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Unbiased Screening of Marine Sponge Extracts for Anti-Inflammatory Agents Combined with Chemical Genomics Identifies Girolline as an Inhibitor of Protein Synthesis

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Abstract

Toll-like receptors (TLRs) play a critical role in innate immunity, but activation of TLR signaling pathways is also associated with many harmful inflammatory diseases. Identification of novel anti-inflammatory molecules targeting TLR signaling pathways is central to the development of new treatment approaches for acute and chronic inflammation. We performed high throughput screening from crude marine sponge extracts on TLR5 signaling and identified girolline. We demonstrated that girolline inhibits signaling through both MyD88-dependent and –independent TLRs (i.e. TLR2, 3, 4, 5 and 7), and reduces cytokine (IL-6 and IL-8) production in human peripheral blood mononuclear cells and macrophages. Using a chemical genomics approach, we identified Elongation factor 2 as the molecular target of girolline, which inhibits protein synthesis at the elongation step. Together these data identify the sponge natural product girolline as a potential anti-inflammatory agent acting through inhibition of protein synthesis.

INTRODUCTION

The human immune system has two components—the innate immune system and the adaptive immune system—that work in tandem to provide resistance to infection. The innate immune response precedes and directs the adaptive immune response. Innate immunity allows the host to differentiate self from pathogen and generates an acute inflammatory response within minutes (1, 2).

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The innate immune system is an ancient host defense system found in many multicellular organisms from plants to humans. Understanding of innate immunity was accelerated in the mid-1990s when the *Drosophila* protein Toll was shown to be critical for defending the flies against infections (3). This opened the way for the description of similar proteins, called Tolllike receptors (TLRs), in mammalian cells. The human TLR family consists of 10 receptors (4). TLRs allow for recognition and response to diverse microbial epitopes—pathogen-associated molecular patterns or PAMPs (5)—enabling the innate immune system to discriminate among groups of pathogens and to induce an appropriate cascade of effector responses. Individual TLRs recognize a distinct, but limited, repertoire of conserved microbial products. For example, TLR5 recognizes the flagellin protein expressed by flagellated bacteria such as *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (6). Collectively, the complete TLR family allows the host to detect infection by most (if not all) types of pathogens.

TLR signaling pathways have been the focus of considerable attention (7, 8). The emerging model is that ligation of microbial products by TLRs culminates in the activation of the nuclear transcription factor NF-κB and others driving the production of proinflammatory cytokines and additional immunological responses. To date, two predominant intracellular TLR pathways have been identified (7, 9, 10): (a) MyD88-dependent pathway uses the adapter molecule MyD88 leading to early activation of NF-κB and production of cytokines, and (b) MyD88-independent pathway signals through TRIF (TIR-domain-containing adapter inducing interferon-beta). TRIF-dependent pathway activates IRF3 and IRF7, together with NF-κB and AP1, to form a multi-protein complex, which induces transcription of the IFN-β gene. With the exception of TLR3 and TLR4, all TLRs that have been characterized to date signal exclusively through the MyD88-dependent pathway. TLR4 is unique in that it activates both the MyD88-dependent and -independent pathways, while TLR3 signals exclusively through the MyD88-independent one.

The innate immune system and TLR signaling play a vital role in protection from infections. However, TLR signaling is a ‘double-edged sword’ and vigorous innate immune responses can be harmful. Indeed, such harmful responses contribute to pathology in sepsis (11), asthma and atopy (12, 13), and cystic fibrosis (CF) (14–19), as well as a variety of autoimmune disorders including Type 1 diabetes, inflammatory bowel disease and systemic lupus erythematosus (20, 21).

TLRs have emerged as attractive targets for drug development (8, 22, 23). An example is the development of TLR4 antagonists and inhibitors to block the nucleic acid sensing TLRs (e.g., TLR7/9) as potential therapies for severe sepsis and autoimmune diseases (22, 24, 25). Similarly, TLR5 has been identified as a novel therapeutic target to prevent chronic lung-damaging inflammation in cystic fibrosis (19, 26, 27). Together, these data suggest that modulating TLR signaling pathways could provide a novel avenue for clinical intervention in many diseases associated with undesired inflammatory responses.

Natural products have proven to be a rich source of bio-active compounds with therapeutic potential, including anti-infective (macrolides, alkaloids and cyclic depsipeptides) (28) and anti-tumor agents (the indoleamine-2,3-dioxygenase (IDO) inhibitor exiguamine A,

niphatenones, sintokamides and rolloamines) (29–32). In this study, we established a high-throughput platform to screen crude marine sponge extracts for potential anti-inflammatory agents that target TLR signaling. The advantage of examining crude biological extracts is that each extract likely contains a wide variety of primary and secondary metabolites, allowing us to dramatically increase the 'chemical space' being sampled. The primary screening focused on the TLR5 signaling pathway given its relative simplicity and engagement of the canonical TLR signaling cascade involving MyD88 to activate downstream transcription factors (7, 9). The positive 'hits' underwent subsequent cycles of assay-guided fractionation until a pure compound—girolline (**1**)—was identified. We undertook further evaluation of girolline as an anti-inflammatory agent by completing comprehensive structure-activity relationship (SAR) studies, as well as defining the mechanism of action, anti-inflammatory activity and toxicity profile of the compound in a variety of cell types.

Chemical-genetic profiling with yeast has proven to be a powerful new system for determining the mode-of-action and molecular targets of bioactive molecules (33). Because chemical perturbations mimic genetic perturbations, enhanced understanding of genetic networks allows the prediction of the cellular targets of bioactive molecules (34, 35). By scoring the sensitivity of 5000 viable deletion mutant yeast strains, together with 1000 strains carrying hypomorphic mutations in essential genes, using a parallel growth assay followed by barcode sequencing, it was possible to determine the mode-of-action of girolline using the unique pattern of drug sensitivity and resistance of the yeast deletion mutants.

We anticipate that this study will serve as a model in the discovery for new anti-inflammatory therapeutics from the natural product chemical space, as well as identifying girolline as a potential anti-inflammatory agent operating via translation inhibition.

RESULTS and DISCUSSION

The goal of this study was to identify potential anti-inflammatory agents that act on TLR signaling from natural marine sponge products. The work combined chemical extraction, screening, synthesis and characterization of bio-active compounds; together with SAR analysis and the application of chemical genomics to elucidate the inhibitory molecular mechanism.

Screening marine sponge extracts to identify TLR5 inhibitors

Marine sponges have been a rich source of novel bio-active natural products, many of which possess important medicinal properties (36). In this study, we screened a library of methanol extracts from marine sponges collected in temperate and tropical habitats for potential anti-inflammatory agents. The screening process is illustrated in Figure 1. Screening was performed on a TLR5 reporter cell line—CHO-K1 cells stably expressing human TLR5 and a NF-κB luciferase reporter (37). This TLR5 reporter cell line responded only to flagellin (with an optimized concentration of 100 ng ml^{-1}) but not to other TLR ligands (Supplementary Figure S1) allowing us to monitor TLR5 activation in a very specific and sensitive fashion. In the first round of screening, 480 crude extracts (representing a vast

assortment of primary and secondary metabolites) were tested and 40 extracts (8.3% of total), which reduced signaling by at least 70%, were taken to the second round of screening. The representative results are shown in Figure 2a, where the crude extract 47643 was found to be a potent inhibitor of TLR5 signaling. Second-round screening examined the dose-dependency of inhibition and assessed toxicity, and subsequently 5 crude extracts (1% of total) were selected for sub-fractionation and further analysis. This sub-fractionation involved both solvent partitions (i.e., water vs. butanol or ethyl acetate) and high-performance liquid chromatography (HPLC). Again, the extract 47643 stood out as other potential hits exhibited toxicity as indicated by the crosses in Figure 2b. The screening continued on the sub-fractions of the extract 47643, and after 4 cycles, a pure active compound 47643.3.1.1.2.1 was isolated (Figure 2c–e).

We solved the chemical structure of compound 47643.3.1.1.2.1 using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry, and determined that this 2-aminoimidazole containing compound had been previously reported as girolline or girodazole. Girolline was initially isolated from the marine sponge *Pseudoaxinyssa cantharella* for its antitumor activity (38, 39), and compared to many natural products, the structure of this compound is relatively simple ($C_6H_{11}ClN_4O$) (40).

Structure-activity relationship studies examining the TLR inhibitory activity of girolline

Girolline was synthesized with >99% purity based on a previously reported method (Supporting Figure S2) (41). We produced synthetic analogs of girolline for structure-activity relationship analysis of the TLR5 inhibiting pharmacophore. By systematically excluding each of the functional groups of girolline, we generated a panel of analogs demonstrating that every functional group in the molecule was very important for inhibition of TLR5 signaling. This outcome was anticipated, as this natural product is already very small. The activity decreased by several orders of magnitude or disappeared completely in compounds lacking the aromatic amine (**4**), the chlorine (**5**), and both the chlorine and hydroxyl (**6**) (Figure 3, and Supporting Table S1). In addition, we prepared an enantiomer of girolline (**3**) as well as a diastereomer (**2**). While the activity of the enantiomer was attenuated by two orders of magnitude (Figure 3c), the diastereomer of girolline was more potent than girolline in the inhibition of TLR5 signaling (Figure 3b).

Anti-inflammatory activity of girolline in hematopoietic cells, including fresh primary human mononuclear cells

In the next series of experiments, we examined the potential anti-inflammatory activity of girolline. We focused on hematopoietic cells as these cells play a critical role in driving inflammatory innate immune responses. To quantify the anti-inflammatory activity of girolline on macrophage responses, the human acute monocytic leukemia cell line (THP1) transfected with NF-κB/AP-1 reporter was cultured and differentiated into a macrophage-like phenotype. THP1-derived macrophages were pre-treated with girolline (2 μg ml^{-1}) for 1 h and then stimulated with the TLR5 ligand flagellin (100 ng ml^{-1}) for 6 h. We found that girolline significantly reduced ($P<0.001$) flagellin-induced IL-8 secretion (Figure 4a), and almost completely abolished NF-κB/AP-1 activity (Figure 4b).

To confirm this observation and to enhance the clinical relevance of our studies, similar inhibition experiments were performed on freshly isolated human peripheral blood mononuclear cells (PBMCs). Encouragingly, girolline decreased the secretion of the pro-inflammatory cytokines IL-8 and IL-6 to baseline following flagellin stimulation ($P<0.001$) (Figure 4c and d).

Girolline does not cause significant cytotoxicity

Because girolline was originally discovered as an antitumor agent, it was critical to examine the toxicity of girolline in a variety of cell types. To address this issue, two different cytotoxicity assays were performed. Cytosolic lactate dehydrogenase (LDH) is released when cell membrane integrity is compromised, and therefore the LDH release assay was conducted to study cell death directly. In addition, mitochondrial metabolic activity was monitored with the MTS assay, which works on the principle that the mitochondrial dehydrogenase enzyme in viable cells converts the substrate to a soluble colored formazan product.

As shown in Figure 5a, different concentrations of girolline (0.08–2 μg ml^{-1}) in the presence or absence of flagellin stimulation did not cause LDH release from the CHO-K1-TLR5 cells, whereas the MTS assay showed that at higher concentrations girolline modestly (~20%) reduced mitochondrial activity in CHO cells (Figure 5b). The same test was conducted on several other cell types, including HEK reporter cells, THP1 derived macrophages and fresh primary peripheral blood mononuclear cells (PBMCs). As shown in Figure 5c–e, girolline had no effect on the mitochondrial activity of the THP1 derived macrophages and PBMCs, but did slightly decrease mitochondrial metabolic activity of HEK reporter cells at concentrations higher than 0.4 μg ml^{-1}. Overall, girolline did not exhibit significant toxicity on various cell types within the experimental periods (4–6 h).

Girolline acts as a global inhibitor of TLR signaling

We next asked the question whether girolline was a specific inhibitor of the TLR5 pathway or a more global inhibitor of TLR signaling. To address this important question, we used 4 additional reporter cell lines to examine both MyD88-dependent (TLR2, TLR4, TLR7) and MyD88-independent (TLR3, TLR4) signaling, as well as signaling triggered by cell surface (TLR2, TLR4) and endosomal (TLR3, TLR7) TLRs. The ligands and corresponding TLRs examined in this study are listed in Supporting Table S2.

Using this comprehensive approach, we found that girolline acted as a global inhibitor of all the TLR pathways tested as shown in Figure 6. Girolline decreased TLR induced $_{NF\text{-}KB/AP\text{-}1}$ activation in a dose-dependent fashion with pronounced inhibition at concentrations higher than 0.2 μg ml^{-1}. These data suggest that girolline acts on a downstream element common to many TLRs.

Ligands that activate different aspects of the host innate immune pathway were used to stimulate cells and gain further insights into the molecular targets of girolline. Specifically, stimulating girolline-treated cells with IL-1β (via IL1R) and TNF-α (via TNFR) provided useful insights into molecules inhibited by girolline. IL-1β interacts with IL-1 receptor (IL1R) to trigger MyD88-dependent or independent signaling (42), whereas TNF-α

signaling occurs through TRAF2/5 (bypassing MyD88) to activate downstream transcription factors (43). Our results showed that girolline was capable of inhibiting both IL-1β and TNF-α induced NF-KB activation, and this inhibitory activity was dose-dependent (Figure 6a–d). Again these results suggest that girolline inhibited TLR signaling at the level of a common downstream element.

Defining the molecular target of girolline

Our data suggested that the target of girolline was a downstream signaling element common to many TLRs and an obvious candidate was NF-KB. To test this hypothesis, we performed immunoblotting to probe NF-KB activation in THP1-derived macrophages after flagellin stimulation in the presence or absence of girolline. Interestingly, we did not observe direct effects of girolline on NF-KB activation by examining IKBA degradation, NF-KB subunit p65 nuclear translocation and p65 phosphorylation (Supporting Figure S3).

To predict the molecular target of girolline, we then employed an unbiased discovery approach by defining the chemical genomic profile of girolline in comparison with its inactive variant, des-chloro girolline (**5**). The chemical genomic assays were performed by screening each compound against a set of 5000 yeast mutants (the non-essential gene deletion collection), together with a set of 1000 hypomorphic mutants of essential genes, and quantifying the abundance of the mutant strains relative to a solvent control (44). The results showed that girolline had a distinct chemical genomic profile against our 5000 mutant collection at a concentration of 2.2 μg ml^{-1} (toxicity to yeast: IC_{50} = 5 μg ml^{-1}). The top 10 mutant strains (Supporting Table S3) sensitive to girolline were significantly enriched for genes involved in peptidyl-diphthamide biosynthetic process (P value = 3.42×10^{-8}). This enrichment was driven by sensitive mutants with deletions of the genes *DPH1, DPH2, RRT2, JJJ3*. The top sensitive mutant of an essential gene was *RBP7*, RNA polymerase II subunit B16 involved in translation initiation. To identify protein complexes sensitive to girolline, we calculated the sensitivity scores of mutants within known protein complexes, and the most sensitive protein complex to girolline was the dipthamide complex. In contrast, the deletion mutants sensitive to des-chloro girolline (the inactive variant of girolline) did not show any functional enrichment for any biological process, and the girolline-sensitive diphthamide mutants were not sensitive or even slightly resistant to this compound.

In principle, deletion of a gene that encodes the target of an inhibitory compound should cause cellular effects that are similar to inhibition of the target by drug treatment. Therefore, the chemical-genetic interaction profile of a compound can recapitulate the genetic interactions of its gene target (44). By comparing the chemical genomic profile of girolline to the genetic interaction network of *S. cerevisiae* allows us to predict its molecular target (35). In both experimental replicates, the predicted target for girolline—the deletion mutant with the most similar genetic interaction profile to the chemical genomic profile of girolline—was *eft2*Δ (Figure 7a). Elongation factor 2 (EF-2) catalyzes ribosomal translocation during protein synthesis, and this protein requires the unique diphthamide post-translational modification.

Given the predicted target of Eft2 and the sensitivity of the dipthamide complex, this suggests that girolline may target the ribosome and inhibit translation specifically by

affecting Eft2 function. The anti-fungal agent sordarin and the anti-tumor compound Mycalamide B are examples of protein translation inhibitors that target the elongation step (45, 46). Indeed mutants in the diphthamide pathway are sensitive to both sordarin and girolline (47). To validate these chemical genomics findings and to test whether girolline could inhibit new protein synthesis, we conducted a fluorescence-based protein synthesis inhibition assay. As shown in Figure 7b, the total new protein synthesis (visualized in green) in the macrophages treated with girolline was significantly lower than in non-treated and solvent control cells. Quantitative analysis (Figure 7c) revealed that girolline strongly inhibited protein synthesis similar to the known protein synthesis inhibitor, cycloheximide. This inhibitory activity was also confirmed using a luciferase-based, rabbit reticulocyte assay (Supporting Figure S4).

Potential biological activity of girolline

TLR signaling is an attractive target for drug development, as TLRs contribute to damaging inflammation in a large variety of human diseases (8, 22, 23, 48). Through high-throughput screening, we successfully identified a small bioactive compound—girolline—from crude marine sponge extracts. Girolline inhibits both MyD88-dependent and MyD88-independent signaling, as well as signaling through TLRs located on the cell surface and in endosomes. Importantly, girolline reduces TLR-mediated pro-inflammatory cytokine secretion in a variety of cell types, including fresh human PBMCs, without significant cytotoxicity. Together, these data identify girolline as a potential anti-inflammatory agent.

Intriguingly, although we identified girolline (from Stylissa aff. carter) through an unbiased screen of marine sponge extracts, girolline was previously identified as an anticancer agent from the marine sponge *Pseudoaxinyssa cantharella* (38). Girolline showed significant antitumor activity on cultured cells and in murine tumor models (49). Toxicological studies in mice and dogs did not reveal any major toxic effects. However, Phase I clinical trial in 12 human subjects with advanced refractory solid tumors was discontinued when girolline showed no antitumor activity and was found to cause hypotension and lethargy (50). Since then, limited studies of girolline have been reported, primarily focusing on its mechanisms (39, 40). For the first time our screening identifies girolline as a potential anti-inflammatory agent. This discovery opens new venues for girolline to be re-evaluated as a therapeutic agent in other diseases associated with undesired inflammation, such as sepsis, cystic fibrosis and a variety of autoimmune diseases.

Although girolline was first discovered as an anti-tumor agent, several modes of action of girolline are reported, including cell cycle arrest, recruitment of polyubiquitinated p53 to the proteasome, and inhibition of protein synthesis (39, 40, 51). Through our unbiased screening of marine sponge extracts on TLR signaling, to our surprise, girolline stood out as a potential TLR5 inhibitor based on our reporter system. We then found that girolline had a broad inhibitory spectrum on multiple TLRs as well as other inflammatory pathways (Figure 6e). This led to the hypothesis that the molecular target affected by girolline would be in the common downstream signaling machinery distal to TRAF6. One possible target was the NF-κB complex, which is the central orchestrator of many inflammatory responses (52–54). However, our results indicated that NF-κB was not the direct target of girolline.

To solve this puzzle, we employed a more global approach by looking at the chemical genomic profiles of girolline in comparison with the known genetic interaction network in yeast. An inactive girolline analog was tested in parallel as a control to allow better identification of the target by removing the unrelated hits. The top identified genes sensitive to girolline (but not its inactive form) are central in the protein synthesis process, especially the diphthamide biosynthetic process. Based on comparison of the chemical genomic profile with the genetic interaction network of yeast, the predicted target of girolline is Elongation factor 2 (EF-2), which promotes ribosomal translocation during protein translation elongation. This finding is somewhat consistent with the earlier reported mechanism of protein synthesis inhibition by Colson *et al*. (51), where they suggested that the major effect of girolline (or girodazole) was on the termination step of protein synthesis. In contrast, our chemical-genetic approach (vs. biochemical approach) identified EF-2 as the molecular target, indicating that the inhibition may actually be at the elongation step of protein synthesis. Crystal structure analysis by Schroeder *et al*. (55) also revealed that girolline can bind the 50 S ribosomal subunit near the E site, resulting in the conformational change and interfering with tRNA binding.

It is worth noting that inhibition of protein synthesis is a common downstream element of many signaling pathways, including TLR signaling. Thus, identifying girolline as an inhibitor of protein translation is entirely consistent with the observations that girolline decreased TLR-mediated NF-κB reporter signals in multiple cell types (CHO, HEK and THP1) and the secretion of NF-κB-regulated cytokines (IL-6 and IL-8). This also serves as a timely reminder that when enzyme-based reporter systems are used for drug screening, they are susceptible to the detection of protein synthesis inhibitors.

CONCLUSIONS

In this study, a high throughput screening platform was successfully established to identify TLR inhibitors from crude marine sponge extracts. Through this process, we identified girolline which inhibits signaling through MyD88-dependent and –independent TLRs (i.e., TLR2, 3, 4, 5 and 7). Our structure-activity relationship (SAR) analysis indicates that the inhibitory activity of girolline likely relies on its amino, hydroxyl and chlorine groups. Importantly, girolline inhibited TLR signaling in human hematopoietic cells (including primary mononuclear cells) without significant cytotoxicity. Chemical genomics studies revealed that girolline targets Elongation factor 2 to inhibit protein synthesis at the elongation step.

METHODS

Materials and reagents

The TLR5 reporter cell line (CHO-K1-TLR5), obtained as a gift from Dr. Kelly D. Smith (University of Washington), stably expresses human TLR5 and a $_{NF\text{-}KB}$ luciferase reporter (6). HEK 293 reporter cell lines used (HEK-TLR2, HEK-TLR3, HEK-TLR4 and HEK-TLR7) and THP1-XBlue cells were from InvivoGen (San Diego, CA). All the culture media and reagents were either from HyClone (Logan, UT) or Invitrogen (Life Technologies, Burlington, ON, Canada) unless otherwise stated. The selective antibiotics to maintain the

stable transfection of the reporter cell lines and the ligands for TLR 2, 3, 4 and 7 listed in Supporting Table S2 were purchased from InvivoGen (San Diego, CA). The TLR5 ligand flagellin (from *Salmonella typhimurium)* was obtained from Cedarlane Laboratories (Burlington, ON, Canada) while the recombinant TNFα and IL-1β were from eBioscience (San Diego, CA). Chemicals were either from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada) unless otherwise specified.

Preparation of marine sponge extracts

Marine sponges (100 g to 1000 g each) were collected by hand using SCUBA at depths between 1 and 30 m in tropical and temperate marine habitats in the North Eastern Pacific, South Western Pacific, and Caribbean Oceans. Freshly collected sponge specimens were deep frozen on site and transported to Vancouver. A small portion of each sponge sample was homogenized with methanol and then concentrated *in vacuo* to give a gummy extract. Approximately 1 mg portion of the gummy extract from each sponge was transferred to a 96 well plate to give a master screening library. The gums were dissolved in DMSO and portions were transferred to daughter plates to give the desired screening concentration.

Isolation and identification of girolline

Sponge specimen #47643, an orange rope sponge with branching fingers, was collected at Mont pass in Pohnpei (Federated States of Micronesia) at a depth of 12 m. It was identified as *Stylissa aff. carteri* and a voucher sample has been deposited at the Netherlands Centre for Biodiversity Naturalis in Leiden, The Netherlands. Frozen sponge was lyophilized and then extracted first with methanol and subsequently with dichloromethane. The combined extracts were concentrated *in vacuo*, suspended in water and portioned against ethyl acetate and butanol to give a bioactive aqueous fraction. The aqueous soluble material was sequentially fractionated using Sephadex LH20 chromatography (eluent: methanol) followed by a step gradient (water to methanol) C-18 open column reversed phase chromatography and gradient C-18 revered phase HPLC (water to 40% water/acetonitrile) to give a highly purified bioactive material (≈ 90% pure). A HRESI-TOFMS measurement on this sample gave a $[M + H]^+$ ion at *m/z* 191.0611 appropriate for a molecular formula of $C_6H_{11}ON_4{}^{35}Cl$ (calcd for $C_6H_{12}ON_4{}^{35}Cl$ 191.0700) and an isotope pattern characteristic of one chlorine atom. Analysis of the ^1H NMR, ^{13}C NMR, COSY and HMBC data obtained for this material identified it as the known sponge metabolite girolline. Because the natural sample was not 100% pure, we synthesized the natural product using literature methods to confirm that girolline was the active component in the mixture. Natural Giroline: ^1H NMR (600 MHz, MeOD-d_4) δ 3.4, dd (J= 13.8, 9.6 Hz), 3.54 dd (J = 13.8, 3.2 Hz), 4.53 dt (J = 9.6, 3.3 Hz), 5.09 dd (J = 3.3, 0.8 Hz), 6.83 d (J = 0.8 Hz); ^{13}C NMR (150 MHz, MeOD-d_4) δ 42.8, 59.8, 66.0, 124.2, 110.9, 147.1.

Synthesis of girolline and its derivatives

Flash column chromatography was carried out with EcoChrom ICN SiliTech 32–63 D 60Å silica gel, as indicated. Reactions and chromatography fractions were monitored with Merck silica gel 60 F_{254} TLC plates and visualized using charring solutions of potassium permanganate or ninhydrin stain. Reactions were carried out under inert gas atmosphere in

oven-dried glassware and reaction solutions were magnetically stirred. All reagents and solvents were used without further purification from commercial sources unless otherwise noted. The synthetic scheme for preparation of girolline is illustrated in the Supporting Figure S2a. The detailed synthetic procedures of each analog in Figure 3 were described in the Supporting Experimental Section. Synthetic girolline and its analogs were characterized by nuclear magnetic resonance (NMR) spectroscopy (Supporting Figures S2b).

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were measured by the UBC NMR facility on a Bruker 400 MHz spectrometer and calibrated using residual solvent signal. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, app. = apparent, br. = broad. Where consistent coupling constants have been observed in the NMR spectrum, the apparent multiplicity of the proton signal concerned is reported. High resolution mass spectra (HRMS) were obtained by the UBC Mass Spectrometry facility using electrospray ionization (ESI).

Cell culture

The CHO-K1-TLR5 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 2 mM L-glutamine. Cells were regularly passaged with 100 μg ml^{-1} G418 antibiotics to maintain the stable transfection.

HEK cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 2 mM L-glutamine and selective antibiotics according to the supplier's instructions and our previous work (56). THP1 cells were cultured in RPMI 1640 medium containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and the selective antibiotics zeocin (100 μg ml^{-1}) based on the reported standard procedures (57).

Inhibitor screening on TLR5 reporter cells

CHO-K1-TLR5 cells were primarily used to screen for potential TLR inhibitors derived from marine sponge extracts. They were seeded into 96-well plates at a cell density of 5×10^4 cells per well and incubated overnight. Cells were pretreated with various crude marine sponge extracts 1 h before the flagellin stimulation (100 ng ml^{-1}), and after 3.5 h, cells were assayed for luciferase activity following previous published procedures (19). The screening continued on the sub-fractions of the potential inhibitory hits until a pure compound was identified. The screening procedure is illustrated in Figure 1b.

NF-κB/AP-1 activity assay for HEK reporter cells

HEK-TLR reporter cells were used to test spectrum of inhibitory activity of the target compound. HEK reporter cells were seeded in 96-well plates at a density of 5×10^4 cells per well and incubated overnight. Cells were pretreated with girolline at different concentrations (8–5000 ng ml^{-1}) for 1 h, followed by stimulation with various TLR ligands, TNFα and IL-1β for 6 h. The stimulants used, their concentrations and corresponding targets are list in Supporting Table S2. At the end of stimulation, 20 μl of the medium from each well was transferred to another 96-well plate and mixed with 180 μl of Quanti-Blue substrate solution (InvivoGen). The substrate underwent dephosphorylation by the secreted alkaline

phosphatase (SEAP) in proportion to the NF-KB/AP-1 activity. The plate was incubated at 37°C overnight and read on a SpectraMax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA) at 655 nm.

Cellular toxicity tests

Cellular toxicity of girolline was examined by measuring both the released lactate dehydrogenase (LDH) from cells and mitochondrial activity, using a LDH detection kit (Roche Diagnostics, Laval, QC, Canada) and the MTS assay, respectively. For the LDH release assay, culture medium (100 μl) was collected for the measurement of LDH release following the manufacturer's instruction. 0.1 % Triton-X was used as the positive control. For the MTS assay, cells were seeded in a 96-well plate overnight (CHO, HEK and THP1 cells: 5×10^4 cells per well; PBMCs: 1×10^5 cells per well). The medium was replaced with or without girolline (3.2–10000 ng ml^{-1}) and incubated for 4 h. The mitochondrial activity was then measured by a commercial kit (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Madison, WI) following the supplier's instruction and the previously published work (58). The results were normalized to the cells-only control (set as 1) to generate the mitochondria activity index.

THP1 derived macrophages and PBMCs

THP1 reporter cells were suspended in the culture medium containing 50 ng ml^{-1} of phorbol 12-myristate 13-acetate (PMA), seeded in 6-well plates (2×10^6 cells per well) and incubated for 24 h to allow differentiation into a macrophage-like phenotype. Cells were then washed with PBS (2 ml/well) and rested in fresh culture medium for 2 days before experiments. THP1-derived macrophages were pretreated with girolline (2 μg ml^{-1}) or DMSO control (0.04%) for 1 h and stimulated with flagellin (100 ng ml^{-1}) for various time periods (15 min to 6 h). Culture medium was collected for cytokine measurements and NF-κB activity assay, while the cells were processed for immunoblotting.

Blood samples were obtained with informed written consent from healthy volunteers at the Child & Family Research Institute with protocols approved by the University of British Columbia Clinical Research Ethics Board (H04–0534). PBMCs were isolated based on our previously described method (59). Isolated PBMCs in RPMI 1640 medium (10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate) were seeded in a 96-well round-bottom plate at a density of 1×10^5 cells per well overnight. PBMCs were treated with girolline (2 μg ml^{-1}) for 1 h and stimulated with flagellin (100 ng ml^{-1}) for 6 h. At the end of the experiment, cells were spun down at 400xg for 5 min, and aliquots of the supernatant (100 μl per well) were collected and stored at −80°C until cytokine quantification.

Chemical genomic analysis

The chemical genomic analysis was carried out as described in Parsons *et al*. (44) with slight modifications. Briefly, the optimal inhibitory concentration of girolline for chemical genomic profiling (70–80% growth vs. solvent control in YEP-Galactose media) was first determined to be 2.2 μg ml^{-1}. We then grew 200 μl cultures containing a pooled *S. cerevisiae* mutant collection in the presence of girolline (2.2 μg ml^{-1}), des-chloro girolline (2.2 μg ml^{-1}), or a DMSO control in triplicate for 48 h at 30 °C. We challenged both the

non-essential, haploid deletion collection and the DAmP collection of hypomorphic mutations in essential genes (60). After 48 h, we extracted the genomic DNA using the Epicentre MasterPure™ Yeast DNA purification kit (Epicentre, Madison, WI). Strain-specific molecular barcodes were amplified with specially designed multiplex primers (61), and sequenced using an Illumina MiSeq platform. We sequenced 2 replicates of each condition (girolline, des-chloro girolline, and DMSO). The barcode counts for each yeast deletion mutant in the presence of girolline or des-chloro girolline were normalized against the DMSO control to generate a Z-score to define sensitivity or resistance of individual strains. A Bonferroni corrected hypergeometric distribution test was used to search for significant enrichment of GO terms among the 10 most sensitive deletion mutants (62). Furthermore, we correlated the chemical genomic profile of both compounds with the yeast genetic interaction network to predict the molecular target, as described in Costanzo *et al*. (35).

Protein Synthesis Inhibition Assay

A fluorescence-based protein synthesis inhibition assay (Click-iT AHA Alexa Fluor 488 Protein Synthesis HCS kit, Invitrogen, ON, Canada) was performed to confirm whether girolline inhibits protein synthesis in cultured cells. Experiments were done following the product instruction. Briefly, THP1 cells were seeded (3×10^6 cells) in an 8-chambered coverglass (Nunc Lab-Tek II, Thermo Scientific), stimulated (50 ng ml^{-1} PMA) for 24 h to differentiate into macrophages, and rested for 2 days. Cells were treated with 2 μg ml^{-1} girolline for 4 h, and then incubated in a special medium, where L-methionine was replaced with a mimic (L-azidohomoalanine), for 30 min. After cell fixing and permeabilization, proteins containing L-azidohomoalanine were labeled with Alexa 488, and the nucleus was stained with Hoechst 33342. Cells were imaged on a laser scanning confocol microscope (Leica SP5II). Non-treated, solvent (0.5% DMSO) and positive (1 μM cycloheximide, 30 min) controls were tested for comparison. Alexa 488 signals were quantified using a freeware (LAS AF Lite) by dividing the total intensity of the green channel with cell numbers (number of nuclei) for each image. A total of 250–350 cells were analyzed for each condition with 4 repeated experiments.

Cytokine quantification

The pro-inflammatory cytokines IL-6 and IL-8 in the cell supernatants were quantified using the human ELISA Ready-Set Go kits (eBioscience). The experimental procedures followed the manufacture's instruction.

Statistical analysis

Statistical analysis was performed using one-way ANOVA with the Bonferroni post-test where applicable. Differences were considered significant only when the *p* value was less than 0.05.

Supporting information available

CHO-TLR5 reporter cells respond to flagellin in a sensitive (> 10 ng ml^{-1}) and specific fashion. Effects of girolline on NF-κB signaling via immunobloting. Effects of girolline on

protein translation via a luciferase-based, rabbit reticulocyte assay. Synthetic procedures and characterization of girolline and related analogs for SAR studies. Top 10 sensitive deletion mutants of non-essential and essential deletion mutant strains for girolline and des-chloro girolline. These materials are available free of charge via the Internet at http://pubs.acs.org.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Palm NW, Medzhitov R. Pattern recognition receptors and control of adaptive immunity. Immunol Rev. 2009; 227:221–233. [PubMed: 19120487]
2. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010; 140:805–820. [PubMed: 20303872]
3. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996; 86:973–983. [PubMed: 8808632]
4. Moresco EM, LaVine D, Beutler B. Toll-like receptors. Curr Biol. 2011; 21:R488–R493. [PubMed: 21741580]
5. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat.Immunol. 2010; 11:373–384. [PubMed: 20404851]
6. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001; 410:1099–1103. [PubMed: 11323673]
7. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34:637–650. [PubMed: 21616434]
8. Hedayat M, Netea MG, Rezaei N. Targeting of Toll-like receptors: a decade of progress in combating infectious diseases. Lancet Infect Dis. 2011; 11:702–712. [PubMed: 21719349]
9. Arancibia SA, Beltran CJ, Aguirre IM, Silva P, Peralta AL, Malinarich F, Hermoso MA. Toll-like receptors are key participants in innate immune responses. Biol Res. 2007; 40:97–112. [PubMed: 18064347]
10. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical microbiology reviews. 2009; 22:240–273. Table of Contents. [PubMed: 19366914]
11. Cinel I, Opal SM. Molecular biology of inflammation and sepsis: a primer. Crit Care Med. 2009; 37:291–304. [PubMed: 19050640]
12. Kim HY, DeKruyff RH, Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat Immunol. 2010; 11:577–584. [PubMed: 20562844]
13. Holt PG, Strickland DH. Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. J Allergy Clin Immunol. 2010; 125:963–972. quiz 973–964. [PubMed: 20394979]

14. Machen TE. Innate immune response in CF airway epithelia: hyperinflammatory? Am J Physiol Cell Physiol. 2006; 291:C218–C230. [PubMed: 16825601]
15. Moraes TJ, Plumb J, Martin R, Vachon E, Cherepanov V, Koh A, Loeve C, Jongstra-Bilen J, Zurawska JH, Kus JV, Burrows LL, Grinstein S, Downey GP. Abnormalities in the pulmonary innate immune system in cystic fibrosis. Am J Respir Cell Mol Biol. 2006; 34:364–374. [PubMed: 16293782]
16. Bals R, Weiner DJ, Wilson JM. The innate immune system in cystic fibrosis lung disease. J Clin Invest. 1999; 103:303–307. [PubMed: 9927489]
17. Greene CM, Branagan P, McElvaney NG. Toll-like receptors as therapeutic targets in cystic fibrosis. Expert Opin Ther Targets. 2008; 12:1481–1495. [PubMed: 19007318]
18. Doring G, Gulbins E. Cystic fibrosis and innate immunity: how chloride channel mutations provoke lung disease. Cell Microbiol. 2009; 11:208–216. [PubMed: 19068098]
19. Blohmke CJ, Victor RE, Hirschfeld AF, Elias IM, Hancock DG, Lane CR, Davidson AG, Wilcox PG, Smith KD, Overhage J, Hancock RE, Turvey SE. Innate immunity mediated by TLR5 as a novel antiinflammatory target for cystic fibrosis lung disease. J.Immunol. 2008; 180:7764–7773. [PubMed: 18490781]
20. Turvey SE, Broide DH. Innate immunity. J Allergy Clin Immunol. 2010; 125:S24–S32. [PubMed: 19932920]
21. Midwood KS, Piccinini AM, Sacre S. Targeting Toll-like receptors in autoimmunity. Curr Drug Targets. 2009; 10:1139–1155. [PubMed: 19715534]
22. O'Neill LA, Bryant CE, Doyle SL. Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol.Rev. 2009; 61:177–197. [PubMed: 19474110]
23. Dunne A, Marshall NA, Mills KH. TLR based therapeutics. Curr.Opin.Pharmacol. 2011; 11:404–411. [PubMed: 21501972]
24. Barrat FJ, Coffman RL. Development of TLR inhibitors for the treatment of autoimmune diseases. Immunol.Rev. 2008; 223:271–283. [PubMed: 18613842]
25. Tidswell M, LaRosa SP. Toll-like receptor-4 antagonist eritoran tetrasodium for severe sepsis. Expert.Rev.Anti.Infect.Ther. 2011; 9:507–520. [PubMed: 21609262]
26. Blohmke CJ, Park J, Hirschfeld AF, Victor RE, Schneiderman J, Stefanowicz D, Chilvers MA, Durie PR, Corey M, Zielenski J, Dorfman R, Sandford AJ, Daley D, Turvey SE. TLR5 as an anti-inflammatory target and modifier gene in cystic fibrosis. J.Immunol. 2010; 185:7731–7738. [PubMed: 21068401]
27. Mohamed FB, Garcia-Verdugo I, Medina M, Balloy V, Chignard M, Ramphal R, Touqui L. A Crucial Role of Flagellin in the Induction of Airway Mucus Production by Pseudomonas aeruginosa. PLoS One. 2012; 7:e39888. [PubMed: 22768318]
28. Donia M, Hamann MT. Marine natural products and their potential applications as anti-infective agents. Lancet Infect.Dis. 2003; 3:338–348. [PubMed: 12781505]
29. Brastianos HC, Vottero E, Patrick BO, Van SR, Matainaho T, Mauk AG, Andersen RJ. Exiguamine A, an indoleamine-2,3-dioxygenase (IDO) inhibitor isolated from the marine sponge Neopetrosia exigua. J.Am.Chem.Soc. 2006; 128:16046–16047. [PubMed: 17165752]
30. Meimetis LG, Williams DE, Mawji NR, Banuelos CA, Lal AA, Park JJ, Tien AH, Fernandez JG, de Voogd NJ, Sadar MD, Andersen RJ. Niphatenones, glycerol ethers from the sponge Niphates digitalis block androgen receptor transcriptional activity in prostate cancer cells: structure elucidation, synthesis, and biological activity. J.Med.Chem. 2012; 55:503–514. [PubMed: 22148427]
31. Sadar MD, Williams DE, Mawji NR, Patrick BO, Wikanta T, Chasanah E, Irianto HE, Soest RV, Andersen RJ. Sintokamides A to E, chlorinated peptides from the sponge Dysidea sp. that inhibit transactivation of the N-terminus of the androgen receptor in prostate cancer cells. Org.Lett. 2008; 10:4947–4950. [PubMed: 18834139]
32. Williams DE, Yu K, Behrisch HW, Van SR, Andersen RJ. Rolloamides A and B, cytotoxic cyclic heptapeptides isolated from the Caribbean marine sponge Eurypon laughlini. J.Nat.Prod. 2009; 72:1253–1257. [PubMed: 19583251]

33. Ho CH, Piotrowski J, Dixon SJ, Baryshnikova A, Costanzo M, Boone C. Combining functional genomics and chemical biology to identify targets of bioactive compounds. Curr Opin Chem Biol. 2011; 15:66–78. [PubMed: 21093351]
34. Parsons AB, Brost RL, Ding H, Li Z, Zhang C, Sheikh B, Brown GW, Kane PM, Hughes TR, Boone C. Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. Nat Biotechnol. 2004; 22:62–69. [PubMed: 14661025]
35. Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS, Ding H, Koh JL, Toufighi K, Mostafavi S, Prinz J, St Onge RP, VanderSluis B, Makhnevych T, Vizeacoumar FJ, Alizadeh S, Bahr S, Brost RL, Chen Y, Cokol M, Deshpande R, Li Z, Lin ZY, Liang W, Marback M, Paw J, San Luis BJ, Shuteriqi E, Tong AH, van Dyk N, Wallace IM, Whitney JA, Weirauch MT, Zhong G, Zhu H, Houry WA, Brudno M, Ragibizadeh S, Papp B, Pal C, Roth FP, Giaever G, Nislow C, Troyanskaya OG, Bussey H, Bader GD, Gingras AC, Morris QD, Kim PM, Kaiser CA, Myers CL, Andrews BJ, Boone C. The genetic landscape of a cell. Science. 2010; 327:425–431. [PubMed: 20093466]
36. Thakur NL, Muller WEG. Biotechnological potential of marine sponges. Curr.Sci. 2004; 86:1506–1512.
37. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat.Immunol. 2003; 4:1247–1253. [PubMed: 14625549]
38. Ahond A, Bedoya-Zurita M, Colin M, Fizames C, Laboute P, Lavelle F, Laurent D, Poupat C, Pusset J, Pusset M, Thoison O, Potier P. La girolline, nouvelle substance antitumorale extraite de l'Ponge, Pseudaxinyssa cantharella n. sp. (Axinellidae). C.R.Acad.Sci.Paris. 1988; II 307:145–148.
39. Tsukamoto S, Yamashita K, Tane K, Kizu R, Ohta T, Matsunaga S, Fusetani N, Kawahara H, Yokosawa H. Girolline, an antitumor compound isolated from a sponge, induces G2/M cell cycle arrest and accumulation of polyubiquitinated p53. Biol.Pharm.Bull. 2004; 27:699–701. [PubMed: 15133248]
40. Diop D, Chauvin C, Salhi S, Poupat C, Ahond A, Jean-Jean O. Girolline interferes with cell-cycle progression, but not with translation. C.R.Biol. 2007; 330:855–860. [PubMed: 18068643]
41. Commerçon A, Paris JM. Enantioselective synthesis of girolline. Tetrahedron Letters. 1991; 32:4905–4906.
42. Oda K, Kitano H. A comprehensive map of the toll-like receptor signaling network. Mol.Syst.Biol. 2006; 2 2006.
43. Dempsey PW, Doyle SE, He JQ, Cheng G. The signaling adaptors and pathways activated by TNF superfamily. Cytokine Growth Factor Rev. 2003; 14:193–209. [PubMed: 12787559]
44. Parsons AB, Lopez A, Givoni IE, Williams DE, Gray CA, Porter J, Chua G, Sopko R, Brost RL, Ho CH, Wang J, Ketela T, Brenner C, Brill JA, Fernandez GE, Lorenz TC, Payne GS, Ishihara S, Ohya Y, Andrews B, Hughes TR, Frey BJ, Graham TR, Andersen RJ, Boone C. Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. Cell. 2006; 126:611–625. [PubMed: 16901791]
45. Dang Y, Schneider-Poetsch T, Eyler DE, Jewett JC, Bhat S, Rawal VH, Green R, Liu JO. Inhibition of eukaryotic translation elongation by the antitumor natural product Mycalamide B. RNA. 2011; 17:1578–1588. [PubMed: 21693620]
46. Dominguez JM, Gomez-Lorenzo MG, Martin JJ. Sordarin inhibits fungal protein synthesis by blocking translocation differently to fusidic acid. J Biol Chem. 1999; 274:22423–22427. [PubMed: 10428815]
47. Botet J, Rodriguez-Mateos M, Ballesta JP, Revuelta JL, Remacha M. A chemical genomic screen in Saccharomyces cerevisiae reveals a role for diphthamidation of translation elongation factor 2 in inhibition of protein synthesis by sordarin. Antimicrob Agents Chemother. 2008; 52:1623–1629. [PubMed: 18285480]
48. Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Tolllike receptors in inflammation, infection and cancer. Int.Immunopharmacol. 2007; 7:1271–1285. [PubMed: 17673142]

49. Lavelle F, Zerial A, Fizames C, Rabault B, Curaudeau A. Antitumor activity and mechanism of action of the marine compound girodazole. Invest New Drugs. 1991; 9:233–244. [PubMed: 1783523]
50. Catimel G, Coquard R, Guastalla JP, Merrouche Y, Le Bail N, Alakl MK, Dumortier A, Foy M, Clavel M. Phase I study of RP 49532A, a new protein-synthesis inhibitor, in patients with advanced refractory solid tumors. Cancer Chemother Pharmacol. 1995; 35:246–248. [PubMed: 7805184]
51. Colson G, Rabault B, Lavelle F, Zerial A. Mode of action of the antitumor compound girodazole (RP 49532A, NSC 627434). Biochem Pharmacol. 1992; 43:1717–1723. [PubMed: 1575768]
52. Pasparakis M. Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. Nat Rev Immunol. 2009; 9:778–788. [PubMed: 19855404]
53. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest. 2001; 107:7–11. [PubMed: 11134171]
54. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. Immunol Rev. 2006; 210:171–186. [PubMed: 16623771]
55. Schroeder SJ, Blaha G, Tirado-Rives J, Steitz TA, Moore PB. The structures of antibiotics bound to the E site region of the 50 S ribosomal subunit of Haloarcula marismortui: 13-deoxytedanolide and girodazole. J Mol Biol. 2007; 367:1471–1479. [PubMed: 17321546]
56. Marr N, Turvey SE. Role of human TLR4 in respiratory syncytial virus-induced NF-kappaB activation, viral entry and replication. Innate.Immun. 2012
57. Tang A, Sharma A, Jen R, Hirschfeld AF, Chilvers MA, Lavoie PM, Turvey SE. Inflammasome-Mediated IL-1beta Production in Humans with Cystic Fibrosis. PLoS One. 2012; 7:37689.
58. Bawa R, Fung SY, Shiozaki A, Yang H, Zheng G, Keshavjee S, Liu M. Self-assembling peptide-based nanoparticles enhance cellular delivery of the hydrophobic anticancer drug ellipticine through caveolae-dependent endocytosis. Nanomedicine. 2012; 8:647–654. [PubMed: 21889478]
59. Hirschfeld AF, Bettinger JA, Victor RE, Davidson DJ, Currie AJ, Ansermino JM, Scheifele DW, Orange JS, Turvey SE. Prevalence of Toll-like receptor signalling defects in apparently healthy children who developed invasive pneumococcal infection. Clin.Immunol. 2007; 122:271–278. [PubMed: 17157070]
60. Yan Z, Costanzo M, Heisler LE, Paw J, Kaper F, Andrews BJ, Boone C, Giaever G, Nislow C. Yeast Barcoders: a chemogenomic application of a universal donor-strain collection carrying bar-code identifiers. Nat Methods. 2008; 5:719–725. [PubMed: 18622398]
61. Smith AM, Heisler LE, Mellor J, Kaper F, Thompson MJ, Chee M, Roth FP, Giaever G, Nislow C. Quantitative phenotyping via deep barcode sequencing. Genome Res. 2009; 19:1836–1842. [PubMed: 19622793]
62. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G. GO::TermFinder--open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics. 2004; 20:3710–3715. [PubMed: 15297299]

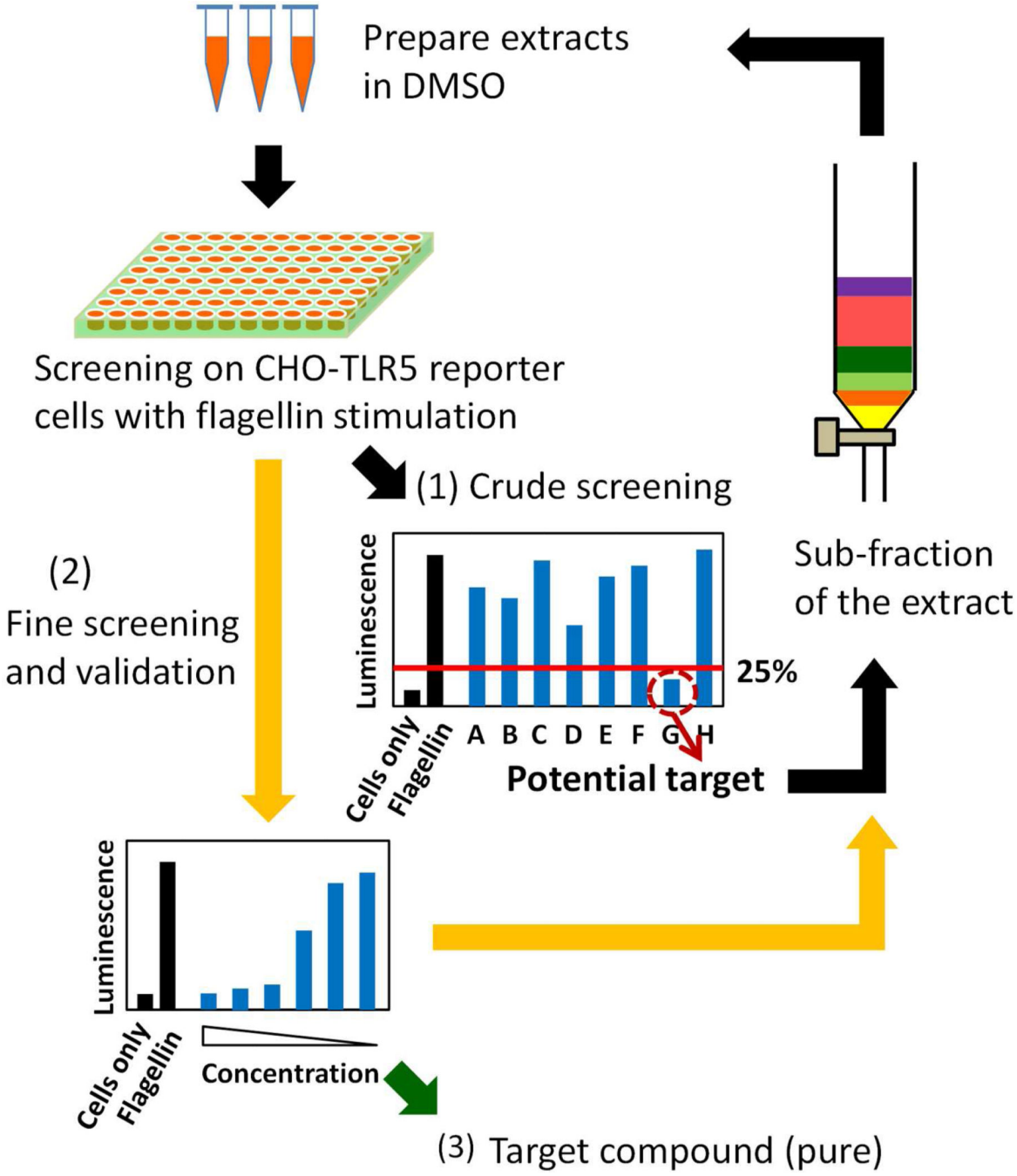

Figure 1.
Screening marine sponge extracts for TLR inhibitors.

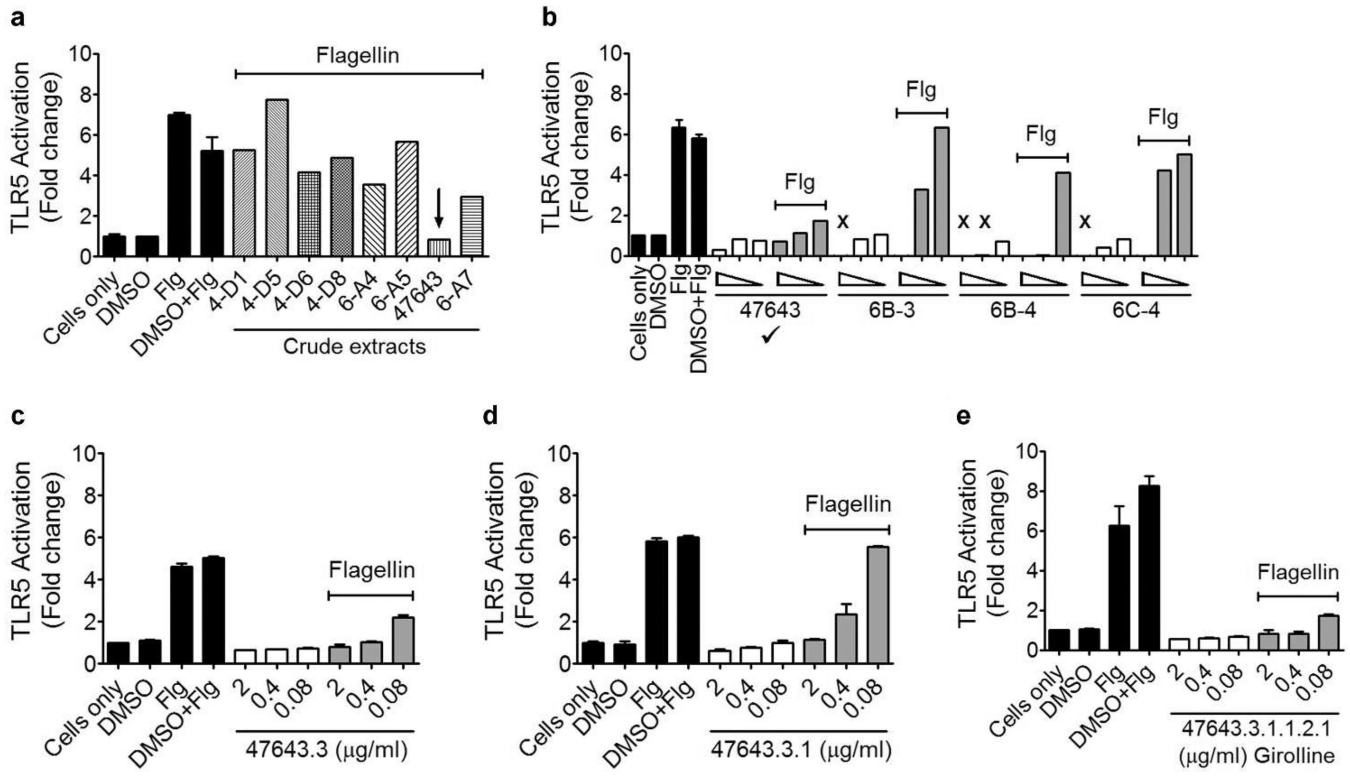

Figure 2.

Discovery of girolline as a potential inhibitor for TLR5 signalling from marine sponge extracts. (a) The first round of screening was performed on the crude extracts to identify potentially active extract fractions. (b) The second round was confirmatory examining dose-responsiveness and non-flagellin stimulated samples were used to investigate false positive results due to compound toxicity. X denotes extracts excluded from further analysis due to potential toxicity. (c–d) The confirmed positive crude extracts underwent further assay-guided fractionation until a pure compound was identified (e). Flg: flagellin.

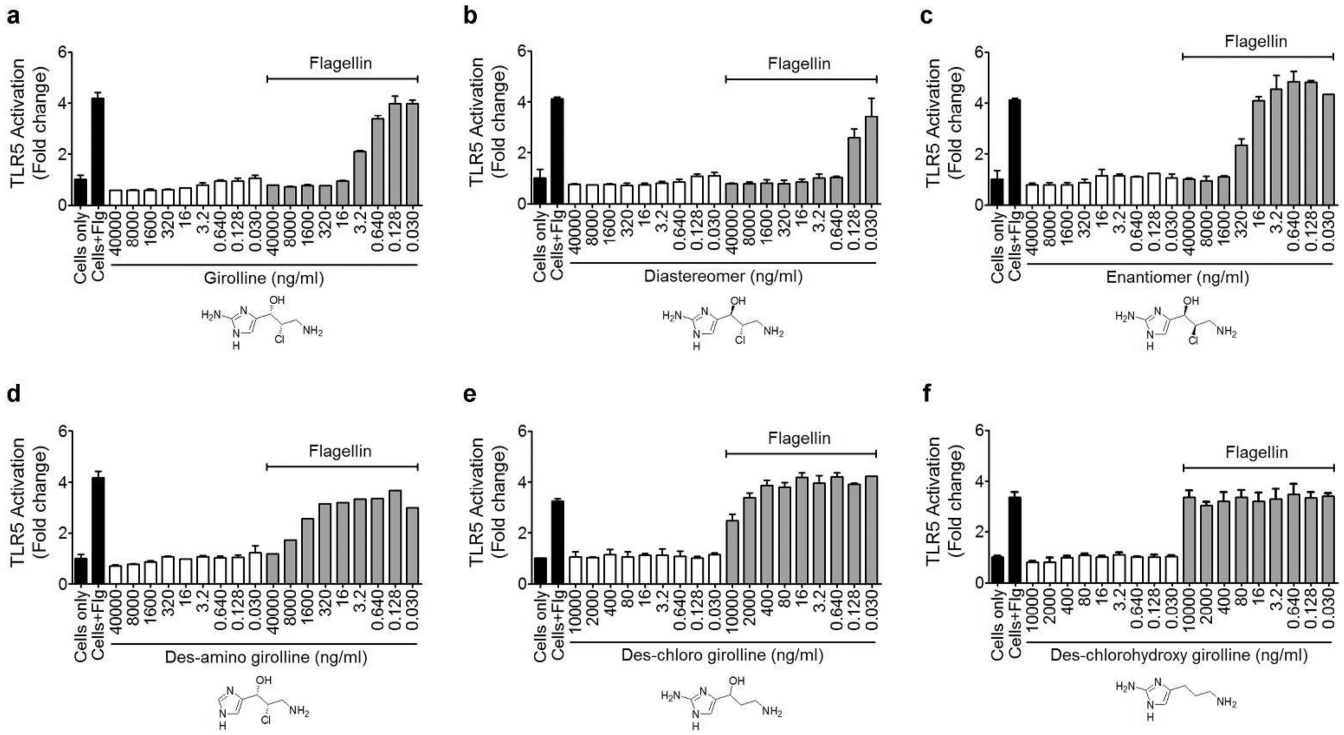

Figure 3.

Structure-activity relationship studies examining the inhibitory activity of girolline derivatives. (a) girolline; (b) diastereomer; (c) enantioner; (d) des-amino girolline; (e) des-chloro girolline; (f) des-chlorohydroxy girolline. Flg: flagellin.

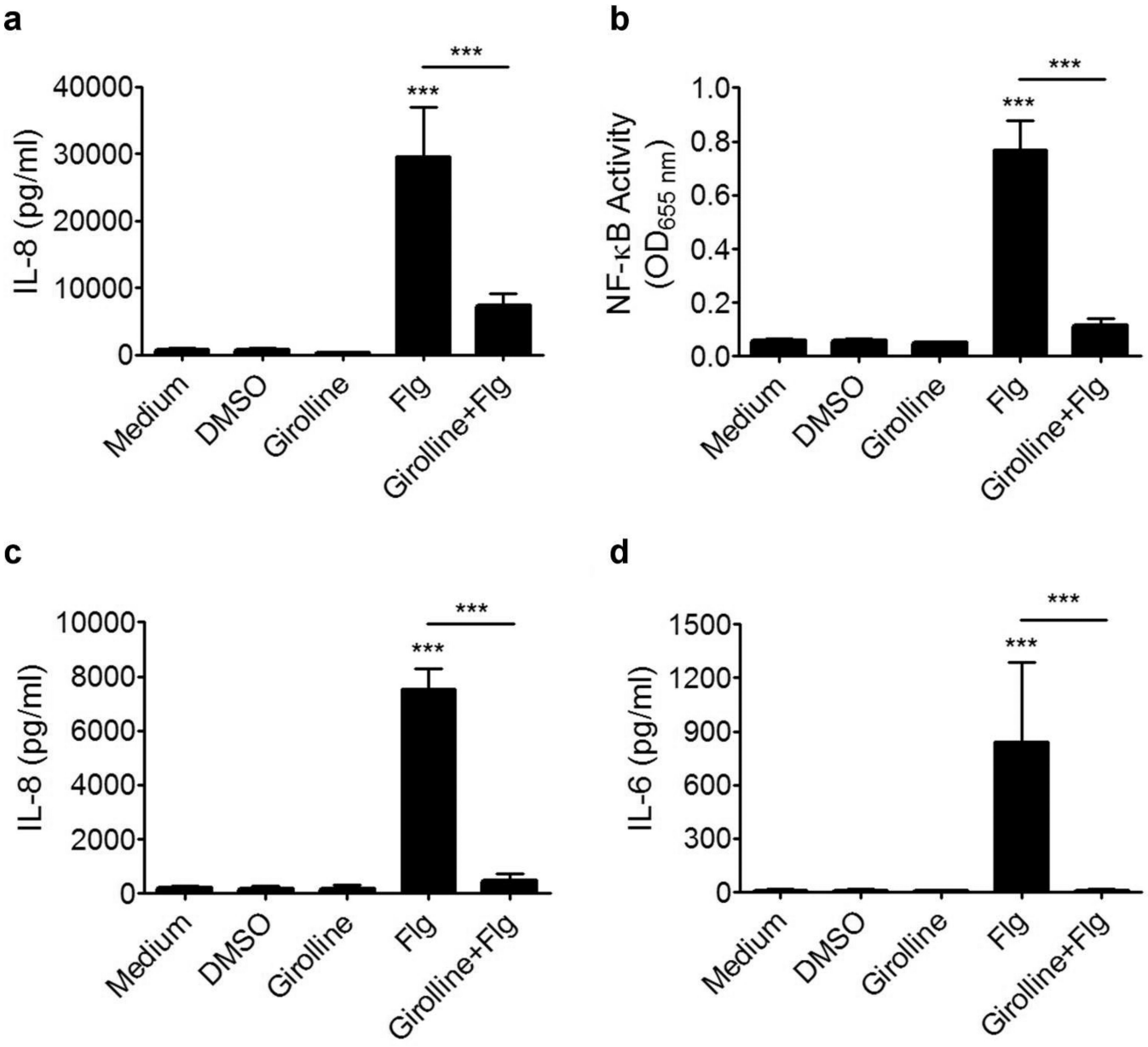

Figure 4.

Inhibitory activity of girolline on flagellin stimulated THP1 derived macrophages and fresh human PBMCs. Girolline pre-treatment decreased IL-8 secretion (a) and NF-κB activity (b) after flagellin stimulation. The same inhibitory activity was observed with PBMCs, where both IL-8 (c) and IL-6 (d) levels were significantly reduced after girolline pre-treatment. Flg: flagellin.

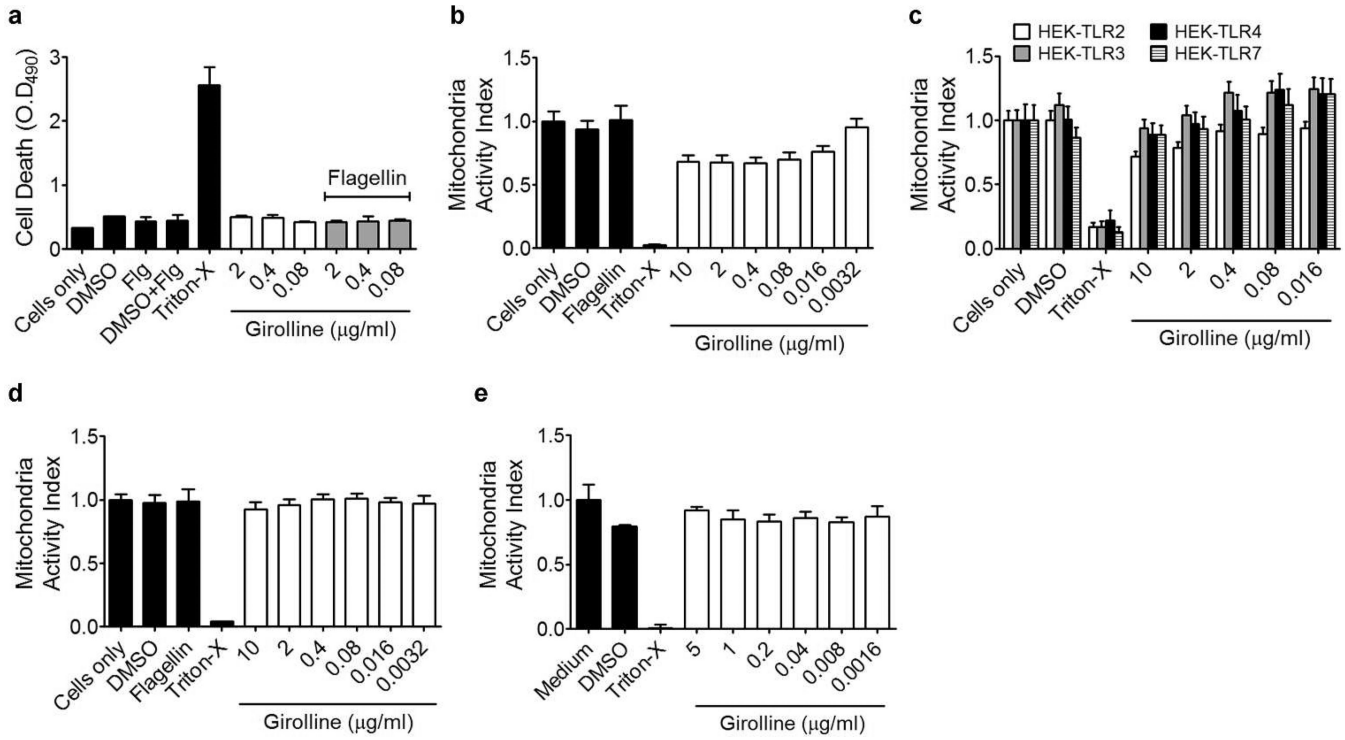

Figure 5.

Cytotoxicity of girolline examined in a number of cell types. Dose-dependent toxicity of girolline on CHO cells examined by (a) LDH release assay and (b) MTS assay. Same MTS assay was performed on HEK cells (c), THP1 derived macrophages (d) and fresh PBMCs (e). Flg: flagellin.

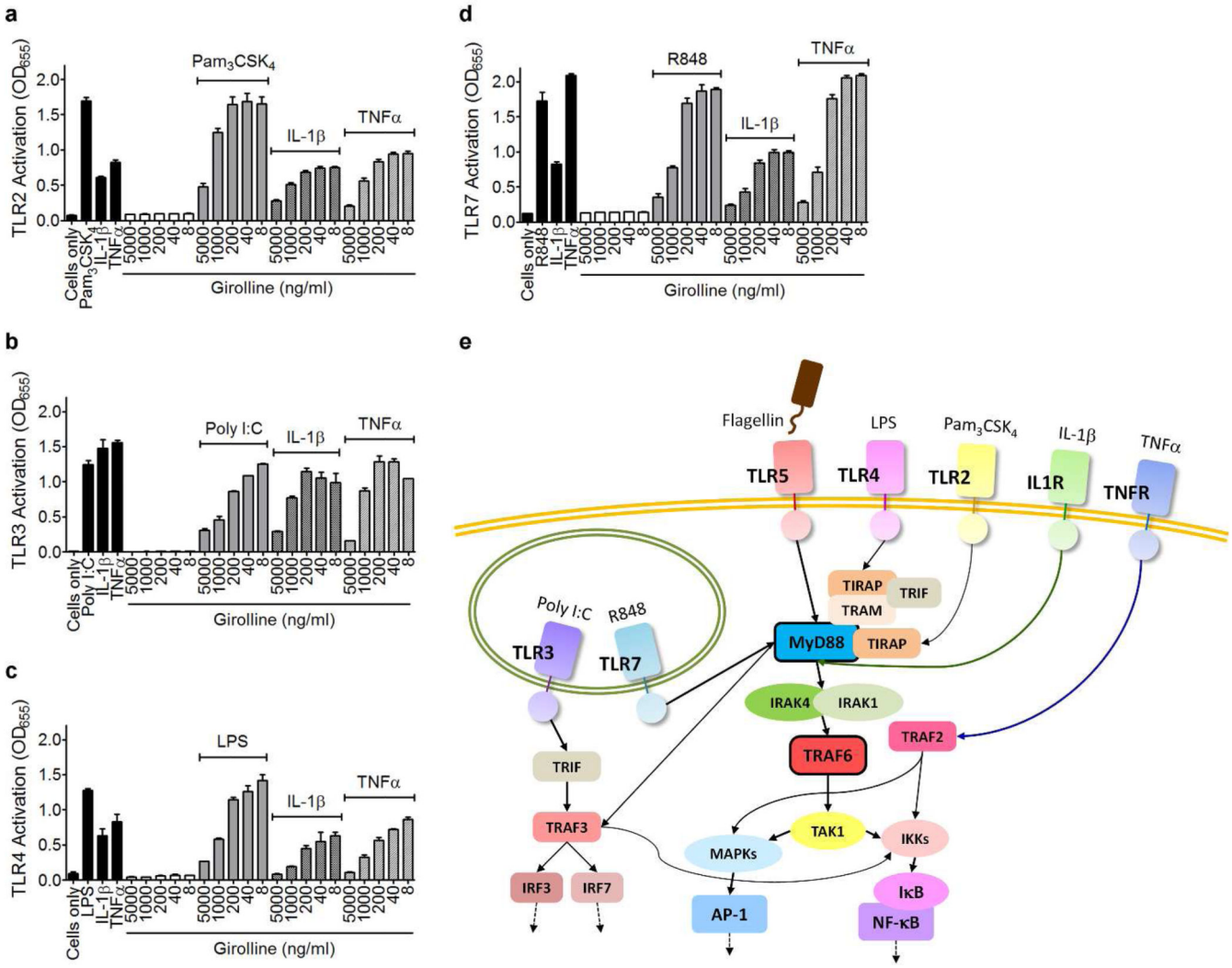

Figure 6.

The inhibitory activity of girolline on TLR signaling. Dose-dependent inhibition profiles were examined following stimulation of a variety of TLRs, as well as IL-1R and TNFR, using HEK-TLR2 (a), HEK-TLR3 (b) HEK-TLR4 (c) and HEK-TLR7 (d) reporter cells. A simplified TLR signaling pathway was illustrated in (e).

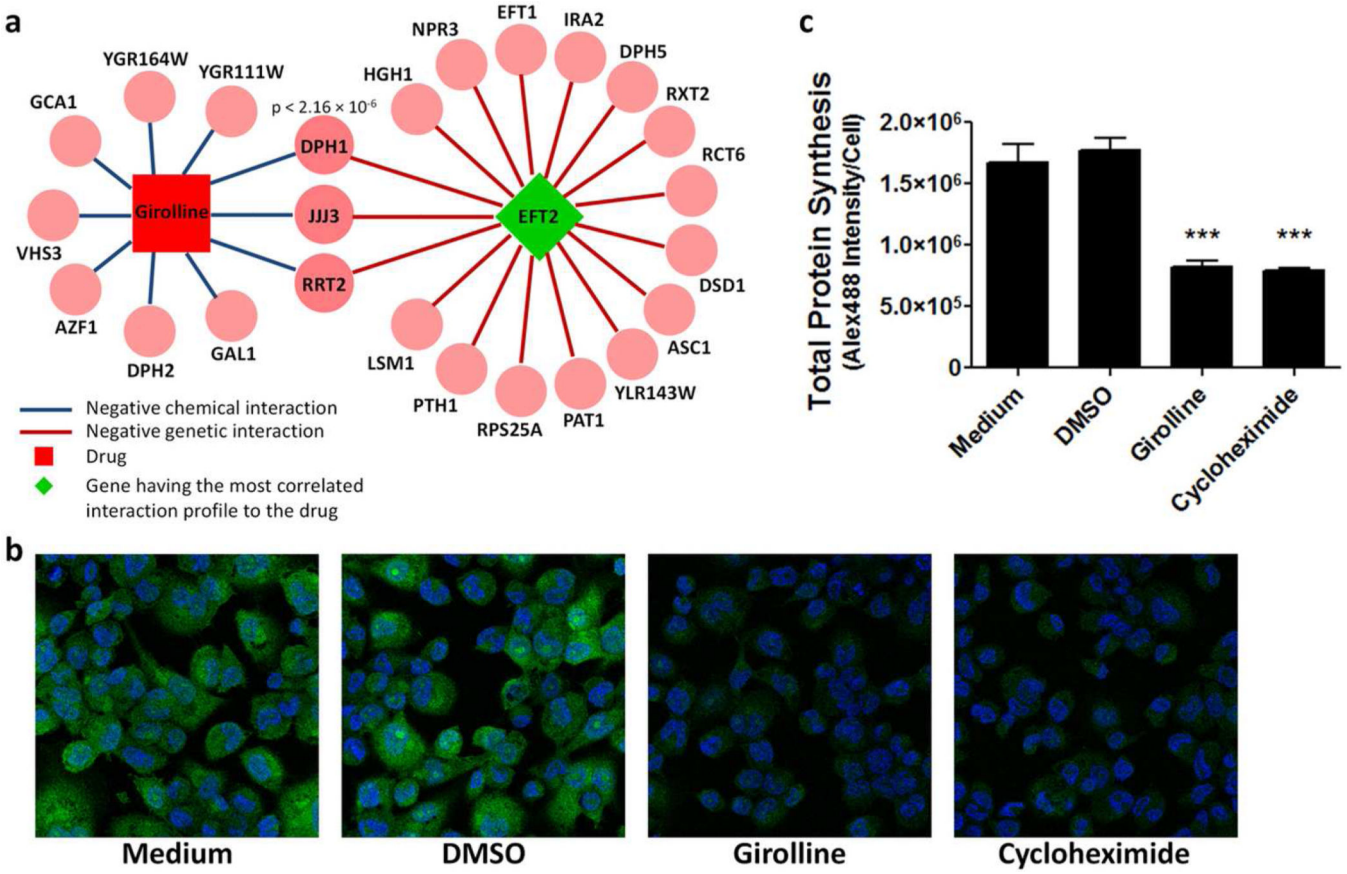

Figure 7.

Girolline acts as a protein synthesis inhibitor targeting Elongation Factor 2. (a) Chemical genomic profile of girolline showing that Elongation Factor 2 (Eft2) is the predicted target of girolline; the deletion mutants of genes that had described genetic interactions with *EFT2* had significant correlation with the gene mutants that had chemical genomic interactions with girolline. (b) Protein synthesis inhibition of girolline (2 μg ml^{-1}, 4 h) in comparison with cycloheximide (1 μM, 30 min); total new synthesized proteins in the cells are stained in green (Alex488) while nuclei are stained in blue (Hoechst 33342). (c) Quantitative analysis of Alex488 signals per cells in (b); analysis of 250–350 cells per sample in each experiment; n = 4, *** $p < 0.001$ vs. medium control. 116×76mm (300 × 300 DPI)