex, whole cell environment (Figure 1D). We also investigated the inhibitory effects of **TH1027** on the mRNA level of pro-inflammatory cytokines by quantitative real-time PCR (RT-PCR) in HEK-Blue hTLR8 cells. The results showed that **TH1027** completely abolished the elevation of TNF-α and IL-8 mRNA levels induced by R848. By contrast, the negative control, compound **2a**, showed negligible inhibition (Figure 1E).

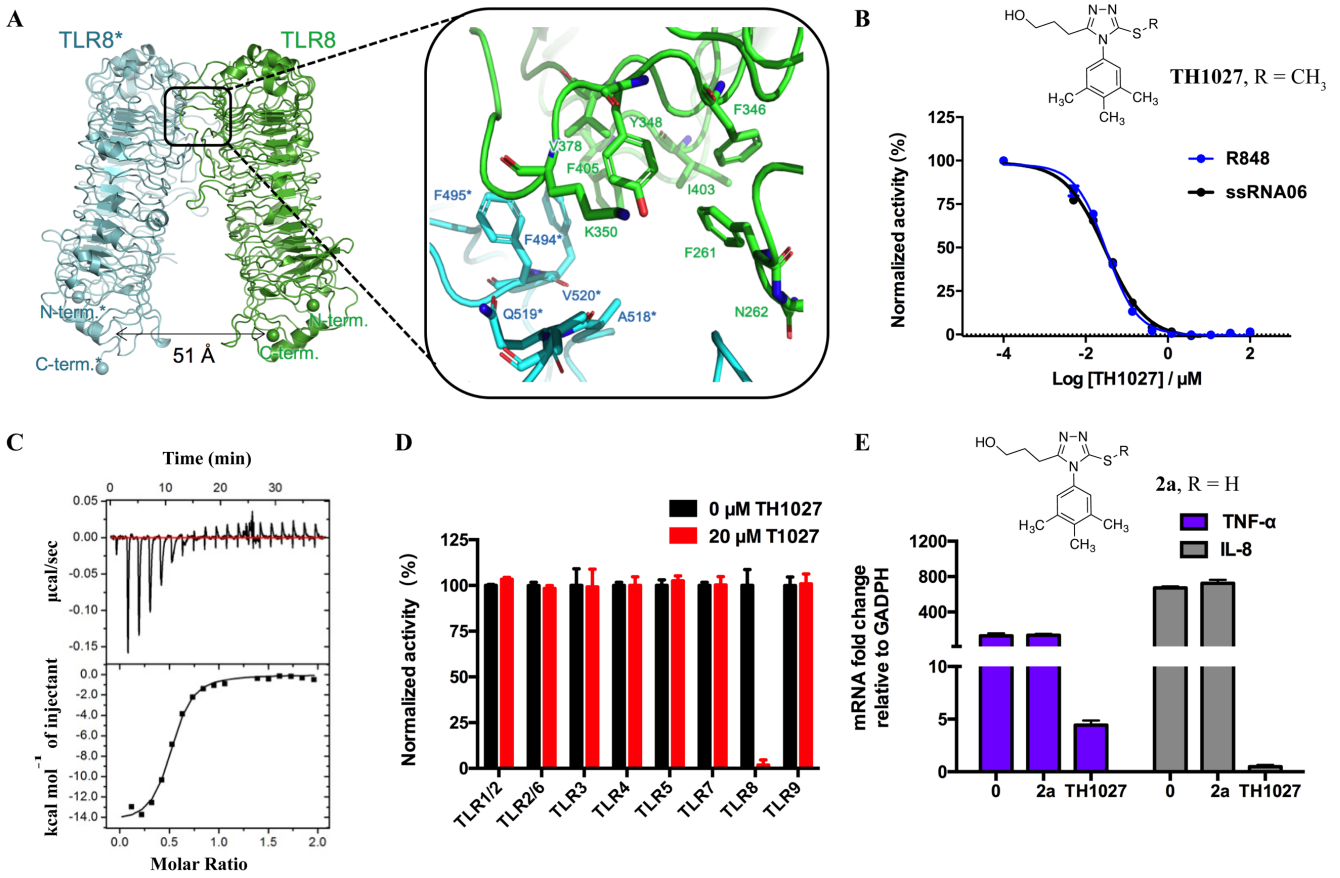


Figure 1. Rational design of small-molecule TLR8 inhibitors. (A) X-ray crystal structure of the TLR8 protein (PDB: 3W3G). The side view of unliganded conformation of the TLR8 ectodomain and the close-up view of the antagonist binding site of unliganded TLR8. (B) **TH1027** dose-dependently inhibited R848- or ssRNA06-induced TLR8 activation with an IC_{50} of 30 nM. (C) ITC thermogram of **TH1027** titrated into human TLR8 (hTLR8) to determine its binding affinity and stoichiometry (K_d = 225 nM). The raw data are presented on top and the integrated peak areas are shown and fitted below. Red line represents baseline for peak integration. (D) Specificity test for **TH1027** using TLR-specific ligands to selectively activate the respective TLRs: 100 ng/mL of Pam_3CSK_4, 100 ng/mL of Pam_2CSK_4, 5 μg/mL of polyriboinosinic : polyribocytidylic acid [poly(I:C)], 20 ng/mL of LPS (lipopolysaccharide), 50 ng/mL of Flagellin, 1 μg/mL of R848, 2 μg/mL of R848 and 0.5 μM of ODN2006 were used to selectively activate hTLR1/2, hTLR2/6, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8 and hTLR9 cells, respectively (Data are mean s.d.). (E) TNF-α and IL-8 mRNA levels in R848-treated HEK-Blue TLR8 cells in the presence or absence of 1 μM **TH1027** or the negative control **2a** (1 μM). Data are the average quantification of three independent biological replicates (Data are mean s.d.).

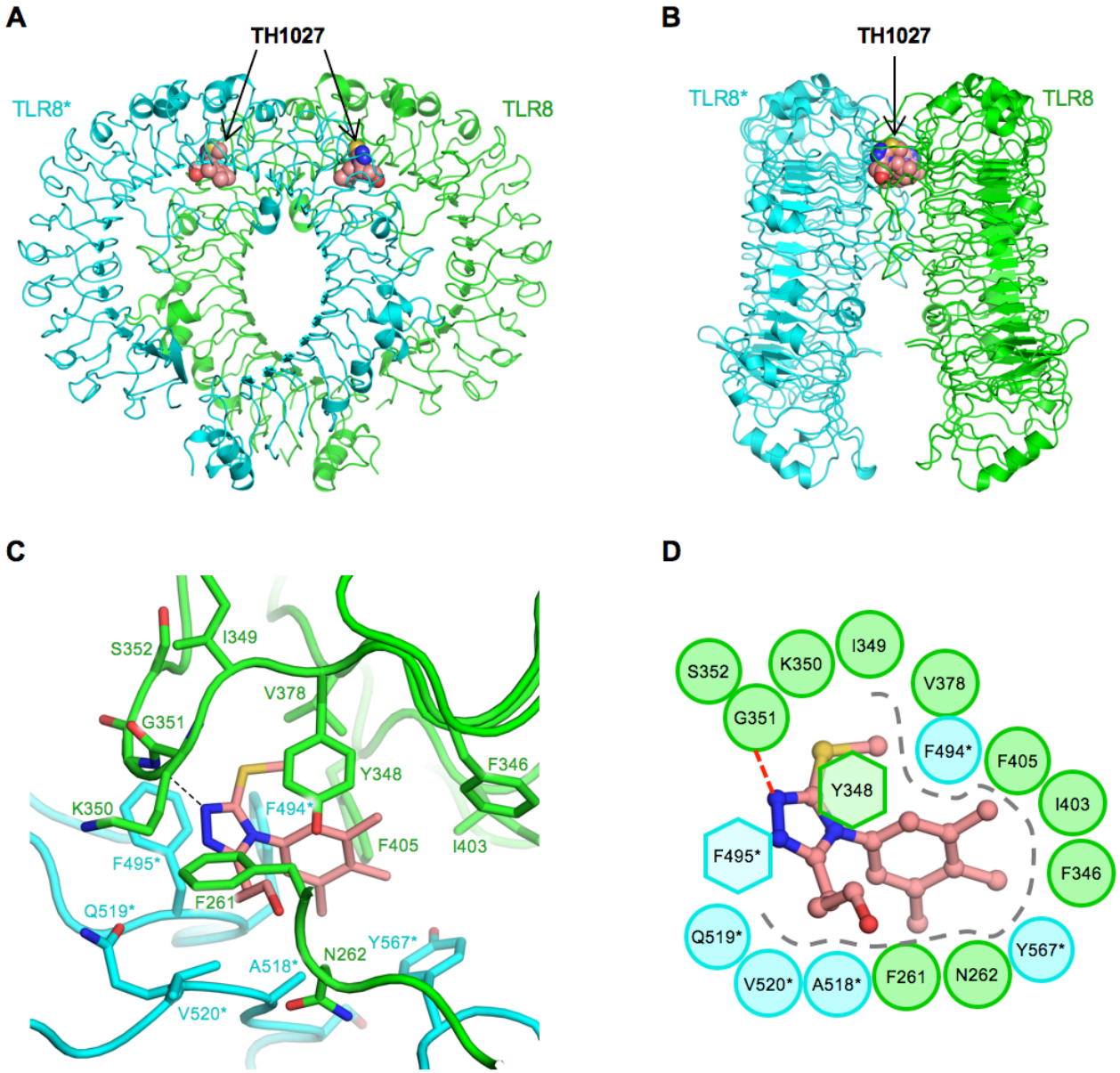


Figure 2. Crystal structure of the TLR8/TH1027 complex (PDB ID: 6KYA, resolution: 2.89 Å). (A) Front view of the TLR8/**TH1027** complex. Two TLR8 monomers, TLR8 and TLR8*, are colored green and cyan, respectively. The ligand molecules are illustrated by space-filling representations. The C, N, O and S atoms of the ligands are colored pink, blue, red and yellow, respectively. (B) Side view of TLR8/**TH1027** complex. (C) Close-up view of the antagonist binding site with **TH1027**. (D) Schematic representation of interactions between **TH1027** and the TLR8 proteins. The hydrophobic pocket and hydrogen bonds are shown as dashed gray arcs and a dashed red line, respectively.

X-ray crystal structure of the TLR8/TH1027 complex

In order to elucidate the molecular mechanism of inhibition, we successfully obtained the crystal structure of the TLR8/**TH1027** complex (PDB: 6KYA). Clearly, **TH1027** bound to the target pocket and located between two copies of TLR8 monomers, validating our rational design (Figure 2A and 2B). **TH1027** formed a hydrogen bond with the polypeptide backbone of the G351 residue and was sandwiched by Y348 and F495*, contributing π-π interactions at this pocket (Figure 2C and 2D). In addition, hydroxyl group at the R^1 position of **TH1027** made another hydrogen bond with Y348 only in one protomer of the dimer, and its density was rather poor, suggesting the hydrogen bond is weak. Moreover, no hydrogen bond was observed in another protomer due to the different orientation of hydroxyl group at the R^1(Figure S5). Considering these structural features, it was difficult to estimate the significance of this interaction for the affinity from the structure. Therefore we did not mark a dotted line to repre-

sented a hydrogen bond between TH1027 and Y348. Interestingly, these structural results also were in good agreement with the results of SAR studies discussed above. The proper length of linker and hydroxyl group at R^1 position was essential for the formation of hydrogen bonds. For example, the hydrophobic pocket around R^2 position is relatively small. Therefore, **TH1027** with a methyl substitution showed better activity compared with compounds with larger or more polar groups at R^2 (**2a** - **2c**). Further, hydrophobic residues including V520*, A518*, F261, F346, F405, F494* and F495*, formed a hydrophobic pocket around R^3 position which fitted well with 3,4,5-trimethyl benzene substitution. With 3-pyridyl- (**3n**) and cyclohexyl- (**3o**) substitutions at R^3 position, the inhibitory activities of **TH1027** totally disappeared, indicating the π-π interaction is vital for the activities. Furthermore, our experimental results were in good agreement with Lu lab.[29] Interestingly, superimposition of the structure of TLR8 derived from the TLR8/**TH1027** complex onto the unliganded TLR8 produced a root mean square deviation (RMSD) value of 2.2 Å. By comparison, its superimposition with the agonist-bound, active form of TLR8 produced a RMSD value of 7.5 Å, indicating that the conformation of TLR8/**TH1027** complex was closer to the resting state. Also, its superimposition with the structure of TLR8/**CU-CPT8m** complex, produced a RMSD value of 0.39 Å, indicating that the conformation of TLR8/**TH1027** complex was very similar to TLR8/**CU-CPT8m**. So we developed a series of TLR8 inhibitor with a triazole scaffold showing almost the same binding mode, but the interactions with surrounding amino acids were slightly different. These results further confirmed the previous strategy we had proposed,[27] locking the resting state of TLR8 by targeting a feasible pocket on the TLR8-TLR8* interface.

TH1027 Inhibited TLR8 downstream signaling in cultured cells or patient specimens

Next, we carried out on-target validation for **TH1027**, in an attempt to confirm that **TH1027** indeed functioned by selectively targeting TLR8 in the complex, cellular environment. The phosphorylated IRAK4 (p-IRAK4), TRAF3 and p65 (component of NF-κB) are all signature downstream proteins for the TLR8 signaling transduction (Figure 3A). In a good agreement that **TH1027** targeted TLR8, the expression of p-IRAK4 and TRAF3 was elevated upon R848 treatment in human monocyte cell lines and this elevation can be suppressed by **TH1027** in a dose-dependent manner. Furthermore, the translocation of p65 into the nucleus induced by the R848 could also be inhibited by **TH1027** (Figure 3A). The blots were further analyzed with image J software for quantification (Figure S6).

Finally, we employed more physiologically- and pathologically-relevant assays to confirm the activities of **TH1027**. Results showed that **TH1027** dose-dependently inhibited the TLR8-mediated production of pro-inflammatory cytokines TNF-α (Figure 3B), IL-6 (Figure 3C) and IL-1β (Figure 3D) in human monocyte cell lines. Besides cultured cell lines, we also investigated whether **TH1027** could inhibit TLR8 signaling pathway in primary cells. Human PBMCs (include lymphocytes, monocytes, and dendritic cells expressing various TLRs) were isolated from the fresh blood of healthy donators using established methods of density gradient centrifugation.[30] The R848 treatment of PBMCs induced TNF-α secretion, which could be inhibited by **TH1027** in a dose-dependent manner (Figure 3E), but not by the negative control, compound **2a**. It was worth noting that TNF-α secretion was not inhibited to the baseline by **TH1027** even at the concentration up to 20 μM, most likely because both TLR7 and TLR8 were activated by R848 but **TH1027** only reversed the TLR8 activation. This result further proved that **TH1027** was a TLR8-specific inhibitor and did not affect TLR7-mediated signaling.

Previous studies showed that TLR8 deficiency can lead to rheumatoid arthritis (RA).[31] Murine TLR8 had mutants at the ligand binding site and therefore could not be activated by either ssRNA or R848, making it impossible to use wild type mice to test the efficacy of these TLR8 inhibitors.[32] As an alternative to the mouse animal models, we tested **TH1027** by monitoring its ability to reduce TNF-α production in patients' primary cells. Fresh blood from six RA patients were collected and their PBMCs were isolated from these blood samples by density gradient centrifugation[30]. Immediately after separation, cells were co-cultured with various concentrations of **TH1027** or inactive compound **2a** for 24 hours. Then the culture media were collected to measure the concentration of TNF-α. The results indicated that **TH1027** significantly inhibited spontaneous TNF-α production from these cultures compared to untreated cells, pointing to remarkable therapeutic potential of **TH1027** and its derivatives (Figure 3F). Moreover, the cell viability experiment showed that **TH1027** had no toxicity in PBMCs from RA patients (Figure S7).

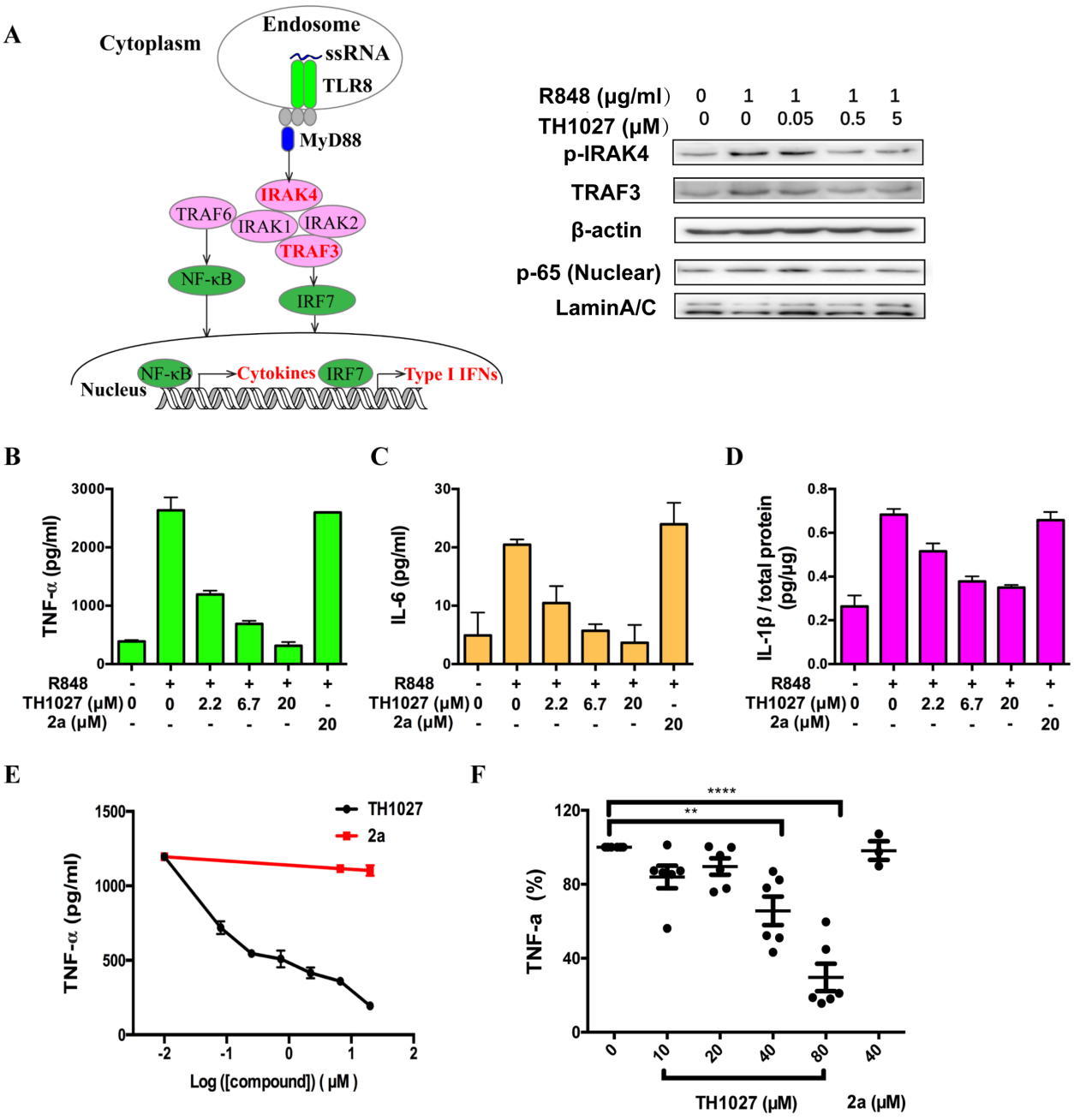


Figure 3. **Inhibition of downstream signaling in cultured cells or human specimens.** (A) The brief diagram of the TLR8 signaling pathway. **TH1027** downregulated the expression levels of proteins in the TLR8 signaling pathway. (B-D) ELISA assay results showed that **TH1027** dose-dependently inhibited the TNF-α, IL-6 and IL-1β production activated by 1 μg/ml R848 in human monocyte cell lines (20 μM **2a** as a negative control). (E) Dose-dependent inhibitory effects of R848-induced TLR8 activation by **TH1027** (**2a** as a negative control) in human PBMCs. (F) **TH1027** dose-dependently inhibited the production of TNF-α in PBMCs harvested from six rheumatoid arthritis patients (**2a** as a negative control). Each data point represents an independent sample read. Center lines indicate means, and whiskers indicate s.e.m. (P values were determined using one-way ANOVA; **P < 0.01; ****P < 0.0001).

CONCLUSIONS

In conclusion, our rational designs targeting an unconventional pocket on the dimeric interface of TLR8 led to the discovery of a next generation of highly potent and specific TLR8 inhibitor, **TH1027**, with a novel scaffold. The X-ray crystallographic structure of TLR8/**TH1027** complex validated the contacts within the binding pocket. A variety of biological evaluations in cultured cell lines, human PBMCs from healthy donators and RA patients further demonstrated high efficacy and specificity of **TH1027**, suggesting strong therapeutic potential against autoimmune diseases such as RA. Overall, the identification of novel small-molecules as TLR8 inhibitors not only further confirmed the feasibility of targeting interface of protein-protein with small molecules, but also provided more

candidate compounds for drug development in autoimmune diseases.

EXPERIMENTAL SECTION

Chemistry Method. All solvents and chemicals were used as purchased without further purification. NMR spectra were recorded on a Bruker AVANCE III HD 400 (^{1}H at 400 MHz, ^{13}C at 101 MHz) nuclear magnetic resonance spectrometer. ^{1}H NMR spectra were recorded at 400 MHz in $CDCl_3$ or $(CD_3)_2SO$ using residual $CHCl_3$ (7.26 ppm) and $(CH_3)_2SO$ (2.50 ppm) as the internal standard. ^{13}C NMR spectra were recorded at 101 MHz in $CDCl_3$ or $(CD_3)_2SO$ using residual $CHCl_3$ (77.16 ppm) and $(CH_3)_2SO$ (39.6 ppm) as internal reference. High-resolution mass spectra were acquired on a Thermo Scientific QExactive mass spectrometer (ESI). The purity was determined by high performance liquid chromatography (HPLC) on SHIMAZU (LC-20AP) except for **3o**. Purity of all final compounds was more than 95%. The column was a Inertsil C8 (Lot: SB5-1449), 5 μm particle size (150 mm * 4.6 mm). The binary solvent system (A/B) was as follows: water (A) and acetonitrile (B), A/B = 5/95 or 100% (B). The flow rate was 0.15 - 0.5 mL/min depending on the pressure of pumps. Of note, compounds **2a**, **2d** and **2e** required MeOH to ensure proper dissolution. All compounds were named using ChemDraw 15.0.

3-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol(56-G5). A solution of 2,4-dichloroaniline (1.52 g, 9.39 mmol) in anhydrous chloroform (10 mL) was added sodium carbonate (1.27 g, 11.98 mmol) and pre-cooled in an ice bath. Then thiophosgene (660 μL, 8.66 mmol) was slowly added and the solution was allowed to stir in an ice bath for 2 hours. After this time, 2M sodium hydroxide solution (10 mL) was added to quench the reaction and the mixture was extracted with dichloromethane (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The residue obtained was purified via flash SiO_2 chromatography (eluted with petroleum ether/ethyl acetate 20:1) to give 2,4-dichloro-1-isothiocyanatobenzene (17.61 g, 99%) as pale yellow liquid.^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.39 (d, *J* = 2.2 Hz, 1H), 7.20 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 1H). A solution of 2,4-dichloro-1-isothiocyanobenzene (251 mg, 1.23 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (160 mg, 1.35 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl solution (9 mL)was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2,4- dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (110 mg, 76%) as a white solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 13.80 (s, 1H), 7.98 (d, *J* = 2.2 Hz, 1H), 7.72 – 7.63 (m, 2H), 3.36 (s, 2H), 2.36 (dd, *J* = 8.6, 6.8 Hz, 2H), 1.66 (p, *J* = 6.7 Hz, 2H). A solution of 3-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.33 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (31 μL, 0.5 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4- Triazol-3-yl)propan-1-ol (79 mg, 75%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, *J* = 2.1 Hz, 1H), 7.72 – 7.63 (m, 2H), 4.42 (t, *J* = 5.2 Hz, 1H), 3.36 – 3.32 (m, 2H), 2.50 (s, 3H), 2.45 – 2.38 (m, 2H), 1.65 (dq, *J* = 8.2, 6.4 Hz, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 151.0, 136.6, 133.3, 132.1, 130.8, 130.1, 129.7, 60.2, 29.8, 21.8, 15.3. HPLC purity analysis: 95.1% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{13}Cl_2N_3OS[M+H]^+$: 318.0235; found: 318.0230.

4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-ol (1a) A solution of 2,4-dichloro-1-isothiocyanobenzene (400 mg, 1.95 mmol) in anhydrous ethanol (8 mL) was added methyl carbazate (223 mg, 2.48 mmol) and then the mixture was reflux overnight. After the reaction was completely converted, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) (9 mL) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazole-3-ol (200 mg, 39%) as white solid without further purification. Next, a solution of 4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-ol (200 mg, 0.76 mmol) in acetonitrile (8 mL) was added potassium carbonate (276 mg , 2.00 mmol) and then methyl iodide (57 μL, 0.92 mmol) was added dropwise. The mixture was reacted at room temperature overnight. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 30:1) to give 4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazole- 3-alcohol (79 mg, 85%) as a white solid powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 8.03 – 7.86 (m, 1H), 7.64 (d, J = 1.7 Hz, 2H), 2.43 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 154.2, 144.2, 136.1, 134.2, 133.0, 130.4, 129.5, 129.2, 13.8. HPLC purity analysis: 95.6% (229 nm). HRMS (ESI) calcd. for $C_9H_7Cl_2N_3OS[M+H]^+$: 275.9765; found: 275.9751.

Synthesis of (4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)methanol (1b). A solution of methyl 2-hydroxyacetate (200 mg, 2.22 mmol) in anhydrous ethanol (10 mL) was added hydrazine hydrate (166.7 mg, 2.66 mmol, 80% in purity) and reflux for 3h. A large amount of white solid was precipitated when the mixture cooled in room temperature. After filtration, the precipitation was washed with water and then dried to obtain 2-hydroxyacetohydrazide (160 mg, 80%) as a white solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.83 (s, 1H), 4.55 (s, 2H), 4.34 – 4.00 (m, 1H), 3.90 – 3.78 (m, 2H). A solution of 2,4-dichloro-1-isothiocyanobenzene (350 mg, 1.71 mmol) in anhydrous ethanol (8 mL) was added 2-hydroxyacetohydrazide (200 mg, 2.22 mmol) and reflux overnight. Then ethanol was evaporated, 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) (9 mL) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain (4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)methanol (235 mg, 50%) as a white solid. A solution of (4-(2,4-dichlorophenyl)-5-mercapto-

4H-1,2,4-triazol-3-yl)methanol (235 mg, 0.86 mmol) in acetonitrile (8 mL) was added potassium carbonate (276 mg, 1.00 mmol) and methyl iodide (80 μL, 1.28 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give (4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)methanol (198mg, 81%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (t, J = 1.3 Hz, 1H), 7.66 (d, J = 1.3 Hz, 2H), 5.36 (t, J = 5.7 Hz, 1H), 4.38 (ddd, J = 47.9, 13.5, 5.8 Hz, 2H), 2.57 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.7, 152.4, 136.4, 133.2, 132.1, 130.5, 130.3, 129.3, 54.1, 15.2. HPLC purity analysis: 98.2% (229 nm). HRMS (ESI) calcd. for $C_{10}H_9Cl_2N_3OS$ $[M+H]^+$: 289.9922; found: 289.9909.

2-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)ethan-1-ol (1c). A solution of oxetan-2-one (300 mg, 4.16 mmol) in diethyl ether (10 mL) was added sodium methoxide (337.1 mg, 6.24 mmol) and reflux for 2 h, followed by addition of hydrazine monohydrate (395 mg, 6.24 mmol, 80% in purity), anhydrous ethanol (10mL) and the mixture was refluxed overnight. Then 2,4-dichloro-1-isothiocyanobenzene (204 mg, 1.00 mmol) was added and refluxed overnight. After the reaction was completely converted, the ethanol solution was spun dry, and then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. After the reaction was completely converted, 2 M HCl solution was slowly added until a large amount of white solid was appeared. After suction filtration, it was washed with water, and then dried to obtain 2-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)ethan-1-ol (147 mg, 48%) as a white solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 13.84 (s, 1H), 7.98 (d, J = 2.3 Hz, 1H), 7.69 (dd, J = 8.5, 2.3 Hz, 1H), 7.62 (d, J = 8.6 Hz, 1H), 4.78 (t, J = 5.6 Hz, 1H), 3.53 (dq, J = 8.5, 2.9 Hz, 2H), 2.58 (dd, J = 15.3, 6.6 Hz, 1H), 2.45 (dt, J = 15.3, 6.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.2, 150.5, 136.1, 133.7, 133.3, 130.8, 130.5, 129.2, 58.2, 29.6. A solution of 2-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)ethan-1-ol (116mg, 0.38 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (40 μL, 0.64 mmol) and the mixture was allowed to reacted at room temperature overnight. Then water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 2-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)ethan-1-ol (198mg, 81%) as a white solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 (s, 1H), 7.70 (s, 2H), 4.72 (t, J = 5.5 Hz, 1H), 3.64 – 3.50 (m, 2H), 2.66 (dt, J = 13.9, 6.8 Hz, 2H), 2.54 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 154.1, 151.2, 136.6, 133.2, 132.3, 130.7, 130.1, 129.6, 59.0, 28.9, 15.3. HPLC purity analysis: 98.3% (229 nm). HRMS (ESI) calcd. for $C_{11}H_{11}Cl_2N_3OS$ $[M+H]^+$: 304.0078; found: 304.0070.

4-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)butan-1-ol (1d). A solution of 2,4-dichloro-1-isothiocyanobenzene (200 mg, 0.977 mmol) in anhydrous ethanol (8 mL) and 5-hydroxypentanehydrazide (181 mg, 1.37 mmol) was added. The mixture was refluxed overnight. Then mixture was concentrated to dryness and 2 M NaOH (aq) (8 mL) was added and reflux overnight. After the mixture was cooled, 2 M HCl solution was slowly added until a large amount of white was appeared. The solid is filtered, washed with water, and then dried to give 4-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)butan-1-ol (108 mg, 33%) as a white solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 13.81 (s, 1H), 7.98 (d, J = 2.2 Hz, 1H), 7.73 – 7.66 (m, 2H), 3.31 (s, 2H), 2.35 (td, J = 7.4, 3.2 Hz, 2H), 1.61 – 1.48 (m, 2H), 1.40 (dt, J = 8.6, 6.3 Hz, 2H). A solution of 4-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)butan-1-ol (100 mg, 0.30 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (28 μL, 0.45 mmol) and the mixture was allowed to reacted at room temperature overnight. Then water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 4-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)butan-1-ol (85mg, 85%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (d, J = 1.9 Hz, 1H), 7.75 – 7.67 (m, 2H), 4.32 (t, J = 5.1 Hz, 1H), 3.33 (m, 2H), 2.54 (s, 3H), 2.44 (t, J = 7.5 Hz, 2H), 1.61 – 1.50 (m, 2H), 1.44 – 1.33 (m, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.4, 150.5, 136.0, 132.6, 131.5, 130.2, 129.6, 129.1, 60.1, 31.3, 24.0, 22.8, 14.8. HPLC purity analysis: 95.2% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{15}Cl_2N_3OS$ $[M+H]^+$: 332.0391; found: 332.0389.

5-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)pentan-1-ol (1e). A solution of tetrahydro-2H-pyran-2-one (500 mg, 4.38 mmol) in ethanol(8 mL) was added hydrazine monohydrate (329 mg, 5.26 mmol, 80% in purity) and reflux overnight, and spin dry to give crude product. Then crude product (320 mg, 1.23 mmol) was added into a new 25 mL round bottom flask, 2,4-dichloro-1-isothiocyanobenzene (324 mg, 1.58 mmol) and ethanol (8 mL) was added and refluxed overnight. The ethanol was spun dry, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. After the reaction was completely converted, 2 M HCl (aq) was slowly added until the solution became weakly acidic, and a large amount of white solid appeared. After suction filtration, it was washed with water and then dried to give a crude 5-(4-(2,4-dichloro) Phenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)pentan-1-ol (white solid, 147 mg). Next, 5-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)pentan-1-ol (314 mg, 0.95 mmol) and potassium carbonate solid (276 mg, 2 mmol) were added in 8 mL of acetone, and methyl iodide (89 μL, 1.42 mmol) was added dropwise with stirring under a rotor, and allowed to react at room temperature overnight. After rotary evaporation, a silica gel column was eluted using dichloromethane/methanol 20:1 to 10:1 to give 5-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1. 2,4-Triazol-3-yl)pentan-1-ol as yellow oily liquid (280 mg, 85%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (d, J = 2.1 Hz, 1H), 7.76 – 7.67 (m, 2H), 4.29 (t, J = 5.2 Hz, 1H), 3.33 (d, J = 5.5 Hz, 2H), 2.54 (s, 3H), 2.43 (t, J = 7.5 Hz, 2H), 1.50 (p, J = 7.2 Hz, 2H), 1.37 – 1.21 (m, 4H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 151.1, 136.6,

133.2, 132.1, 130.8, 130.2, 129.6, 60.9, 32.4, 26.5, 25.3, 24.9, 15.3. HPLC purity analysis: 95.6% (229 nm). HRMS (ESI) calcd. for $C_{14}H_{17}Cl_2N_3OS$ $[M+H]^+$: 346.0548; found: 346.0532.

4-(2,4-dichlorophenyl)-3-(methylthio)-5-propyl-4H-1,2,4-triazole (1f). A solution of 2,4-dichloro-1-isothiocyanobenzene (200 mg, 0.98 mmol) in anhydrous ethanol (8 mL) and butyrohydrazide (102 mg, 1.00 mmol) was added. The mixture was refluxed overnight. Then mixture was concentrated to dryness and 2 M NaOH (aq) (8 mL) was added and reflux overnight. After the mixture was cooled, 2 M HCl solution was slowly added until a large amount of white was appeared. The solid is filtered, washed with water, and then dried to give 4-(2,4-dichlorophenyl)-5-propyl-4H-1,2,4-triazole-3-thiol (108 mg, 37%) as a white solid. Next 4-(2,4-dichlorophenyl)-5-propyl-4H-1,2,4-triazole-3-thiol (100 mg, 0.30 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (28 μL, 0.45 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 4-(2,4-dichlorophenyl)-3-(methylthio)-5-propyl-4H-1,2,4-triazole as a white solid (50mg, 48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J=2.2 Hz, 1H), 7.75 (d, J=8.5 Hz, 1H), 7.71 (dd, J=8.5, 2.2 Hz, 1H), 2.55 (s, 3H), 2.42 (t, J=7.5 Hz, 2H), 1.54 (h, J=7.2 Hz, 2H), 0.86 (t, J=7.4 Hz, 3H); ^{13}C NMR(101MHz, DMSO-d_6) δ 155.2, 150.6, 136.1, 132.6, 131.6, 130.3, 129.6, 129.2, 26.3, 19.6, 14.8, 13.4. HPLC purity analysis: 99.9% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{14}Cl_2N_3S$ $[M+H]^+$: 302.0286; found: 302.0271.

4-(2,4-dichlorophenyl)-3-(3-methoxypropyl)-5(methylthio)-4H-1,2,4-triazole (1g). 3-(4-(2,4-Dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (60 mg, 0.19 mmol) was added to a 25 mL round bottom flask, and 5 mL of anhydrous tetrahydrofuran was added, followed by addition of sodium hydride (60% dispersion in mineral oil, 15 mg, 0.38 mmol) at room temperature, then methyl iodide (19 μL, 0.30 mmol) was added, the mixture was stirred overnight. Then add 10 mL of water and dichloromethane (10 mL×3), the organic phase is combined, washed with saturated brine, dried over anhydrous sodium sulfate, and then filtered over silica gel 4-Dichlorophenyl)-3-(3-methoxypropyl)-5-(methylthio)-4H-1,2,4-triazole as a white solid (58 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, J = 2.3 Hz, 1H), 7.42 (dd, J = 8.4, 2.3 Hz, 1H), 7.22 (d, J = 8.3 Hz, 1H), 3.38 (t, J = 6.1 Hz, 2H), 3.22 (s, 3H), 2.63 (s, 3H), 2.52 (tt, J = 15.6, 7.6 Hz, 2H), 1.94 (p, J = 6.7 Hz, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 155.8, 152.7, 137.4, 133.8, 131.0, 130.6, 129.8, 128.8, 71.3, 58.5, 26.7, 22.0, 15.0. HPLC purity analysis: 98.3% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{15}Cl_2N_3OS$[M+H]$^+$: 332.0391; found: 322.0387.

3-(4-(2,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propyl acetate (1h). 3-(4-(2,4-Dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (60 mg, 0.19 mmol) into a 25 mL round bottom flask, 5 mL of anhydrous dichloromethane, acetic anhydride (19 mg, 0.19 mmol) and 4-dimethylaminopyridine (4.61 mg, 0.038 mmol) were added and allowed to react at room temperature overnight. Then added 10 mL of water and dichloromethane (10 mL×3), the organic phase was combined, washed with saturated brine, dried over anhydrous sodium sulfate and then filtered over silica gel to abtain (2,4-Dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propyl acetate as a white solid (58mg, yield: 88%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, J = 2.2 Hz, 1H), 7.48 (dd, J = 8.4, 2.2 Hz, 1H), 7.28 (d, J = 1.9 Hz, 1H), 4.10 (t, J = 6.1 Hz, 2H), 2.68 (s, 3H), 2.59 (m, 2H), 2.10 – 2.02 (m, 2H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.9, 155.0, 152.3, 137.4, 133.7, 131.0, 130.5, 129.5, 128.8, 63.1, 25.8, 21.8, 20.9, 14.9. HPLC purity analysis: 99.7% (229 nm). HRMS (ESI) calcd. for $C_{14}H_{15}Cl_2N_3O_2S$ $[M+H]^+$: 360.0340; found: 360.0327.

3-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (2a). 2,4-dichloroaniline (1.52 g, 9.39 mmol) was added into a 25 mL bottle, 10 mL anhydrous chloroform and sodium carbonate (1.27 g, 11.98 mmol) were added and stirred in an ice bath. Sulfur phosgene (660 μL, 8.66 mmol) was slowly added and stirred for 2 h in an ice bath. When the reactant was completely transformed, 2M sodium hydroxide aqueous solution was slowly added to quench the reaction, and then dichloromethane and saturated carbonic acid were added. The organic phase was extracted with saturated aqueous sodium chloride, dried over anhydrous sodium sulfate and evaporated. The residue obtained was purified via flash SiO_2 chromatography (10 g silica gel, gradient of hexanes to 5% ethyl acetate/95% hexanes) to give isothiocyanobenzene was an oily pale yellow liquid (1.91 g, 99%). ^{1}H NMR (400 MHz, chloroform-d) δ 7.39 (d, J = 2.2 Hz, 1H), 7.20 (dd, J = 8.6, 2.2 Hz, 1H), 7.14 (d, J = 8.6 Hz, 1H). Next 2,4-dichloro-1-isothiocyanobenzene (251 mg, 1.23 mmol) and 4-hydroxybutanehydrazide (160 mg, 1.35 mmol) were added to 8 mL of anhydrous ethanol and refluxed overnight. After the reaction was completely converted, the ethanol solution was spun dry, and then 8 mL 2M aqueous sodium hydroxide solution was added and refluxed overnight. After the reaction was completely converted, 2M aqueous HCl solution was slowly added until the solution became acidic, and a large amount of white solid was appeared. After suction filtration, washed with water and then dried to give 3-(4-(2,4-dichlorophenyl) 5-5-Mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid (110 mg, 76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.80 (s, 1H), 7.98 (d, J = 2.2 Hz, 1H), 7.72 - 7.63 (m, 2H), 3.36 (s, 2H), 2.36 (dd, J = 8.6, 6.8 Hz, 2H), 1.66 (p, J = 6.7 Hz, 2H). HPLC purity analysis: 99.7% (229 nm). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.1, 152.5, 136.1, 133.7, 133.1, 130.8, 130.5, 129.3, 59.9, 40.2, 28.9, 22.4. HRMS (ESI) calculated for $C_{11}H_{11}CI_2N_3OS$ $[M+H]^+$: 304.0078; found: 304.0068.

3-(4-(2,4-dichlorophenyl)-5-(ethylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (2b). 2a (50 mg, 0.16 mmol) was added in 25 mL flask, 5 mL of acetone, ethyl iodide (54 mg, 0.32 mmol) and potassium carbonate (36 mg, 0.26 mmol) were added and stirred at room temperature overnight. Then add 10 mL of distilled water and dichloromethane (10 mL × 3), the organic phase was combined, washed with a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and then passed through a silica gel column, eluting with dichloromethane: methanol 20:1. 3-(4-(2,4-dichlorophenyl)-5-(ethylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol was obtained as a white solid

(37 mg, 68%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 2.1 Hz, 1H), 7.77 – 7.69 (m, 2H), 4.49 (t, J = 5.2 Hz, 1H), 3.38 (q, J = 6.1 Hz, 2H), 3.04 (qd, J = 7.3, 2.8 Hz, 2H), 2.49 – 2.44 (m, 2H), 1.69 (dt, J = 13.4, 6.5 Hz, 2H), 1.26 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.5, 149.6, 136.1, 132.7, 131.8, 130.3, 129.9, 129.2, 59.7, 29.5, 27.1, 21.5, 15.0. HPLC purity analysis: 95.7% (229 nm). HRMS (ESI) HRMS (ESI) calculated for $C_{13}H_{15}CI_2N_3SO[M+H]^+$: 332.0391; found: 332.0389.

3-(4-(2,4-dichlorophenyl)-5-(isopropylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (2c). 2a (80 mg, 0.26 mmol) was added in 25 mL flask, 5 mL of acetone was added, then 2-iodopropane (54 mg, 0.32 mmol) and potassium carbonate (72 mg, 0.52 mmol) were added and the mixture was stirred at room temperature overnight. After this time, 10 mL water was added and the mixture was extracted with dichloromethane (3 x 5 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over magnesium sulfate, filtered and concentrated to dryness. The residue obtained was purified via flash SiO_2 chromatography (10 g silica gel, gradient of hexanes to 50% ethyl acetate/50% hexanes) to give 3-(4-(2,4-dichlorophenyl)-5-(isopropylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as a pale yellow oily liquid (45 mg, 49%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (dd, J = 2.1, 1.1 Hz, 1H), 7.72 – 7.67 (m, 2H), 4.46 (td, J = 5.2, 1.1 Hz, 1H), 3.38 (t, J = 5.7 Hz, 2H), 2.49 – 2.44 (m, 2H), 1.75 – 1.64 (m, 2H), 1.26 (d, J = 6.7 Hz, 6H), 1.14 (d, J = 1.1 Hz, 1H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.4, 148.9, 135.9, 132.6, 131.6, 130.2, 130.1, 129.1, 59.7, 29.4, 23.1, 23.1, 21.4. HPLC purity analysis: 99.2% (229 nm). HRMS (ESI) calcd. for $C_{14}H_{17}Cl_2N_3OS[M+H]^+$: 346.0548; found: 346.0525.

***S*-(4-(2,4-dichlorophenyl)-5-(3-hydroxypropyl)-4H-1,2,4-triazol-3-yl) carbamothioate (2d). 2a** (100 mg , 0.33 mmol), trichloroacetyl chloride (120 mg, 0.66mmol), 5mL acetonitrile and 1mL DMF were added, then stirred for one hour. When the reactants were completely converted, the solvent was removed by rotary evaporation, then 7M ammonia methanol solution(10 mL) was added and reflux for 3h. After the reaction was completed, the reaction was concentrated to dryness. After this time, the residue obtained was purified via flash SiO_2 chromatography (10 g silica gel, gradient of hexanes to 25% ethyl acetate/70% hexanes) to give *S*-(4-(2,4-dichlorophenyl)-5-(3-hydroxypropyl)-4H-1,2,4-triazol-3-yl) carbamothioate as white solid (77mg, 67%).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 2.2 Hz, 1H), 7.71-7.62 (m, 2H), 4.51 (t, J = 5.1 Hz, 1H), 2.51 (m, 2H) , 2.36 (dd, J = 8.5, 6.8 Hz, 2H), 1.65 (dq, J = 8.1, 6.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.1, 152.6, 136.2, 133.7, 133.1, 132.5, 130.8, 130.5, 129.3, 59.9, 28.9, 22.4. HPLC purity analysis: 99.7% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{12}Cl_2N_4O_2S$ $[M+H]^+$: 347.0134; found: 347.0541.

2-((4-(2,4-dichlorophenyl)-5-(3-hydroxypropyl)-4H-1,2,4-triazol-3-yl)thio)acetamide (2e). 2a (80 mg , 0.26 mmol), 2-iodoacetamide (59 mg, 0.66 mmol) and potassium carbonate (138 mg, 1.00 mmol) were added in 10 mL acetonitrile, then stirred overnight. After the reaction was completed, the reaction was concentrated to dryness. After this time, the residue obtained was purified via flash SiO_2 chromatography (10 g silica gel, gradient of hexanes to 10% methanol in dichloromethane) to give 2-((4-(2,4-dichlorophenyl)-5-(3-hydroxypropyl)-4H-1,2,4-triazol-3-yl)thio)acetamide (110mg, yield: 90%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 (s, 1H), 7.71 (d, J = 1.8 Hz, 2H), 7.63 (s, 1H), 7.22 (s, 1H), 4.48 (td, J = 5.1, 1.7 Hz, 1H), 3.90 – 3.78 (m, 2H), 3.38 (s, 2H), 2.46 (d, J = 8.4 Hz, 2H), 1.75 – 1.62 (m, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.5, 155.6, 149.6, 136.2, 132.7, 131.7, 130.4, 129.5, 129.2, 59.7, 36.5, 29.5, 21.3. HPLC purity analysis: 96.2% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{14}Cl_2N_4O_2S$ $[M+H]^+$: 361.0293; found: 361.0275.

3-(4-(4-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3a). A solution of 4-chloro-1-isothiocyanobenzene (300 mg, 1.77 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (219 mg, 1.86 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) (9 mL)was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2,4- dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (350 mg, 73%) as a white solid without further purification. Next a solution of 3-(4-(2,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (150 mg, 0.56 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (38 µL, 0.61 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(4-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (87 mg, 55%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.68 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H), 4.47 (t, J = 5.2 Hz, 1H), 3.37 (q, J = 6.1 Hz, 2H), 2.54 (m, 2H), 2.54 (s, 3H), 1.74 – 1.64 (m, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 156.0, 151.0, 135.1, 132.5, 130.5, 129.8, 60.2, 29.9, 21. 9, 15.1. HPLC purity analysis: 95.4% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{14}ClN_3OS$ $[M+H]^+$: 284.0624 ; found: 284.0612.

3-(4-(4-methoxyphenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3b). A solution of 1-isothiocyanato-4-methoxybenzene (227 mg, 1.37 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (186 mg, 1.00 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(4-methoxyphenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid without further purification (150 mg, 38%).^{1}H NMR (400 MHz, DMSO-d_6) δ 13.63 (s, 1H), 7.35 – 7.28 (m, 2H), 7.12 – 7.06 (m, 2H), 3.83 (s, 3H), 3.35 (t, J = 6.2 Hz, 2H), 2.42 (t, J = 7.6 Hz, 2H), 1.65 (dt, J = 8.3, 6.5 Hz, 2H). Next, a solution of 3-(5-mercapto-4-(4-methoxyphenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.39 mmol) in acetone (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (64 µL, 1.02 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The

combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(4-methoxyphenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (87 mg, yiled: 45%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.36 (d, J = 8.8 Hz, 2H), 7.12 (d, J = 8.8 Hz, 2H), 4.49 (t, J = 5.1 Hz, 1H), 3.84 (s, 3H), 2.54 (m, 4H), 1.68 (d, J = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.4, 156.3, 151.5, 129.1, 126.0, 115.5, 60.2, 56.0, 30.0, 21.9, 14.9. HPLC purity analysis: 97.5% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{17}N_3O_2S$ $[M+H]^+$: 280.1120, $[M+Na]^+$:302.0930; found: $[M+H]^+$:280.1107, $[M+Na]^+$: 302.0924.

3-(5-(methylthio)-4-(4-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3c). A solution of 1-isothiocyanato-4-(trifluoromethyl)benzene (305 mg, 1.00 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (186 mg, 1.00 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(4-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as orange solid without further purification (128 mg, 40%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.81 (s, 1H), 7.98 (d, J = 8.2 Hz, 2H), 7.73 (d, J = 8.2 Hz, 2H), 3.35 (d, J = 6.1 Hz, 2H), 2.47 (t, J = 7.6 Hz, 2H), 1.66 (p, J = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.9, 152.5, 137.9, 130.0, 127.0, 125.6, 122.9, 59.9, 28.8, 22.6. Finally, a solution of 3-(5-mercapto-4-(4-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (120 mg, 0.38 mmol) in acetone (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (35 μL, 0.56 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(5-(methylthio)-4-(4-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid (90 mg, 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (d, J = 8.3 Hz, 2H), 7.70 (d, J = 8.3 Hz, 2H), 4.42 (t, J = 5.2 Hz, 1H), 3.32 (m, 2H), 2.48 (m, 2H), 2.46 (s, 3H), 1.66 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 150.8, 137.2, 130.6 (J = 32.32), 128.9, 127.6, 124.2 (J = 273.7 Hz), 60.1, 29.9, 21.9, 15.2. HPLC purity analysis: 97.4% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{14}F_3N_3OS$ $[M+H]^+$:318.0888; found: 318.0866.

3-(4-(2-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3d). A solution of 2-chloro-1-isothiocyanobenzene (200 mg, 1.18 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (139 mg, 1.18 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (140 mg, 39%) as a pale yellow solid without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.79 (s, 1H), 7.74 (d, J = 7.7 Hz, 1H), 7.59 (dt, J = 15.2, 4.5 Hz, 3H), 3.35 (t, J = 6.2 Hz, 2H), 2.34 (t, J = 7.7 Hz, 2H), 1.65 (p, J = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.2, 152. 5, 132.8, 132.0, 131.1, 131.0, 129.8, 128.5, 61.8, 29.3, 22.4. Next, A solution of 3-(4-(2-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (80 mg, 0.30 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (28 μL, 0.45 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(2-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (52 mg, 61%) as brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (dd, J = 8.0, 1.5 Hz, 1H), 7.52 (td, J = 7.8, 1.7 Hz, 1H), 7.45 (td, J = 7.6, 1.5 Hz, 1H), 7.30 (dd, J = 7.7, 1.7 Hz, 1H), 3.69 (dq, J = 8.2, 5.1 Hz, 2H), 2.86 (s, 1H), 2.64 – 2.43 (m, 2H), 1.92 (qd , J = 6.9, 5.0 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.2, 152. 5, 132.8, 132.0, 131.1, 131.0, 129.8, 128.5, 61.8, 29.3, 22.4, 15.0. HPLC purity analysis: 98.9% (229 nm). HRMS (ESI) Calculated as $C_{12}H_{14}ClN_3OS$ $[M+H]^+$: 284.0624; found: 284.0601.

3-(4-(3-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3e). A solution of 3-chloro-1-isothiocyanobenzene (339 mg, 2.00 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (248 mg, 2.10 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(3-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (166mg, 31%) as a white solid without further purification. Next, a solution of 3-(4-(2-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.35 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (33 μL, 0.53 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(3-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (56 mg, 56%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 (d, J = 2.0 Hz, 1H), 7.69 – 7.66 (m, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.47 (dt, J = 7.6, 1.6 Hz, 1H), 4.50 (t, J = 5.2 Hz, 1H), 3.39 (d, J = 6.1 Hz, 2H), 2.57 (m, J = 4.8 Hz, 2H), 2.55 (s, 3H), 1.70 (m, J = 8.1, 6.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 151.0, 134.9, 134.4, 132.0, 130.6, 127.9, 126.8, 60.2, 29.9, 21.9, 15.1. HPLC purity analysis: 97.9% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{14}ClN_3OS$ $[M+H]^+$: 284.0624; found: 284.0614.

3-(5-(methylthio)-4-(3-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3f). A solution of 1-isothiocyanato-3-(trifluoromethyl)benzene (305 mg, 1.00 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (186 mg, 1.00 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(3-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid without further purification (260 mg, 82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.78 (s, 1H), 7.94 (d, J = 11.8 Hz, 2H), 7.87 – 7.76 (m, 2H), 2.46 (t, J = 7.6 Hz, 2H), 1.66 (p, J = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.0, 152.6, 135.1, 133.3, 131.2, 130.7, 130.4, 126.8, 126.1, 59.9, 28.7, 22.6. Then a solution of 3-(5-mercapto-4-(3-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (120 mg, 0.38 mmol) in acetone(8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (35 µL, 0.56 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 15:1) to give 3-(5-(methylthio)-4-(3-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as a colorless liquid (92 mg, 77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 – 7.96 (m, 2H), 7.89 – 7.80 (m, 2H), 4.49 (t, J = 5.2 Hz, 1H), 2.55 (m, 4H), 1.75 – 1.66 (m, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 156.0, 151.0, 134.5, 132.3, 131.8, 131.0 (J = 32.3 Hz), 127.3, 123.9 (J = 273.7 Hz), 125.1, 60.1, 29.8, 21.9, 15.2. HPLC purity analysis: 99.9% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{14}F_3N_3OS$ $[M+H]^+$: 318.0888, $[M+Na]^+$: 340.0707; found: $[M+H]^+$:318.0869, $[M+Na]^+$: 340.0687.

3-(5-(methylthio)-4-(m-tolyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3g). A solution of 1-isothiocyanato-3-methylbenzene (298 mg, 2.00 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (284 mg, 2.10 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(*m*-tolyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid without further purification (260 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.65 (s, 1H), 7.46 (t, J = 7.7 Hz, 1H), 7.35 (d, J = 7.7 Hz, 1H), 7.25 – 7.12 (m, 2H), 4.47 (t, J = 5.2 Hz, 1H), 3.36 (t, J = 5.8 Hz, 2H), 2.43 (t, J = 7.6 Hz, 2H), 2.38 (s, 3H), 1.65 (p, J = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.1, 152.7, 139.5, 134.2, 130.6, 129.7, 129.1, 125.8, 60.0, 28.9, 22.6, 21.2. Next, a solution of 3-(5-mercapto-4-(*m*-tolyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (150 mg, 0.60 mmol) in acetone (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (56 µL, 0.90 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 15:1) to give 3-(5-(methylthio)-4-(*m*-tolyl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid (231 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (t, J = 8.1 Hz, 1H), 7.33 (d, J = 7.8 Hz, 1H), 7.11 – 6.97 (m, 2H), 3.70 (q, J = 5.4 Hz, 2H), 3.27 (t, J = 5.4 Hz, 1H), 2.69 (t, J = 7.0 Hz, 2H), 2.64 (s, 3H), 2.43 (s, 3H), 1.98 – 1.87 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.1, 152.4, 140.4, 133.1, 130.9, 129.8, 127.5, 124.0, 61.8, 29.4, 22.6, 21.3, 14.6. HPLC purity analysis: 97.8% (229 nm). HRMS (ESI) calcd. for $C_{13}H_{17}N_3OS$ $[M+H]^+$: 264.1171; found: 264.1173.

3-(4-(3,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3h). A solution of 3,4-dichloro-1-isothiocyanobenzene (306 mg, 1.50 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (186 mg, 1.57 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(3,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (166mg, 31%) as a white solid without further purification. Next, a solution of 3-(4-(3,4-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.33 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (33 µL, 0.53 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(3,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (55 mg, 52%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, J = 8.5 Hz, 1H), 7.41 (d, J = 2.4 Hz, 1H), 7.14 (dd, J = 8.5, 2.4 Hz, 1H), 3.72 (t, J = 5.7 Hz, 2H), 2.92 – 2.74 (m, 1H), 2.70 (d, J = 7.0 Hz, 1H), 2.67 (s, 3H), 2.00 – 1.92 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.7, 152.2, 134.9, 134.3, 132.4, 131.8, 129.1, 126.5, 61.6, 29.2, 22.4, 14.7. HPLC purity analysis: 97.6% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{13}Cl_2N_3OS$ $[M+H]^+$: 318.0235; found:318.0222.

3-(4-(2,3-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3i). A solution of 2,3-dichloro-1-isothiocyanobenzene (314 mg, 1.54 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (190 mg, 1.61 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2,3-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (400mg, 80%) as a white solid without further purification. Next, a solution of 3-(4-(2,3-dichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.33 mmol) in acetonitrile (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (31 µL, 0.49 mmol) and the mixture was allowed to reacted at

room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(3,4-dichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (64 mg, 61%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (dd, J = 8.0, 1.5 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.24 (d, J = 1.5 Hz, 1H), 3.71 (dq, J = 10.1, 5.3 Hz, 2H), 2.94 (s, 1H), 2.66 (s, 3H), 2.64 – 2.52 (m, 2H), 1.95 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.8, 152.1, 147.2, 135.1, 132.6, 131.9, 128.2, 127.9, 61.7, 29.1, 22.3, 14.9. HPLC purity analysis: 99.6% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{13}Cl_2N_3OS$ $[M+H]^+$: 318.0235; found: 318.0222.

3-(5-(methylthio)-4-(2,4,6-trichlorophenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3j). A solution of 2,4,6-trichloro-1-isothiocyanobenzene (314 mg, 1.32 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (248 mg, 2.10 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2,4,6-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (330mg, 49%) as a white solid without further purification. Next, a solution of 3-(4-(2,4,6-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (186 mg, 0.82 mmol) in acetonitrile (8 mL) was added potassium carbonate (276 mg, 2.00 mmol) and methyl iodide (51 μL, 0.82 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(2,4,6-trichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid (87 mg, 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12 (d, J = 11.4 Hz, 2H), 4.48 (p, J = 5.1 Hz, 1H), 3.41 (m, 2H), 2.59 (s, 3H), 2.45 (m, 2H), 1.72 (qd, J = 7.9, 3.4 Hz, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.6, 150.9, 137.3, 134.9, 130.6, 128.0, 60.1, 30.0, 21.5, 15.3. HPLC purity analysis: 99.9% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{12}Cl_3N_3OS$ $[M+H]^+$: 351.9845; found: 351.9830.

3-(5-(methylthio)-4-(2,3,4-trichlorophenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3k). A solution of 2,3,4-trichloro-1-isothiocyanobenzene (358 mg, 1.50 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (186 mg, 1.58 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(2,3,4-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (170mg, 33%) as a white solid without further purification. Next, a solution of 3-(4-(2,3,4-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (107 mg, 0.32 mmol) in acetone (8 mL) was added potassium carbonate (276 mg, 2.00 mmol) and methyl iodide (30 μL, 0.48 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-(2,3,4-trichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid (35 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (d, J = 8.6 Hz, 1H), 7.80 (d, J = 8.6 Hz, 1H), 4.48 (t, J = 5.2 Hz, 1H), 3.41 – 3.37 (m, 2H), 2.55 (s, 3H), 2.47 (t, J = 7.8 Hz, 2H), 1.77 – 1.65 (m, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 151.0, 135.8, 132.8, 132.4, 131.5, 130.6, 130.1, 60.1, 30.0, 21.7, 15.4. HPLC purity analysis: 97.8% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{12}Cl_3N_3OS$ $[M+H]^+$: 351.9845; found: 351.9828.

3-(5-(methylthio)-4-(3,4,5-trichlorophenyl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3l). A solution of 3,4,5-trichloroaniline (786 mg, 4.00 mmol) in anhydrous chloroform (10 mL) was added sodium carbonate (1.27 g, 11.98 mmol) and pre-cooled in an ice bath. Then thiophosgene (335.5 μL, 5.37 mmol) was slowly added and the solution was allowed to stir in an ice bath for 2 h. After this time, 2M sodium hydroxide solution (10 mL) was added to quench the reaction and the mixture was extracted with dichloromethane (3 x 10 mL) . The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The residue obtained was purified via flash SiO_2 chromatography (eluted with petroleum ether/ethyl acetate 20:1) to give 3,4,5-trichloro-1-isothiocyanatobenzene as a white solid (400 mg, 42%).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (s, 2H). Then a solution of 3,4,5-trichloro-1-isothiocyanobenzene (350 mg, 1.47 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (209 mg, 1.76 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(3,4,5-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid without further purification (198 mg, 39%).^{1}H NMR (400 MHz, DMSO-d_6) δ 13.81 (s, 1H), 7.94 (d, J = 1.7 Hz, 2H), 4.52 (dt, J = 5.3, 2.5 Hz, 1H), 3.41 (s, 2H), 2.50 – 2.45 (m, 2H), 1.68 (dq, J = 8.1, 6.3 Hz, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.0, 152.5, 134.1, 133.9, 132.0, 130.2, 60.0, 28.7, 22.5. Then a solution of 3-(4-(3,4,5-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (190 mg, 0.56 mmol) in acetone (8 mL) was added potassium carbonate (258 mg, 1.86 mmol) and methyl iodide (38 μL, 0.61 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 30:1) to give 3-(4-(3,4,5-trichlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid (120 mg, 61%). ^{1}H NMR (400 MHz,

DMSO-d_6) δ 8.03 (s, 2H), 4.49 (t, J = 5.2 Hz, 1H), 3.42 – 3.37 (m, 2H), 2.63 – 2.56 (m, 2H), 2.55 (s, 3H), 1.73 (p, J = 6.6 Hz, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 150. 9, 134.4, 133.5, 132.4, 129.1, 60.2, 29.8, 21.8, 15.4. HPLC purity analysis: 97.2% (229 nm). HRMS (ESI) calcd. for $C_{12}H_{12}Cl_3N_3OS[M+H]^+$: 351.9845; found: 351.9825.

3-(5-(methylthio)-4-(3,4,5-trimethylphenyl)-4*H*-1,2,4-triazol-3-yl)propan-1-ol (3m, TH1027). A solution of 3,4,5-trimethyaniline (541 mg, 4.00 mmol) in anhydrous chloroform (10 mL) was added sodium carbonate (1.27 g, 11.98 mmol) and pre-cooled in an ice bath. Then thiophosgene (335.5 μL, 5.37mmol) was slowly added and the solution was allowed to stir in an ice bath for 2 h. After this time, 2M sodium hydroxide solution (10 mL) was added to quench the reaction and the mixture was extracted with dichloromethane (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The residue obtained was purified via flash SiO_2 chromatography (eluted with petroleum ether/ethyl acetate 20:1) to give 3,4,5-trimethy-1-isothiocyanatobenzene as a white solid (443mg, 63%).^{1}H NMR (400 MHz, chloroform-*d*) δ 6.85 (s, 2H), 2.23 (s, 6H), 2.13 (s, 3H). Then a solution of 3,4,5-trimethy-1-isothiocyanobenzene (430 mg, 2.43 mmol) in anhydrous ethanol (8 mL) was added 4-hydroxybutanehydrazide (295mg, 2.50 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-(3,4,5-trichlorophenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid without further purification (500 mg, 45%).^{1}H NMR (400 MHz, DMSO-d_6) δ 13.60 (s, 1H), 7.00 (s, 2H), 3.35 (t, J = 6.2 Hz, 2H), 2.43 – 2.35 (m, 2H), 2.29 (s, 6H), 2.18 (s, 3H), 1.66 (dq, J = 8.0, 6.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.8, 152.5, 137.5, 136.6, 130.8, 126.9, 59.7, 28.6, 22.4, 20.3, 15.2. Then a solution of 3-(4-(3,4,5- trimethyphenyl)-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (300 mg, 1.32 mmol) in acetone (8 mL) was added potassium carbonate (300 mg, 1.32 mmol) and methyl iodide (93 μL, 1.49 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 30:1) to give 3-(4-(3,4,5-trimethyphenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as a white solid (200 mg, 64%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (s, 2H), 4.49 (t, J = 5.2 Hz, 1H), 3.42–3.37 (m, 2H), 2.63–2.56 (m, 2H), 2.55 (s, 3H), 2.27(s, 6H), 2.16(s, 3H), 1.73 (p, J = 6.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.8, 151.0, 138.2, 137.2, 130.2, 125.8, 60.0, 29.8, 21.7, 15.3, 14.6. HPLC purity analysis: 98.5% (229 nm). HRMS (ESI) calcd. for $C_{15}H_{21}N_3OS[M+H]^+$: 292.1484; found: 292.1480.

3-(4-(4-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3n). A solution of 3-isothiocyanatopyridine (300 mg, 2.20 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (260 mg, 2.20 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq)(8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid without further purification (323 mg, 62%). Then a solution of 3-(5-mercapto-4-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.42 mmol) in acetone (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (40 μL, 0.64 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3x10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 15:1) to give 3-(4-(4-chlorophenyl)-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid (231 mg, 68%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (d, J = 4.8 Hz, 1H), 8.66 (d, J = 2.2 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.62 (dd, J = 8.0, 4.8 Hz, 1H), 4.42 (t, J = 5.1 Hz, 1H), 3.33 (q, J = 6.0 Hz, 2H), 2.53 (m, 2H), 2.50 (s, 3H), 1.66 (p, J = 6.5 Hz, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ 156.2, 151.4, 151.2, 148.5, 135.9, 130.7, 125.1, 60.1, 29.9, 21.9, 15.3. HPLC purity analysis: 96.3% (229 nm). HRMS (ESI) calcd. for $C_{11}H_{14}N_4OS[M+H]^+$: 251.0967; found: 251.0968.

3-(4-cyclohexyl-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol (3o). A solution of isothiocyanatocyclohexane (283 mg, 2.00 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (248 mg, 2.10 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(4-cyclohexyl-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid without further purification (230 mg , 48%). Then a solution of 3-(4-cyclohexyl-5-mercapto-4H-1,2,4-triazol-3-yl)propan-1-ol (100 mg, 0.41 mmol) in acetone (8 mL) was added potassium carbonate (138 mg, 1.00 mmol) and methyl iodide (39 μL, 0.62 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(4-cyclohexyl-5-(methylthio)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid (40 mg, 38%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.60 (t, J = 5.2 Hz, 1H), 4.02 (ddt, J = 12.4, 8.5, 3.8 Hz, 1H), 3.48 (q, J = 5.9 Hz, 2H), 2.78 (t, J = 7.6 Hz, 2H), 2.51 (s, 3H), 2.01 (qd, J = 12.4, 3.4 Hz, 2H), 1.79 (m, J = 14.4, 9.3, 3.8 Hz, 6H), 1.67 (d, J = 13.2 Hz, 1H), 1.39 (qt, J = 12.9, 3.4 Hz, 2H), 1.18 (ttd, J = 12.9, 9.6, 4.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.8, 149.1, 60.3, 55.5, 31.1, 30.8, 25.8, 25.1, 22.2, 15.9. Purity＞95% (by ^{1}H-NMR). HRMS (ESI) calcd. for $C_{12}H_{21}N_3OS$ $[M+H]^+$: 256.1484, $[M+Na]^+$: 278.1303; found $[M+H]^+$: 256.1471, $[M+Na]^+$: 278.1289.

3-(5-(methylthio)-4-(naphthalen-1-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol (3p). A solution of 1-isothiocyanatonaphthalene (370 mg, 2.00 mmol) in anhydrous ethanol (6 mL) was added 4-hydroxybutanehydrazide (248 mg, 2.10 mmol) and reflux overnight. After this time, the ethanol was evaporated, then 2 M NaOH (aq) (8 mL) was added and refluxed overnight. Then 2 M HCl (aq) was slowly added until a large amount of white solid was precipitated. After filtration, the precipitation was washed with water and then dried to obtain 3-(5-mercapto-4-(naphthalen-1-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid without further purification (352 mg, 62%). Then a solution of 3-(5-mercapto-4-(naphthalen-1-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol (180 mg, 0.60 mmol) in acetone (8 mL) was added potassium carbonate (276 mg, 2.00 mmol) and methyl iodide (55 µL, 0.87 mmol) and the mixture was allowed to reacted at room temperature overnight. After this time, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with saturated aqueous sodium chloride (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated to dryness. The resulting residue was purified via flash SiO_2 chromatography (eluted with dichloromethane/methanol 20:1) to give 3-(5-(methylthio)-4-(naphthalen-1-yl)-4H-1,2,4-triazol-3-yl)propan-1-ol as white solid (97 mg, 54%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 (ddd, J = 28.4, 8.1, 3.6 Hz, 2H), 7.75 – 7.57 (m, 4H), 7.11 (dd, J = 8.6, 3.1 Hz, 1H), 4.41 (q, J = 4.7 Hz, 1H), 3.36 (d, J = 3.5 Hz, 2H), 2.52 (s, 3H), 2.48 – 2.29 (m, 2H), 1.65 (ddt, J = 15.0, 7.7, 3.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.8, 152.1, 134.3, 131.2, 129.5, 129.2, 128.8, 127.7, 127.1, 126.3, 121.7, 60.1, 30.1, 21.9, 15.0. HPLC purity analysis: 98.6% (229 nm). HRMS (ESI) calcd. for $C_{16}H_{17}N_3OS$ $[M+H]^+$: 300.1171; found: 300.1155.

Cell culture

HEK-Blue TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 cells were purchased from InvivoGen and cultured in complete culture medium: Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 mg/mL normocin, and 2 mM L-glutamine. Human monocyte cell lines were purchased from ATCC and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin and 0.05 mM 2-mercaptoethanol. All cultured cells were grown at 37 °C in a humidified incubator containing 5% CO_2. The cultures were checked periodically and found to be free of mycoplasma contamination.

SEAP reporter assay

HEK-Blue TLR8 cells were plated at the density of 4 × 10^4 cells per well in a 96-well plate in DMEM and treated with 1µg/mL R848 (Invivogen) and varying concentrations of appropriate compounds. After incubation at 37 °C for 24 h, 50 µL of supernatant was collected and placed in a new 96-well plate and then 50 µL of Quanti-Blue (Invivogen) was added to the media, and incubated at 37 °C until color change was observed (within 1 h). The plate were then measuring absorbance at 620 nm. Data was normalized as readout of ligand-treated cells are 100% activation and untreated cells are 0% activation.

Cell viability assay

HEK-Blue TLR8 cells (4 × 10^4 cells/well) or human PBMCs (3 × 10^6 cells/mL) were placed in a 96-well plate and co-incubated with indicated compounds at 37℃ for 24 h. Next Cell Counting Kit-8 (Bimake) was added to each well (1:10 dilution). Then cells were incubated at 37 °C until a color change was observed (within 2 h) and absorbance was read at 450 nm. Data was normalized with the untreated cells control as 100% survival.

TLR Selectivity assay

The selectivity of TH1027 against the TLR family was examined in HEK-Blue cells overexpressing TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9. The assay was performed in the same manner as "SEAP reporter assay", using their selective ligand: 100 ng/mL of Pam3CSK4 (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine3HCl), 5 µg/mL of polyriboinosinic : polyribocytidylic acid (poly(I:C)), 20 ng/mL of LPS (lipopolysaccharide), 50 ng/mL of Flagellin, 100 ng/mL of Pam2CSK4 (S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S] lysine3CF_3COOH), 0.5 µg/mL of R848, 1 µg/mL of R848 and 0.5 µM ODN2006.

RT-qPCR analysis

HEK-Blue TLR8 cells were seeded at a density of 1 × 10^6 cells per well in a 12-well plate and the plate was incubated at 37°C in 5% CO_2. After incubation for 24 h, the medium was replaced by serum-free medium, and then the cells were left untreated, treated with R848 (4 µg/mL), R848 (4 µg/mL) with TH1027 (1 µM) or compound 2a (1 µM), respectively. After incubation for 8 h, cells were scraped and re-suspended in PBS and then centrifuged at 2000 rpm, 5 min to remove PBS. Total RNA was extracted with the TRIZOL reagent (Invitrogen, Lot number: 15596018) using standard protocols. Reverse transcription was performed using iScriptTM cDNA Synthesis Kit (Bio-Rad, Lot number: 1708890) according to manufacturer's instructions using an Applied Biosystems. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Lot number: 1725124) following the manufacturer's protocol. TNF-α, IL-8 and GAPDH primers were obtained from Ruibiotech company. Data was analyzed using the ΔΔCt method using GAPDH gene as a housekeeping gene and normalized to cell control.

Protein expression, purification and crystallization

The extracellular domain of human Toll-like receptor 8 (hTLR8, residues 27–827) was prepared as described previously[33] and was concentrated to 8.2 mg/mL in 10 mM Tris-HCl pH 8.0 and 150 mM NaCl. The protein solutions for the co-crystallization of hTLR8 and TH1027 contained hTLR8 (7.0 mg/mL) and a ten-fold excess of TH1027 in a crystallization buffer containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 5% dimethyl sulfoxide (DMSO). Crystallization experiments were performed with sitting-drop vapor-diffusion methods at 293 K. Crystals of hTLR8/TH1027 were obtained with reservoir solutions containing 12-20% PEG 4000, 0.2 M calcium chloride, 0.1 M Tris-HCl pH 8.0, and 25% ethylene glycol.

Data collection and structure determination

Diffraction dataset for hTLR8/TH1027 crystals was collected on beamline PF-AR NE12A (Ibaraki, Japan) under cryogenic condition at 100 K. The wavelength was set to 1.0000 Å. The dataset was processed with the HKL2000 package[34]. hTLR8/TH1027 structure was determined by the molecular replacement method using the Molrep program[35] with the hTLR8/CUCUT8m structure (PDB ID: 5WYX) as a search model. The model was further refined with stepwise cycles of manual model building with the COOT program[36] and restrained refinement with REFMAC[37] until the R factor was converged. TH1027 molecules, N-glycans were modeled into the electron density map at the latter cycles of the refinement. The quality of the final structure was validated with the PDB validation server (http://wwpdb-validation.wwpdb.org/). The favored and the allowed regions in the Ramachandran plot contain 93% and 6% residues for TLR8/TH1027, respectively. The statistics of the data collection and refinement are summarized in Supplementary Table 1. The figures representing structures were prepared with PyMOL (http://www.pymol.org). Coordinate and structure factor of TLR8/TH1027 have been deposited in the Protein Data Bank with PDB ID: 6KYA.

Isothermal Titration Calorimetry (ITC) assay

ITC experiments were done in a buffer composed of 25 mM MES pH 5.5, 0.20 M NaCl, and 5% DMSO at 298 K using a MicroCal iTC200 (GE Healthcare). The titration sequence included a single 0.4 μL injection followed by 18 injections, 2 μL each, with a spacing of 120 seconds between the injections. The titration conditions were as follows: 100 μM TH1027 into 10 μM hTLR8; 100 μM R848 into 10 μM hTLR8; 100 μM R848 into 10 μM hTLR8 in the presence of 50 μM TH1027. OriginLab software (GE Healthcare) was used to analyze the raw ITC data.

Western blot

Human monocyte cell lines were added in 6-well plates with a density of $2x10^6$ cells/well in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol. Then cells were treated with phorbol-12-myristate-13-acetate (PMA) (100 ng/mL) and incubated in humidified incubators containing 5% CO_2 at 37 C for 24 h. After differentiation, the supernatant was removed and cells were washed three times by PBS and then replaced with unsupplemented RPMI, these cells were then treated with R848 (1 μg/mL) along with various concentrations of TH1027. After incubation for 2 h, cells were collected, nuclear protein fraction was extracted using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Massachusetts, USA). BCA assay was then used to determine protein concentration. Protein samples were loaded and run in 10% Tris-glycine SDS-PAGE, and then transferred onto a nitro-cellulose membrane (Bio-Rad, California, USA) using electroblotting. P65 (CST, 1:1000), TRAF3 (CST, 1:1000) and p-IRAK4 (CST, 1:1000) was then used as primary antibody, and peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) antibody (CST, 1:10000) as secondary antibody, then blots were visualized using Thermo SuperSignal West Pico Plus kit (Thermo Fisher Scientific, Massachusetts, USA). Lamin A/C (transgen, 1:1000) was used as internal controls for nuclear fractions and β-actin (CST, 1:1000) was used as internal controls for cytoplasmic fractions.

Enzyme-Linked Immunosorbent Assay (ELISA) in human monocyte cell lines

Human monocyte cell lines were seeded at 1×10^6 per well in 1 mL RPMI medium supplemented with (10% (v/v) FBS, 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.05 mM 2-mercaptoethanol in a 12-well plate and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and cells were washed three times using PBS buffer. Then 1 mL of RPMI medium was added to each well and cells were treated with R848 (1 μg/mL) and various concentrations of compounds TH1027 or left untreated. After incubation for 24 h, supernatants of the culture medium were collected, and the levels of TNF-α, IL-6 and IL-1β were determined using BD OptEIATM human TNF-ELISA kit (BD Biosciences) , BD OptEIATM human IL-6-ELISA kit (BD Biosciences) and BD OptEIATM human IL-1β-ELISA kit (BD Biosciences) respectively, according to the manufacturer's instructions.

ELISA in human PBMCs from healthy donors and rheumatoid arthritis patients

Human whole blood was collected by venipuncture from healthy human volunteers and rheumatoid arthritis patients. All experiments performed on human PBMCs have been described and approved by the IRB of PUMCH (No. S-478) and are consistent with Institutional Guidelines. Diagnosis of rheumatoid arthritis (RA) was confirmed by senior consultant rheumatologists according to 2010 American College of Rheumatology (ACR) criteria for RA. Human PBMCs from healthy donors and six RA patients were isolated using Density Gradient Centrifugation. Immediately after separation, cells were cultured at a density of $3 \times l0^6$ cells/mL in 0.2 mL of RPMI 1640 in 96-well round-bottom plates (Thermo Scientific). Cells were then treated with indicated TH1027 or 2a as a negative control. After incubating for 24 h, the supernatants were collected after centrifuged for 10 min at 4,000 r.p.m. at 4 °C and frozen at -80 °C until ready for TNF-α measurement. The levels of TNF-α were determined using BD OptEIATM human TNF-ELISA kit (BD Biosciences) according to the manufacturer's instructions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figure S1. Crystal structure of inhibition and activation state of TLR8 protein

Figure S2. The activity, toxicity and structure of three compounds screened from a 14,400-membered commercial library

Figure S3. Toxicity of **TH1027** in HEK-Blue hTLR8 cells

Figure S4. ITC experiments

Figure S5. Close-up view of each antagonist binding site

Figure S6. Quantitative data analyses of one representative Western Blot experiment

Figure S7. Toxicity of **TH1027** in PBMCs from RA patients

Table S1. Data collection and refinement statistic for hTLR8/**TH1027**

NMR Spectra of compounds

HPLC traces of representative compound(TH1027)

Molecular formula strings (CSV)

Accession Codes

PDB code for TLR8/**TH1027** complex is 6KYA. Authors will release the atomic coordinates upon article publication.

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Author Contributions

H.Y. supervised the overall project. S.J. designed the experiments in consultation with H.Y. S.Z. performed the high throughput screening. S.J., K.Y., and Y.Y. performed chemical synthesis of compounds. S.J. performed SEAP reporter assay, cell viability assay, selectivity assay, RT-qPCR, ELISA, Western Blot. H.T., K.S., U.O., and T.S. expressed protein, solved the crystal structure, performed isothermal titration calorimetry experiments. J.L. contributed to provide the patient specimens. S.J. performed PBMC isolation and patient specimens studies. H.Y. and S.J. wrote the manuscript with input from U.O., K.S., J.H., Y.Y. and K.Y.

Notes

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

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ABBREVIATIONS

TLRs, toll-like receptors; PBMCs, peripheral blood mononuclear cells; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; SEAP, secreted alkaline phosphatase; THF, tetrahydrofuran; DMF, dimethyl formamide; SAR, structure−activity relationship; RT-qPCR, real-time quantitative polymerase chain reaction; ITC, isothermal titration calorimetry; K_d, dissociation constant; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; RMSD, root mean square deviation; p-IRAK4, phosphorylated interleukin-1 receptor-associated kinase 4; TRAF3, tumor necrosis factor-alpha receptor-associated factor 3; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; IL-6, interleukin-6; IL-1β, interleukin-1 beta; RA, rheumatoid arthritis; ssRNA, single stranded-RNA.

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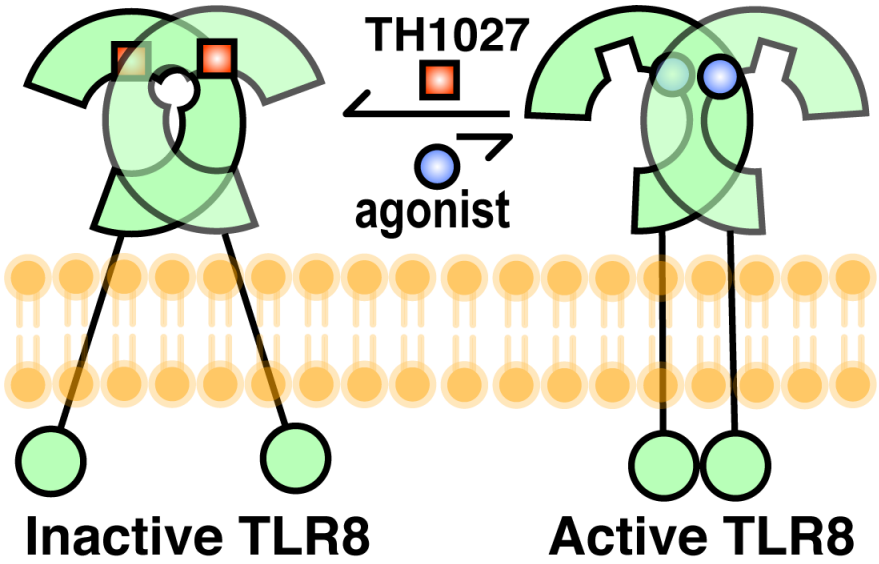

Table of Contents Graphic