A highly selective inhibitor of interleukin-1 receptor-associated kinases 1/4 (IRAK-1/4) delineates the distinct signaling roles of IRAK-1/4 and the TAK1 kinase

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Running Title: Delineation of IRAK and TAK1 inflammatory processes

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ABSTRACT

Interleukin-1 receptor associated kinase-1 (IRAK-1) and IRAK-4, as well as transforming growth factor beta–activated kinase 1 (TAK1), are protein kinases essential for transducing inflammatory signals from interleukin receptors. IRAK family proteins and TAK1 have high sequence identity within the ATP-binding pocket, limiting the development of highly selective IRAK-1/4 or TAK1 inhibitors. Beyond kinase activity, IRAKs and TAK1 act as molecular scaffolds along with other signaling proteins, complicating the interpretation of experiments involving knock-in or knockout approaches. In contrast, pharmacological manipulation offers the promise of targeting catalysis-mediated signaling without grossly disrupting the cellular architecture. Recently, we reported the discovery of takinib, a potent and highly selective TAK1 inhibitor that has only marginal activity against IRAK-4. On the basis of the TAK1/takinib complex structure and the structure of IRAK-1/4, here we defined critical contact sites of the takinib scaffold within the nucleotide-binding sites of each respective kinase. Kinase activity testing of takinib analogs against IRAK-4 identified a highly potent IRAK-4 inhibitor (HS-243). In a kinome-wide screen of 468 protein kinases, HS-243 had exquisite selectivity toward both IRAK-1 (IC₅₀ 24nM) and IRAK-4 (IC₅₀ 20nM), with only minimal TAK1-inhibiting activity (IC₅₀ 0.5µM). Using HS-243 and takinib, we evaluated the consequences of cytokine/chemokine responses after selective inhibition of IRAK-1/4 or TAK1 in response to lipopolysaccharide (LPS) challenge in human rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). Our results indicate that HS-243 specifically inhibits intracellular IRAKs without TAK1 inhibition and that these kinases have distinct, non-redundant signaling roles.

The immune response to pathogens and tissue damage is an essential aspect of our biology. However, sometimes as a consequence of direct immune challenge, the immune system remains hyperactivated, leading to auto-immune disorders, which are maladaptive and induce chronic disease. Rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and ankylosing spondylitis have all been shown to be immune mediated and associated with local release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-6 at sites of tissue damage (1) (2) (3). The development of disease modifying anti-rheumatic drugs (DMARDs) such as anti-TNF drugs (i.e. Enbrel) and JAK inhibitors (Zeljanz) have transformed the therapeutic options available to auto-immune affected patients (4) (5). Nevertheless, despite advances in more molecularly targeted DMARDs, a significant portion of patients still fail to respond to current therapeutic options. Often this lack of response is attributed to individual genetic variations, tolerance and immune sensitization in the case of biologic based antibody therapies, leading to an unmet need for novel DMARD development (6) (7) (8).

One target area of great interest is the downstream kinases involved in the inflammatory signaling. In particular, there has been recent interest in the serine/threonine kinase interleukin-1 receptor associated protein kinases IRAK-1 and IRAK-4, as well as transforming growth factor beta-activated kinase 1 (TAK1). IRAK-4 is an essential protein kinase in mediating pathogen recognition and local cytokine release (i.e. IL-1, IL-6, TNF) through Toll-like receptor (TLR) signaling...
in response to prokaryotic lipopolysaccharides (LPS) (9) (10). Following inflammatory agonist binding (i.e. LPS, IL-1, TNF), MyD88 is recruited to the cytosolic receptor domain where it triggers downstream phosphorylation of IRAK-1/4, which further stimulates downstream NF-κB signaling and pro-inflammatory cytokine production (11). It has been shown that aberrant IRAK-4 signaling through hyperactive cytokine signaling is a key regulator of innate and adaptive immune cells in auto-immune diseases (12) (13) (14). Due to its role in inflammatory signaling, IRAK-4 has been the focus of numerous drug development platforms and recently advanced to clinical studies for auto-immune diseases (15) (16) (17) (18) (19).

Although previous groups have designed IRAK-4 inhibitors with modest potency, their selectivity within the human kinome has not been examined, begging the question of off-target effects in vitro and in vivo (20). In particular, off-target effects through TAK1 are likely, given the high sequence conservation between the binding pockets of IRAK-1/4 and TAK1; the amino acid sequence identity within the nucleotide binding pocket of all three proteins kinases is ~93%. Examination of the co-crystal structure of TAK1/takinib identifies critical binding sites of the N-acyl-aminobenzimidazole scaffold within the protein kinase, which when compared in molecular modeling studies with IRAK-1/4, reveals two amino acid variations that may allow for the development of an IRAK discriminatory molecule from the takinib backbone. Herein we identified a previously developed takinib analog, HS-243, as a potent and super-selective IRAK-1/4 inhibitor with minimal TAK1 activity (IC\textsubscript{50} ~0.5µM). HS-243’s IRAK-1/4 selectivity was further reflected in kinome wide testing against 468 human protein kinases, which showed a 38 and 15 fold change separation from HS-243’s IRAK-1/4 activity compared to the next best kinase inhibited at 10µM. Cellular assays indicate HS-243 has bioactivity and acts as a strong anti-inflammatory agent with distinct biological responses compared to the parent inhibitor, takinib.

RESULTS
Takinib analogs display varying potency against IRAK-4

Overlay of crystal structures of IRAK-4 (PDB 2NRU, shown in blue) and TAK1 (PDB 5V5N, shown in green) using PyMol shows high sequence and structural homology in the ATP binding pocket contributing to the affinity of takinib towards both kinases (Figure 1a). Takinib fit was assessed with both kinases placed in the DFG-in orientation. A surface representation indicates takinib lays within the IRAK-4 binding pocket with minimal steric clash (Figure 1b). However, despite the close 93% overall amino acid identity within the respective ATP binding pockets of TAK1, IRAK-4 (and IRAK-1) when aligned, significant amino acid variations are present, which provide a discriminatory axis for discovery of a takinib analog towards IRAKs over TAK1 (Figure 1c). Specifically, although the catalytic lysine K63 and aspartate D175 of the DFG motif align, and critical hydrophobic interactions of takinib-TAK1 binding mediated by Y106 and G110 are also congruent between IRAK-1/4 and TAK1, residues preceding the DFG motif are quite distinctive. Furthermore, modeling of IRAK-4 inhibitors suggest π-π stacking interactions with Tyr-262 of IRAK-1/4, which is absent in takinib-TAK1 interactions.

To identify a novel IRAK-1/4 inhibitor, we tested a series of takinib analogs against IRAK-4. Figure 2a outlines regions of the parent takinib scaffold
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subjected to modification (see methods). Analogs were tested in parallel against purified recombinant IRAK-4 in an in vitro kinase assay and identified HS-238 (643nM), HS-242 (149nM), and HS-243 (48nM) as more potent IRAK-4 inhibitors compared to the parent compound takinib (Figure 2b,c). When tested at 1μM, HS-243 showed 92% inhibition of IRAK-4 compared to takinib at 56% (Figure 2d). We next sought to compare HS-243 to the previously described N-acylaminobenzimidazole (commercially sourced by Sigma; Sigma IRAK-1/4) by Powers et. al (18,21,22). The potency of HS-243 compared favorably to Sigma IRAK-1/4, which exhibited an IC₅₀ 134nM in our assay (Figure 2e). The improved IRAK-4 potency of HS-243 over the parent compound takinib is related to the R3 position replacement of the carbamide to a nitro group. Other modifications, particularly in the R2 site, reduced potency towards IRAK-4, indicating the addition of functional groups at this position sterically hinders binding (Figure 2b). It is important to note that IRAK-4 has a relatively high Km for ATP at ~500μM (Supplemental 1s). Therefore, to ensure optimal kinase activity in vitro and meaningful inhibition constants for all our analogs, assays for this protein kinase were conducted well above IRAK-4 Km at 2mM. Prior studies by others against IRAK-4 compounds, such as Sigma IRAK-1/4 were performed well below the true IRAK-4 Km for ATP, leading to an overestimation of the Kᵢ, and potentially lack of selectivity and potency in vivo at physiological [ATP] (18,21,22).

**HS-243 displays exquisite selectivity towards IRAK-1 and 4 over all other human kinases**

We next sought to test the selectivity of leading IRAK-4 analogs against TAK1 to identify kinase specific compounds (Figure 3a). As previously reported, HS-206 (takinib) showed greater affinity towards TAK1 than IRAK-4, whereas HS-238 and HS-242 showed improvements in IRAK-4 potency, exhibiting IC₅₀'s of 643nM and 149nM respectively, compared to takinib (Figure 3b). However, they retained significant TAK1 activity and are dual TAK1/IRAK-4 inhibitors (Figure 3c,d). In contrast, HS-243 showed exquisite potency towards IRAK-1/4 (20 and 24nM) with a ~23 fold difference in IC₅₀ towards TAK1 activity (IC₅₀ >500μM), representing a selective IRAK-1/4 inhibitor (Figure 3e).

To understand the structural basis for selectivity we performed unconstrained molecular docking of HS-243 in the ATP binding pocket of IRAK-4 (PDB 2NRU) and compared it with the x-ray structure of takinib bound to TAK1. The binding modes were similar overall but highlight interactions that might explain changes in potency and selectivity. In particular, we note hydrogen bonds between the Tyr-262 and a bridging water that interacts with the nitro group of HS-243. Tyr-262, which is a methionine in TAK1, is also predicted to form π-π stacking interactions with the nitrobenzyl component. This is an interaction that cannot occur in TAK1 because of the sidechain difference, which may account for greater specificity of HS-243 toward IRAK-4 (Fig 4). Furthermore, hydrophobic interaction with residues Val-263, Tyr-264, Met-265, Pro-266 may contribute to drug stability and potency (Figure 4a, Supplemental 2s).

We next sought to further validate the π-π stacking interactions between the nitrobenzyl group of HS-243 and Y262 of IRAK-4. Previous work by Wang et al., has shown Y262 acts as a tyrosine gatekeeper in IRAK-4 and plays a critical role in maintaining the active orientation of the kinase (22). Consistent with previous literature, we found IRAK-4 Y262 mutants showed significantly reduced kinase activity.
compared to wild type IRAK-4 further supporting the position that Y262 plays an integral role in maintaining active kinase orientation (Figure 4c). Additionally, titration of HS-243 against IRAK-4 Y262T or Y262A mutants showed minimal to no % inhibition at 0.3 and 1µM (Figure 4d).

Following identification of a lead selective compound with greater potency towards IRAK-4 (HS-243) over TAK1, we further evaluated its selectivity across 468 distinct human protein kinases at 10µM HS-243. Remarkably, in this study HS-243 showed exquisite selectivity and potency towards IRAK-4 and closely related IRAK-1, inhibiting the kinases 98.6% and 99.35%, respectively (Figure 5a,b,c, Supplemental Table 1s). To tease out the selectivity of HS-243 further, we carried out titrations against the top 5 protein kinases identified in the one shot 10µM screen. The Kd for HS-243 towards IRAK-1 was 24nM and IRAK-4 20nM, followed by 423nM for TAK1, 662nM for CLK4 and 2,278nM for DYKR1B (Figure 5d). This study further highlighted the selectivity of HS-243 to the IRAKs over all other protein kinase, with next best inhibited protein kinases being TAK1, and non-related CLK4 and DYRK1B at 21, 33 and 105-fold less sensitivity, respectively. Importantly, IC₅₀ values for HS-243 against TAK1 determined by our in-house enzymatic assay agreed closely with values determined independently in the commercial kinome wide study. Finally, to rule out non-protein kinase off target binding, we next explored the selectivity of HS-243 against an array of known purine binding receptors/enzymes including 5-HT receptors, opioid receptors, Beta receptors, GABAA, SERT and D, H and M receptors. No significant binding was found within the broader purinome, further highlighting the exquisite selectivity of HS-243 towards IRAK-1 and 4 (Supplemental Figure 3s).

**HS-243 potently reduces the pro-inflammatory response of RA cells and macrophages**

Clinical indications for IRAK-1/4 inhibitors have been in auto-immune diseases where maladaptive pro-inflammatory signaling is a key signature of disease. To test and compare the anti-inflammatory effects of HS-243 (IRAK-1/4) against Sigma 1/4, HS-242 (dual IRAK-1/4, TAK1), and HS-206 (takinib) (TAK1), we stimulated human isolated RA-FLS cells with LPS followed by treatment with indicated drug at 10µM. Cytokine array analysis of 36 various cytokines and chemokines indicated that robust changes in drug effects were seen, highlighted by significant effects of HS-243, Sigma 1/4 and HS-242. IRAK-1/4 inhibitors displayed distinct effects on CCL5, CXCL12, MIF and IL-18 protein expression levels when compared to TAK1 inhibition with takinib (Figure 6a-e). Furthermore, overall distinct cytokine profiles were observed with significant differences in overall cytokine/chemokine effects between (HS-243 – Sigma 1/4; p<0.002), (DMSO – HS-243; p<0.0002), (DMSO – Sigma 1/4; p<0.0001), and (DMSO – HS-242; p<0.0001) (Figure 6f). A general schematic of IRAK-1/4 signaling following pro-inflammatory stimuli is shown (Figure 6g).

Due to the selectivity and potency of HS-243 towards IRAK-1/4, we next explored the potential of this compound to reduce inflammatory signaling in THP-1 human macrophages challenged with LPS (100 ng/mL). Following binding to the TLR4 receptor, LPS stimulates the IRAK-1/4 pathway of pro-inflammatory cytokine signaling which leads to NF-κB activation. Immediately following LPS stimulation, THP-1 macrophages were treated with 10µM HS-243 or vehicle, and supernatant was collected 24 hours later. In a panel of 105 cytokines/chemokines, HS-243
significantly reduced the secretion of 15 cytokines, including IL-8 (p<0.003), CD14 (p<0.002), GRO-α (p<0.001), MIP-1α (p<0.007), MIP-3α (p<0.002), uPAR (p<0.014), Osteopontin (p<0.018), MMP-9 (p<0.001), MCP-1 (p<0.011), I-TAC (p<0.001), TIM-3 (p<0.016), IP-10 (p<0.014), GDF-15 (p<0.006) and RANTES (p<0.015) (Figure 7a-m, Table 2s). This profile contrasts dramatically with that reported previously with takinib, which showed discrete ~9 fold inhibition of TNF release with little impact on any other cytokines (23,24). Whereas the discrete actions of takinib in the LPS challenge model are consistent with selective TAK1 inhibition in cells, the broader actions of HS-243 reported herein are consistent with the expected outcomes of IRAK-1/4 inhibition.

**IRAK-1/4 inhibition reduces % survival in pancreatic and breast cancer cell lines**

Previously TAK1 has been shown to play an integral role in mediating TNF induced apoptosis representing a novel therapeutic axis in certain cancers (19). Due to high homology between TAK1 and IRAK-1/4 we were interested in the effects of IRAK-1/4 inhibition on cancer cells. To evaluate the potential anti-cancer properties of IRAK-1/4 inhibition we tested HS-243 against 7 cancer cell lines. At 10µM, HS-243 inhibited cell survival by 21% for AN3-CA, a pancreatic cancer cell line and 13% for SKOV-3 (Figure 8). Furthermore, previous studies with TAK1 inhibitors have shown that inhibition of inflammatory kinases in cancers in conjunction with exogenous upstream ligand stimulation can induce apoptosis. In an effort to understand IRAK-1/4 role in mediating exogenous interleukin signaling and downstream signaling pathways we stimulated various cancer cell lines with IL-1β (30 ng/mL) and HS-243 (10µM) for 24 hours. Addition of IL-1β in conjunction with HS-243, inceased cell death to 46% in SK-OV-3 (ovarian), 33% in AN3-CA (pancreatic) , and 31% in H460 (colon) (Figure 8).

**DISCUSSION**

Evidence supporting the IRAKs as therapeutic targets in auto-immune diseases stems largely from genetic data around IRAK-4. Mutations within the IRAK-4 locus result in premature stop codons and are associated with loss of functional expression of the protein kinase(25). These cause a unique immunodeficiency in early stages of life, rendering adolescents susceptible to certain bacterial infections(26) (25). Interestingly, this impairment resolves during adulthood. IRAK-4 deficient patients show retention of neutrophil function through TLR-9 signaling mechanism, highlighting the role of the protein kinase in regulating inflammatory pathways (27). At the time of writing, ATP competitive inhibitors targeting IRAK-4 are under investigation in clinical trials due to their potential in auto-immune disorders (28) (29). However, because of the close structural similarities within the ATP binding pocket of the IRAK family and TAK1, the relative contributions and consequences of pharmacological inhibition of both protein kinases in vivo have not been considered. Whereas polypharmacological inhibition of both TAK1 and IRAK-4 may be advantageous, its equally plausible such inhibitors could have a limited therapeutic window or result in off target actions. Development of highly selective pharmacological inhibitors that discriminate between these protein kinase families in vivo is therefore highly desirable. Here, we have shown that the takinib scaffold can serve as an effective starting point to develop inhibitors that are specific for IRAK-1 and 4 while dialing out their closest homolog, TAK1. Significantly, HS-243 retained the
exquisite selectivity across the kinome as observed with the original parent scaffold, takinib (19). In the case of HS-243 only, IRAK-1 and 4 are targeted, rather than TAK1, and no other protein kinase is completely inhibited at 10µM. This study suggests that the core takinib aminobenzimidazole molecule is intrinsically tailored towards TAK1 and closely related IRAK family members and can be effectively utilized to develop super-selective inhibitors for each member, and no other protein kinase. This selectivity can be extended to all members of the larger purinome (all proteins that bind purines), as evidence by the partial purinome screen carried out in this study, and the wider purinome specificity study reported previously with takinib by screening against immobilized gamma-phosphate ATP charged with whole cellular homogenates (19,30). To our knowledge, no other protein kinase inhibitor scaffold has exhibited such intrinsic selectivity towards a specific subset of protein kinases. In support of intracellular selectivity, RA-FLS cells immune challenged with LPS and treated by HS-243 showed reduced pro-inflammatory signaling that is distinct from takinib (23). This data supports the idea that IRAK-1/4 and TAK1, despite participating in similar pathways, have distinct druggable responses, creating discrete patterns of cytokine inhibition. The role of IRAK-1/4 in mediating inflammatory signaling is well established and IRAK-4 inhibitors are advancing through clinical trials. However, the kinome wide selectivity of leading the IRAK-4 inhibitors is not available, opening up the possibility that a significant portion of the observed efficacy with these inhibitors may be due to TAK1 inhibition. Therefore, advancement of HS-243 along the clinical path may provide a second-generation series of inhibitors displaying well defined kinome selectivity potentially reducing off target side effects often conferred upon promiscuous kinase inhibitors.

Experimental Procedures

Synthesis of molecules
See supplemental data.

Kinome Screens
Kinome wide screens were carried out independently by DiscoverX Inc. (CA) and kinase assay by the University of Dundee’s (UK) kinome consortium.

Protein Purification
TAK1-TAB1 (kinase domain residues 31-303 fused with 36 residues of TAB1 c-terminal domain residues 468-504) was expressed and purified as reported previously (30). In summary, His-TAK1-TAB1 was expressed in insect cells and purified using His-affinity column followed by TEV cleavage and finally size exclusion chromatography. Protein purity was verified using a gradient SDS-PAGE gel stained with Coomassie blue. Protein concentration was measured using the Bradford method (31). IRAK-4 N-GST (DU 8853) was purchased from the MRC 1 PPU, College of Life Sciences, University of Dundee, Scotland, mrcppureagents.dundee.c.uk. IRAK-4 Y262T (DU4684) and Y262A (DU8865) human plasmid expression vectors were purchased from MRC 1 PPU at the University of Dundee. HEK293T cells were transfected with 2µg of HA-IRAK-4 Y262A or Y262T plasmid with X-tremeGENE HP DNA transfection reagent for 24 hours and purified using HA-affinity resin.

Kinase Assay
Activity of purified IRAK-4 protein was measured as previously described (32). In brief, IRAK-4 (20 ng/well) was incubated with 2mM ATP containing radiolabeled...
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[32P]-ATP in the presence of 300µM substrate peptide in a final volume of 40µl in the presence of buffer (containing 50mM Tris pH 7.5, 0.1mM EGTA, 0.1% β-Mercaptoethanol, 10mM magnesium acetate, 0.5mM MnCl) and indicated compounds. The reaction was terminated at 20 minutes with 10µL of 1M H3PO4. The remaining activity was measured using a scintillation counter. Dose-response curves were repeated two times and averaged.

Binding and functional assays of purinome binding proteins
Binding of 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT3, 5-HT5A, 5-HT6, 5-HT7A, Alpha 1A, Alpha 1B, Alpha 1D, Alpha 2A, Alpha 2B, Alpha 2C, Beta 1, Beta 2, Beta 3, BZP Rat Brian Site, D1, D2, D3, D4, D5, DAR, DOR, GABAA, H1, H2, H3, H4, KOR, M1, M2, M3, M4, M5, MOR, NET, PBR, SERT, Sigma 1, Sigma 2, NOP were tested by UNC PDSP following previously established radioligand binding assays (33).

Cell culture
THP-1 human macrophages were obtained from American Type Culture Collection (ATCC). Cells were incubated at 37°C in 5% CO2. THP-1 were cultured in RPMI 1640X, 10% FBS, 1% Penicillin-Streptomycin (PS), HEPES, Pyruvate, Glucose and BME. RA-FLS cells were isolated as previously reported and cultured no more than 9 passages in CRML media, 10% FBS and 1% PS. Cancer cell lines, AN3 CA, BxPC-3, SK-UT-1B, ES-2, SKOV-3, H460 and COLO 205 were cultured according to ATCC media guidelines.

Macrophage Differentiation
THP-1 cells were treated with 100nM phorbol 12-myristate 13-acetate (PMA) for 72 hours in RPMI 1640X media. Cells were rested in PMA free media 24 hours prior to treatments. LPS (10 ng/mL) was used for pro-inflammatory stimulation.

Cytokine/Chemokine proteome Profile
RA-FLS cells or THP-1 cells were treated with 10µM HS-243 or vehicle (DMSO) and 24 hours after treatment, supernatant was added to Human Cytokine XL proteome array (R&D Systems) in accordance with manufacturer protocol. Chemiluminescence was used to visualize protein quantities.

Cell Viability Assay
Cells were cultured and treated as previously described. Briefly, cells were plated at 80% confluency ~10^4 in 96 well plates and treated with either takinib at various concentrations or takinib + IL-1β (30 ng/mL) for 24 hours and compared to vehicle treated samples. Cell death was quantified using Cell Titer Glo 2.0 (Promega) according to the manufacturer’s protocol.

Quantification and Statistical Analysis
GraphPad Prism 8 was used for statistical analysis. For each analysis, total n and SEM are presented in the figure legend. An alpha of 0.05 was used for all statistical analysis.

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Psychoactive Drug Screening Program, Contract # HHSN-271-2018-00023-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. TAJH, PFH and SAS have filed patent claims on all molecules related to the takinib scaffold described in this body of work.

Conflicts of Interests:
TAJH, SAS and PH all part owners of EydisBio, Durham, NC.

Author Contributions:
SAS, PFH, KWY contributed the synthesis and biochemical/biological assays. KDW, DG and DAC performed modeling and comparative analysis between kinases. TAJH and SAS wrote the paper and directed all studies. All authors contributed to figure design and manuscript preparation.

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Figures

Figure 1
Structural comparison of TAK1 against IRAK-1 and 4. (a) Overlay of the ribbon structure of IRAK-4 and TAK1. (b) Close up view of the surface representation of takinib in the ATP binding pocket of TAK1 and modeled into IRAK-4. (c) Amino acid sequence alignment of TAK1, IRAK-4, and IRAK-1 highlighting key amino acid differences.
Figure 2
Structure-activity studies of takinib analogs against IRAK-4. (a) Core aminobenzimidazole scaffold showing regions targeted for modification. (b) Outline of modifications made during the SAR campaign and corresponding IC$_{50}$ determined by mean IRAK-4 radioactive [32P]ATP filter-binding assay inhibition from vehicle. (c) Titrations of takinib analogs against IRAK-4, presented as % inhibition from vehicle control, data point represent mean ± SEM (n = 2). (d) Comparison of % inhibition of DMSO, takinib (1µM), and HS-243 (1µM) against IRAK-4. (e) Dose dependent response of HS-243 compared to commercially available Sigma IRAK-1/4 compound. Data points represent mean ± SEM (n = 2).
Figure 3
Structure and in vitro profiling of lead compound and other analogs against IRAK-4 and TAK1. (a) Structures of HS-243 IRAK-1/4 inhibitor, HS-242 dual TAK1/IRAK-4 inhibitor, HS-206 (takinib) TAK1 inhibitor and HS-238 mixed TAK1/IRAK-4 inhibitor. (b) Data shows kinase inhibition generated for each indicated analog against purified IRAK-4 and TAK1. Data points represent mean ± SEM (n = 2).
Figure 4
(a) Docking pose of HS-243 in the ATP binding pocket of IRAK-4. π-π stacking interactions between HS-243 and Tyr-262 are in pink dashes. Other interacting residues are labeled. Hydrogen bonds are shown with green dashes. (b) Comparison with TAK1 (5V5N). (c) Kinase activity of IRAK-4 (WT), IRAK-4 (Y262A) and IRAK-4 (Y262T) tyrosine gatekeeper mutants. (d) Titrations of HS-243 against purified IRAK-4 Y262 mutants (n=2).
Delineation of IRAK and TAK1 inflammatory processes

Figure 5
Selectivity of HS-243 within the human kinome. (a) Kinome wide screening of HS-243 against 468 human kinases. (b) % inhibition of top kinase hits from kinase screen (% of control from vehicle). (c) Dendrogram of human non-oncogenic kinases show selectivity of HS-243 in the human kinome. Size of dot indicative of kinase potency. (d) IC$_{50}$ of top 5 kinases as determined by DiscoverX kinase assay. Data points represent mean ± SEM (n = 2). (e) IC$_{50}$ of HS-243 against IRAK-4, IRAK-1, TAK1, CLK4, and DYKR1B (n=2).

| Kinase   | IC$_{50}$ (nM) | SD  |
|----------|----------------|-----|
| IRAK-4   | 20             | 6.8 |
| IRAK-1   | 24             | 3.3 |
| TAK1     | 423            | 3.9 |
| CLK4     | 662            | 26.2|
| DYKR1B   | 2,278          | 9.5 |
**Figure 6**

IRAK-1/4 inhibition has distinct cytokine profile from TAK1. Human rheumatoid arthritis fibroblast like synoviocytes (RA-FLS) were stimulated with LPS and treated with either DMSO, HS-243, Sigma 1/4, takinib, or HS-242 at 10µM for 24 hours before cytokine profiling. (a) Representative cytokine proteome arrays with indicated cytokine positioning. Quantification of
(b) CCL5, (c) CXCL12, (d) MIF and (e) IL-18 expression. * indicates differences between DMSO and treatment, # different from HS-243. Data points represent mean ± SEM (n = 4). (f) Heat map of 30 cytokines/chemokines expression. (g) Schematic of IRAK-1/4 and TAK1 signaling. *p<0.05, **p<0.01, ***p<0.001.

**Figure 7**

THP-1 human macrophages were differentiated with PMA for 72 hours followed by a 24 hour rest period in PMA free media. Cells were stimulated with LPS (10 ng/mL) immediately followed by 10µM HS-243. 105 cytokines/chemokines were profiled from the supernatant of the media. (a,b) Significant changes seen in MIP-3a, Tim-3, uPAR, RANTES, Osteopontin, MMP-9, MIP-1α, MCP-1, I-TAC, IL-8, GDF-15, IP-10, and chitinase-3 like 1 protein expression. (n = 3-4), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 8
% survival of IRAK-1/4 inhibited cancer cells. (a) % survival of 7 cancer cell lines treated for 24 hours in the presence of 10µM HS-243 or HS-243 (10µM) and IL-1β (30 ng/mL). Data points represent mean (n = 2-4).
A highly selective inhibitor of interleukin-1 receptor-associated kinases 1/4 (IRAK-1/4) delineates the distinct signaling roles of IRAK-1/4 and the TAK1 kinase
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