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Discovery of Dual Inhibitors of the Immune Cell PI3Ks p110δ and p110γ: a Prototype for New Anti-inflammatory Drugs

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SUMMARY

PI3Kδ and PI3Kγ regulate immune cell signaling, while the related PI3Kα and PI3Kβ regulate cell survival and metabolism. Selective inhibitors of PI3Kδ/γ represent a potential class of anti-inflammatory agents lacking the antiproliferative effects associated with PI3Kα/β inhibition. Here we report the discovery of PI3Kδ/γ inhibitors that display up to 1000-fold selectivity over PI3Kα/β and evaluate these compounds in a high-content inflammation assay using mixtures of primary human cells. We find selective inhibition of only PI3Kδ is weakly anti-inflammatory, but PI3Kδ/γ inhibitors show superior inflammatory marker suppression through suppression of lipopolysaccharide-induced TNFα production and T cell activation. Moreover, PI3Kδ/γ inhibition yields an anti-inflammatory signature distinct from pan-PI3K inhibition and known anti-inflammatory drugs, yet bears striking similarities to glucocorticoid receptor agonists. These results highlight the potential of selectively designing drugs that target kinases with shared biological function.

INTRODUCTION

Inflammatory disorders such as rheumatoid arthritis represent an important target for drug development. Therapies include naproxen, indomethacin (Backhouse et al., 1980), and corticosteroids (Gray et al., 1991). Although effective, these agents have significant side effects that limit their utility (Gray et al., 1991; Rainsford, 1993). More recently, antibody therapeutics directed against tumor necrosis factor α (TNFα) have become useful for treatment of refractory chronic inflammation (Feldmann, 2002; Feldmann and Maini, 2001). These agents reduce inflammation and slow disease progression (Feldmann, 2002; Feldmann and Maini, 2001; Imperato et al., 2004), but are expensive and can generate immune-related side effects, including infection and lymphoma emergence (Imperato et al., 2004).

Recently, targeted inhibitors of the phosphoinositide-3-kinase (PI3K) pathway have been suggested as immunomodulatory agents (Hirsch et al., 2008; Rommel et al., 2007). This interest stems from the fact that the PI3K pathway serves multiple functions in immune cell signaling, primarily through the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), a membrane-bound second messenger (Cantley, 2002; Deane and Fruman, 2004; Hirsch et al., 2008; Katso et al., 2001). PIP_3 recruits proteins to the cytoplasmic side of the lipid bilayer, including protein kinases and GTPases (Cantley, 2002; Hirsch et al., 2008; Katso et al., 2001), initiating a complex network of downstream signaling cascades important in the regulation of immune cell adhesion, migration, and cell-cell communication.

The four class I PI3K isoforms differ significantly in their tissue distribution. PI3Kα and PI3Kβ are ubiquitous and activated downstream of receptor tyrosine kinases (RTK) (Hirsch et al., 2008; Katso et al., 2001), whereas PI3Kδ and PI3Kγ are primarily limited to hematopoietic (Deane and Fruman, 2004; Rommel et al., 2007) and endothelial cells (Puri et al., 2004, 2005), and are activated downstream of RTKs, and G protein coupled receptors (GPCR), respectively (Katso et al., 2001). Mouse genetic studies have revealed that PI3Kα and PI3Kβ are essential for normal development (Vanhaesebroeck et al., 2005), whereas loss of PI3Kδ and/or PI3Kγ yields viable offspring with selective immune deficits (Okkenhaug and Vanhaesebroeck, 2003; Swat et al., 2006; Vanhaesebroeck et al., 2005; Webb et al., 2005). The expression pattern and functions of PI3Kδ and PI3Kγ have generated much interest in developing PI3Kδ/γ inhibitors as agents for many diseases, including rheumatoid arthritis, allergies, asthma, chronic obstructive pulmonary disease and multiple sclerosis (Hirsch et al., 2008; Marone et al., 2008; Rommel et al., 2007; Ruckle et al., 2006). Studies using both pharmacologic and genetic methods have shown these two isoforms often demonstrate synergistic interactions with each other (Konrad et al., 2008; Laffargue et al., 2002). In mast cells, for example, PI3Kδ is essential for degranulation in response to IgE cross-linking of Fc-receptors (Ali et al., 2004; Ali et al., 2008), but PI3Kγ plays an important role in amplifying the response

(Laffargue et al., 2002). Similar effects have been seen in other cellular functions, including lymphocyte homing (Reif et al., 2004) and the neutrophil respiratory burst (Condliffe et al., 2005), where PI3Kγ plays a critical role and PI3Kδ amplifies each process.

The nonredundant but related roles of PI3Kδ and PI3Kγ have made it difficult to determine which of the two isoforms (alone or in combination) is best targeted in a particular inflammatory disorder. Studies using mice that lack PI3Kδ and/or PI3Kγ or express kinase-dead variants of PI3Kδ and PI3Kγ have been valuable tools in understanding their roles. For example, PI3Kδ knockout mice demonstrated diminished neutrophil chemotaxis (Puri et al., 2004), diminished antibody production (both T cell dependent and independent) (Jou et al., 2002), and lower numbers of mature B cells (Clayton et al., 2002; Jou et al., 2002), and a decrease in their proliferation in response to anti-IgM (Jou et al., 2002) . This phenotype was replicated in the PI3Kδ kinase-dead variant (Okkenhaug et al., 2002), and with PI3Kδ selective inhibitors (Ali et al., 2004; Puri et al., 2004; Sadhu et al., 2003), along with decreased numbers of and proliferation of mast cells, and an attenuated allergic response (Ali et al., 2004). The PI3Kγ knockout contained higher numbers of, but less responsive, neutrophils (Hirsch et al., 2000), lower numbers of and less responsive macrophages (Hirsch et al., 2000) and dendritic cells (Del Prete et al., 2004), displayed decreased mast cell degranulation (Laffargue et al., 2002), a higher ratio of $CD4^+$ to $CD8^+$ T cells (Rodriguez-Borlado et al., 2003), increased thymocyte apoptosis (Sasaki et al., 2000), diminished induction of CXCR3 on activated T cells (Barbi et al., 2008), and decreased cardiac contractility (Crackower et al., 2002). This latter effect on cardiac tissue was a concern for chronic dosing of patients with PI3Kγ inhibitors. However, this concern was largely mitigated when the PI3Kγ kinase-dead variant (which better mimics inhibition of the kinase rather than loss of the protein) showed similar immune cell phenotypes, but importantly had no cardiac defects (Patrucco et al., 2004). The cardiac effect was later shown to be due to scaffolding effects rather than the catalytic activity of PI3Kγ. The dual PI3Kδ /PI3Kγ knockout was viable but exhibited serious defects in T cell development (Webb et al., 2005) and thymocyte survival (Swat et al., 2006). The PI3Kγ knockout/PI3Kδ kinase-dead combination produced a similar phenotype suggesting that at least within the immune system, the role of PI3Kδ is likely only a catalytic one (Ji et al., 2007).

Interpretation of studies using knockout and kinase-dead mice can be challenging because these models provide only a steady-state picture of the immune system, lack temporal and dose control, and do not permit a full understanding of how a dynamic immune response will react to reversible inhibition. Selective inhibitors with varying profiles (PI3Kδ, PI3Kγ, and PI3Kδ/γ) are necessary for studies of leukocyte signaling in order to assess the relative contributions of each PI3K to immune cell activation.

The key challenge in developing isoform selective PI3K inhibitors is that all class I PI3Ks share nearly identical ATP binding pockets (Knight et al., 2004). However, we and others reported that a quinazolinone-based chemical scaffold binds PI3Kδ with high selectivity (Knight et al., 2006; Puri et al., 2004). This chemotype exploits a unique, noncatalytically active conformation of PI3Kδ (and PI3Kγ) that is not easily adopted by PI3Kα and PI3Kβ (Berndt et al., 2010; Knight et al., 2006). In this work, we report the development of potent PI3Kδ and PI3Kδ/γ inhibitors. We then characterize these compounds using primary human leukocytes stimulated in coculture with different cytokine and chemokine ligands in order to determine if targeting PI3Kγ and PI3Kδ, either alone or in combination, results in a distinct anti-inflammatory fingerprint. Finally, we compare selective PI3Kδ/γ inhibitors to other kinase inhibitors and clinical anti-inflammatory therapeutics.

RESULTS

Design of PI3Kδ and PI3Kδ/γ Inhibitors

The ATP-binding pocket of the four class I PI3Ks (α, β, δ, γ) contains three distinct inhibitor binding elements: a hinge region with two hydrogen bonding contacts, a hydrophobic pocket deep in the protein not occupied by ATP, and a conformationally mobile methionine residue above the adenine ring of ATP (Knight et al., 2006) (Figure 1). The first two binding elements are common to all PI3Ks whereas the third appears to be significantly accessible by only PI3Kδ and PI3Kγ (Berndt et al., 2010; Knight et al., 2006).

In order to maximize the differential binding to PI3Kδ or PI3Kγ compared with PI3Kα and PI3Kβ, we exploited the rearrangement of Met804, which normally forms the roof of the ATP-binding pocket when PI3Kγ is bound to ATP and pan-PI3K inhibitors (Figure 1A, left panel). This rearrangement reveals a new pocket, not found in any ATP bound forms of PI3Ks (Knight et al., 2006). The aryl quinazolinone inhibitor, PIK39 (Figure 1A, right panel), exists in a conformation which projects the quinazolinone moiety into the pocket produced by the Met804 movement, affording highly selective binding to PI3Kδ and PI3Kγ (Berndt et al., 2010; Knight et al., 2006).

Synthesis and Biochemical Characterization

Both PIK39 (Figure 1A, right panel) and the related PIK293 (Figure 1B) form hydrogen bonds with the hinge region of PI3Ks, but do not contain elements that project into the deeper hydrophobic pocket (Knight et al., 2006) (Figure 1B). Despite the sequence similarities in the hydrophobic binding pocket, we reasoned that it was possible to distinguish between PI3Kδ and PI3Kγ with the appropriate binding elements. PIK293 was therefore diversified into two series using palladium-catalyzed routes (Figure 1C). Series 1, synthesized with Suzuki couplings, projected substituted aryl rings into the affinity pocket, while series 2 placed sterically minimal alkyne derivatives into the affinity pocket using Sonogashira couplings (see Figures S1 and S2 available online).

Nearly 50 candidate inhibitors were synthesized and screened against all class I PI3Ks as previously described (Knight et al., 2007). As expected, all compounds were highly selective for PI3Kδ over PI3Kα and PI3Kβ (Figures S1 and S2). Addition of elements that interacted with the hydrophobic-affinity pocket increased potency against PI3Kδ and PI3Kγ without compromising selectivity against PI3Kα and PI3Kβ. SW18, one of the most potent PI3Kδ inhibitors in the series (Figures 2A and 2B), was screened against 219 protein kinases at a concentration of 10 μM. Two hundred eighteen kinases in the panel retained at least 75% activity, and only one, macrophage stimulating 1 receptor (MST1R) kinase, was inhibited to less than 60% activity

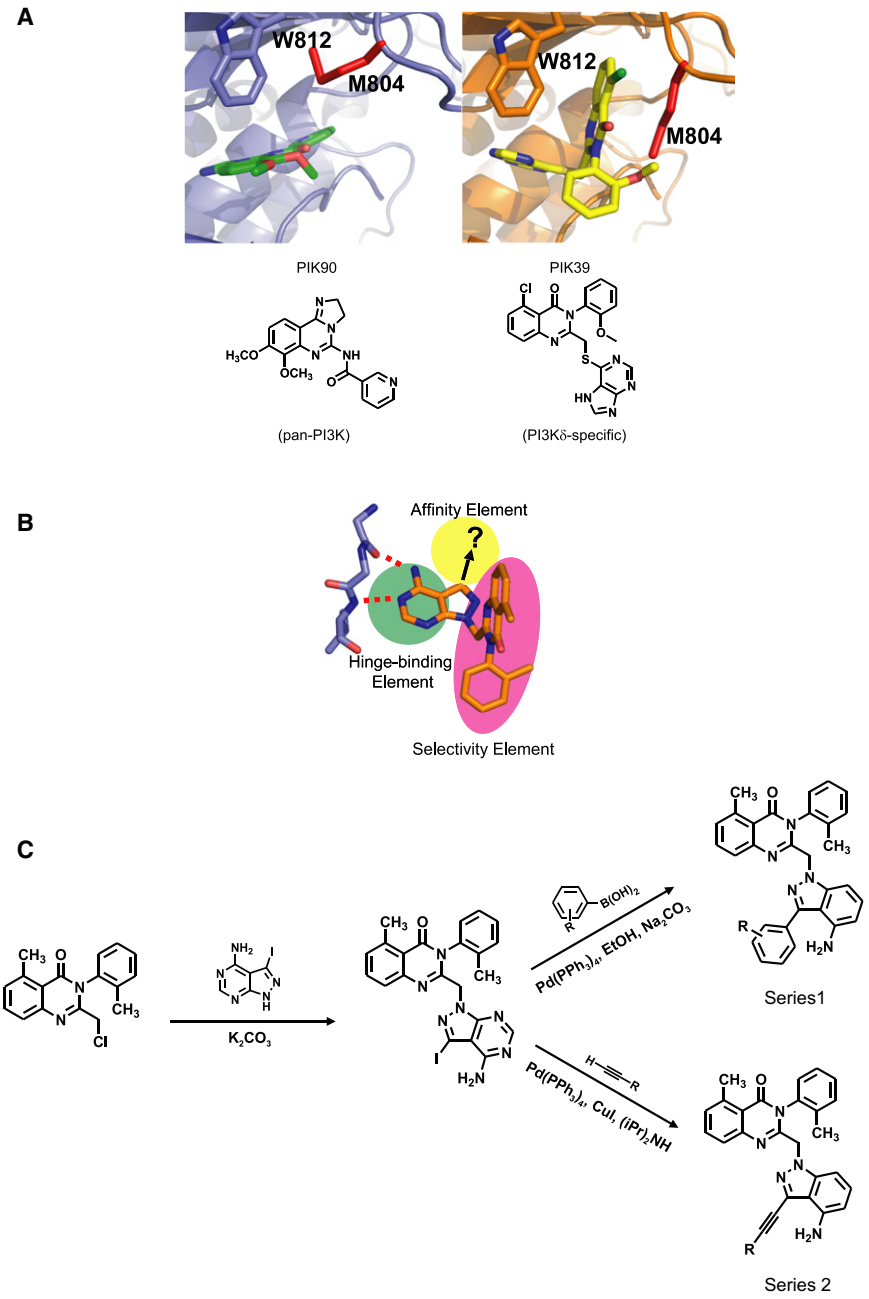


Figure 1. Basis of Selectivity and Potency for Aryl Quinazolinones

(A) Crystal structure of PI3Kγ with PIK90, a pan-PI3K inhibitor (Left) and PIK39, a PI3Kδ inhibitor (Right) (Knight et al., 2006). The biplanar binding mode of PIK39 and alternate positioning of Met804 (red) are illustrated. Chemical compound structures are shown below.

(B) Compounds in this study introduce affinity elements (yellow) onto selective tolyl quinazolinone (red) scaffold maintaining selectivity for PI3Kγ and PI3Kδ.

(C) Diversification of SW series affinity elements was achieved through Suzuki-Miyaura couplings with aryl boronic acids and Sonogashira couplings with terminal alkynes, yielding two distinct chemical series.

(Table S1). These data demonstrate that the tolyl quinazolinone moiety responsible for selectivity within the PI3K family also confers selectivity against protein kinases.

Phenol containing members of Series 1 showed the greatest potency against PI3Kδ (Figures 2A and 2B), and tuning of PI3Kγ affinity was possible by substitution of the phenol ring at the 4 position (SW14, Figures 2A and 2B). Movement of the fluoro-substituent to the 5 position (SW13) afforded the most potent PI3Kδ inhibitor reported to date. Although there is considerable precedent for hydroxyl groups in the hydrophobic affinity pocket of PI3Ks (Palanki et al., 2007; Walker et al., 2000), phenolic substituents are often subject to first-pass metabolism, limiting their oral bioavailability. We therefore explored groups that would preserve hydrogen-bonding interactions but would not contain a phenol. Of the several candidate molecules prepared, only the indole-substituted SW19 was able to retain significant PI3Kδ potency and some activity against PI3Kγ while eliminating the presence of a hydroxyl group (Figure 2B).

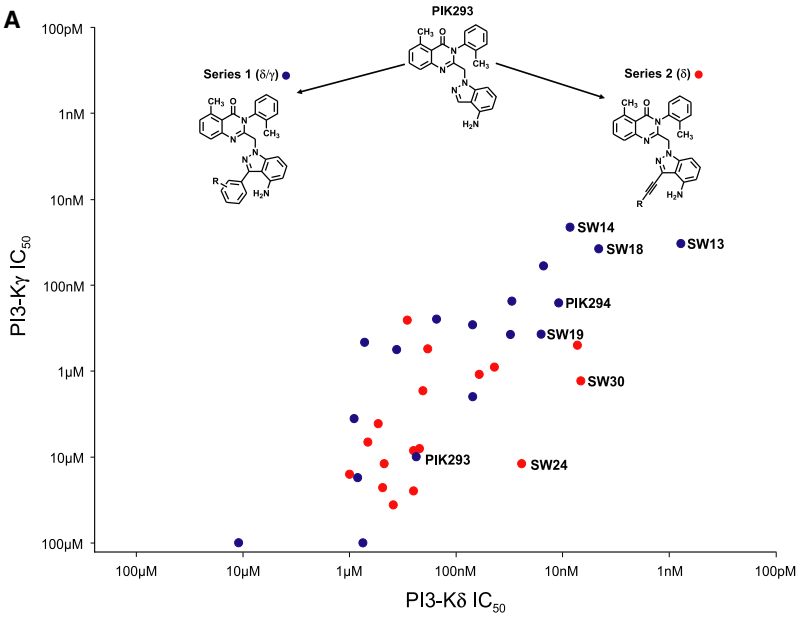
Introducing an aryl-alkynyl linkage in Series 2 reduced the bulk of the affinity pocket binding element and decreased PI3Kγ inhibition, resulting in selectivity for PI3Kδ (Figure S2). As in Series 1, a properly positioned hydroxyl group was critical to maintain potency against PI3Kδ. For example, SW30 contained a hydroxymethyl-substituted alkyne whereas SW41 extended the hydroxyl group by one methylene unit and resulted in > 30-fold loss of binding affinity for PI3Kδ (Figure S2). Together, these structure-activity data suggest that potency against PI3Kδ requires the presence of a properly positioned hydrogen bond donor/acceptor and that the additional bulk provided by aryl groups in Series 1 affords activity against PI3Kγ (Figure 2C).

The PI3Kγ/δ ratio of these compounds illustrates a wide range of biochemical activity against PI3Kγ while preserving potency against PI3Kδ. Out of nearly 50 compounds, SW13, SW14, SW19, and SW30 (Figures 2 and 3) were selected for characterization in cellular assays based on potency for PI3Kδ and PI3Kγ and their range of selectivity between the two targets.

Cellular Signaling Effects

We next assessed the inhibition of PI3Kδ and PI3Kγ in a cell model with a single readout by stimulating THP-1 monocytes with different ligands to activate either PI3Kδ or PI3Kγ. Macrophage colony stimulating factor (M-CSF/CSF-1) is a cytokine that binds to the M-CSF receptor activating RTK-linked PI3Ks (Kelley et al., 1999), whereas the chemokine monocyte

A



B

IC_{50} (nM)

	Compound	Group	α	β	δ	γ	γ/δ ratio
Series 1	PIK293	H	>90,000	>90,000	237	10,000	42
	PIK294		>90,000	486	11	162	15
	SW13		1,240	221	0.7	33	47
	SW14		8,910	697	9	21	2.3
	SW18		6,700	2,400	5	38	7.6
	SW19		>90,000	7,900	16	376	23
Series 2	SW30		85,000	740	7	1,300	186
Benchmark Compounds	PIK90	N/A	11	350	58	18	0.31
	AS605240	N/A	60	270	300	8	0.026

C

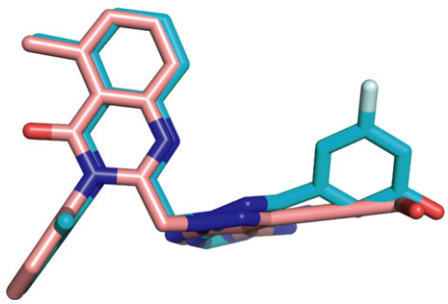


Figure 2. Diversification of Tolyl Quinazolinone Series

(A) Introduction of affinity elements to PIK293. Series 1 (PI3Kδ/γ) introduces aryl groups and Series 2 (PI3Kδ) introduces alkynyl-linked groups. IC_{50}s of both Series 1 (blue) and Series 2 (red) were graphed.

(B) Biochemical IC_{50}s and γ/δ ratios for Series 1 and 2 along with benchmark compounds. All compounds were tested at 10 μM ATP.

(C) Crystal structure of SW13 (cyan), and SW30 (pink) as reported (Berndt et al., 2010). Hydrogen bonding elements for SW series are all aligned.

A

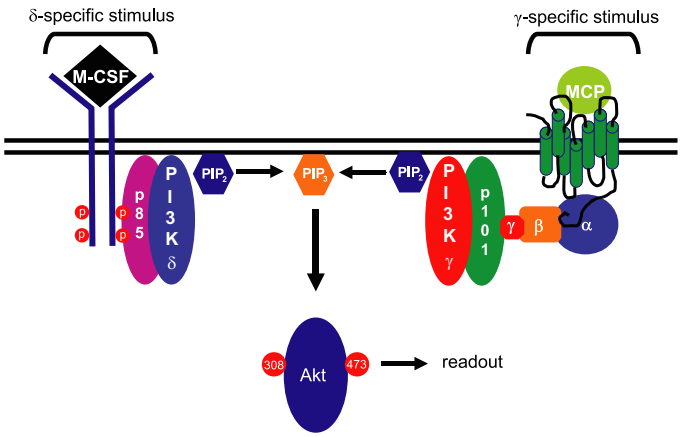


Figure 3. Effect of Selected Compounds on THP-1 Monocyte Signaling

(A) THP-1 monocyte signaling pathway leads to phosphorylation of Akt through either RTK-linked macrophage colony stimulating factor (M-CSF) or GPCR-linked monocyte chemoattractant protein 1 (MCP-1).

(B) pAkt was measured by FACS using fluorescent pSer473 Akt antibodies. Quantitated fluorescence was used to determine an EC_{50} for selected compounds. Biochemical γ/δ ratios are provided for comparison.

B

Compound	EC_{50}(nM)		(γ/δ) Ratio	
	MCP(γ)	CSF(δ)	Cellular	Biochemical[a]
SW13	515	0.7	735	47
SW14	132	4.5	29.3	2.3
SW19	395	35	11.2	23
SW30	2,700	16	168	186
AS605240	117	1,400	0.083	0.026
SW23	>10,000	>10,000	N.D.	N.D.

a - From Figure 2

chemoattractant protein-1 (MCP-1) activates PI3Kγ through a GPCR (Jones et al., 2003). Both stimuli lead to phosphorylation of Akt at Ser473 (Camps et al., 2005; Jones et al., 2003; Pomel et al., 2006), which we monitored by fluorescence-activated cell sorting (FACS) (Figure 3A). We included a literature benchmark compound, AS605240 (Camps et al., 2005; Pomel et al., 2006), and negative control (SW23) in our analysis.

M-CSF stimulated Ser473 p-Akt production was potently blocked by SW13, SW14, SW19, and SW30, but not by SW23 or AS605240 (Figure 3B). We attribute these effects to PI3-Kδ inhibition because SW23 and AS605240, which do not significantly inhibit PI3Kδ at the doses tested, were ineffective. Only the compounds with biochemical IC_{50} values under 25 nM against PI3Kδ were able to inhibit Ser473 p-Akt production in this assay. Further support for the role of PI3Kδ in this assay comes from the consistent rank order (biochemical IC_{50} versus cellular EC_{50}). SW13 was most potent in the biochemical assay and was the most potent in the cellular M-CSF assay as well.

MCP-1 stimulated Ser473 p-Akt production was blocked by AS605240 and SW14, but not significantly by SW30 or SW23. SW19 blocked MCP-1 stimulated Ser473 p-Akt production but only at somewhat higher concentrations, in a manner consistent with its biochemical IC_{50} value (Figure 3B). SW14, the most potent tolyl quinazolinone PI3Kγ inhibitor in the biochemical assays, was of equal potency with AS605240, the most potent PI3Kγ inhibitor included in our studies. Similar to the M-CSF assay, this rank order is consistent with the biochemical potency of these compounds against PI3-Kγ. The PI3Kδ selectivity of SW13 and especially SW30 is highlighted by their low activity in this assay, whereas the inability of SW23 to inhibit p-Akt formation suggests that the activity in this assay is due to inhibition of PI3K rather than chemotype/scaffold-dependent effects.

Modulation of Inflammatory Signaling in Human Peripheral Blood Mononuclear Cells (PBMCs) and Umbilical Vein Endothelial Cells (HUVECs)

The above cellular data motivated us to assess the role of PI3Kδ and PI3Kγ in inflammatory signaling by use of cocultures of primary human cells not adapted to long-term growth in vitro. There are two advantages of this approach. First, human cells rather than rodent cells are used, permitting an accurate assessment of human PI3K immune signaling. Second, the in vitro nature of the assay allows use of the compounds with highly distinct inhibitory profiles and minimizes any pharmacokinetic/pharmacodynamic issues that arise in the transition from cell-based to animal-based models. We compared the activity of selective and pan-PI3K inhibitors using the biologically multiplexed activity profiling (BioMAP) method (Berg et al., 2006; Kunkel et al., 2004). This assay utilizes primary human cells (HUVECs alone or in combination with PBMCs), stimulated with combinations of inflammatory signals (IL-1β, TNFα, IFN-γ, IL-4, histamine, lipopolysaccharide [LPS], and superantigen) (Figure 4A). The resultant expression levels of a panel of 20 receptors, cytokines, cell adhesion molecules and second messengers (Figure 4A) – were measured following stimulation and drug treatment, and compared to the untreated ("no drug") levels. These expression levels yield a distinctive pattern, or profile, unique to each drug that can be used to compare and contrast several types of chemical compounds and drug classes (Figure 4B). Similar profiles are generally observed with similar mechanisms of action, but can also be observed when different chemical compounds share a functional similarity.

We evaluated pan-PI3K (α,β,δ,γ) inhibition and PI3Kγ inhibition using PIK90 and AS605240 as benchmark compounds, whereas PI3Kδ inhibition and dual PI3Kδ/γ inhibition were evaluated using SW30 and SW14, respectively. Each compound was tested at a range of concentrations (Figure 4B) in order to identify potential off-target activities at higher doses. For each marker analyzed, a $\log_{10}$ ratio for drug- treated protein levels versus 0.1% dimethyl sulfoxide (DMSO)-treated cells is indicated (Figure 4B). A $\log_{10}$ ratio of −1.0 indicates a 10-fold decrease in protein level upon treatment with drug as compared with the levels with 0.1% DMSO. The 0.1% DMSO measurement,

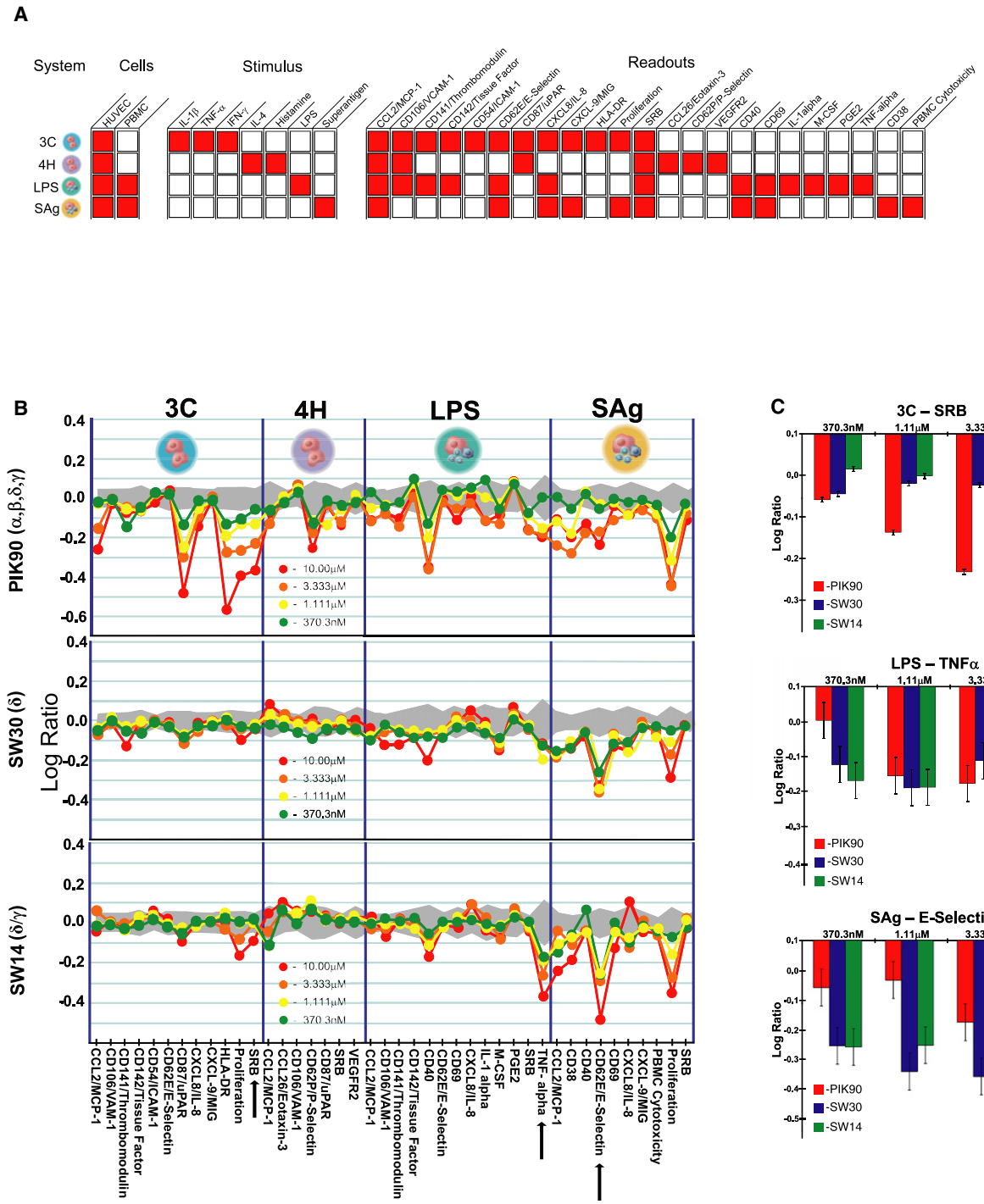


Figure 4. BioMAP Analysis of PI3K Inhibitors

(A) BioMAP systems details.

(B) BioMAP profiles for PIK90 (pan-PI3K) (top), SW30 (PI3Kδ) (middle), and SW14 (PI3Kδ/γ) (bottom). Red dots represent a 10 μM dose, orange corresponds to 3.3 μM, yellow represents 1.1 μM, and green represents 370 nM. Levels of proteins were measured by ELISA and presented as log expression ratios (log_{10}[parameter value with inhibitor/parameter value of 0.1% DMSO]). The gray area represents the 95% prediction interval of the 0.1% DMSO data. Arrows indicate readouts that are expanded in 4C for closer inspection.

(C) Log expression ratios of selected readouts that show divergence between pan-PI3K inhibition and selective PI3K inhibition. Error bars indicate standard deviation from triplicate samples.

compiled over several donor pools provided a benchmark for the inherent variability of the assay, and is represented by the gray shading surrounding $\log_{10}$ ratio = 0.0 in Figure 4 and Figure S3.

Effects of pan-PI3K Inhibition

The pan-PI3K inhibitor PIK90 elicited both anti-inflammatory and antiproliferative effects. In conditions designed to mimic T_H1-driven endothelial cell-based inflammation (HUVECs stimulated with IFNγ, TNFα, and IL1β; Figure 4B, 3C), pan-PI3-K inhibition showed a moderate effect on HUVEC viability (indicated by a reduction in sulforhodamine B (SRB) protein binding < −0.3 log ratio) and inhibited HUVEC proliferation (Figure 4C). PIK90 also strongly inhibited the urokinase receptor (CD87/uPAR), important in tissue remodeling (Solberg et al., 2001) and cell motility (Kjoller and Hall, 2001), and HLA-DR, a surface receptor important in antigen presentation to T cells (Rothbard et al., 1988). In HUVECs stimulated with histamine and IL-4 (4H; Figure 4B), the effects of PIK90 were more subtle, showing only mild inhibition of CD62/P-selectin, a cell adhesion molecule.

When introduced to a PBMC/HUVEC coculture stimulated with LPS, PIK90 exhibited little effect on cell viability but significantly reduced CD40 and mildly reduced TNFα production (Figures 4B and 4C; LPS). In PBMCs and HUVECs stimulated with superantigen, PIK90 showed modest effects on E-selectin (CD62), CCL2/MCP-1, and CD38 production, and a decrease in PBMC (mostly monocytes and T cells) proliferation (Figure 4B; SAg). E-selectin is a cell-adhesion molecule expressed by endothelial cells that recognizes sialylated sugars on leukocytes, and is involved in the first stages of leukocyte recruitment (McEver, 1991). CCL2/MCP-1 is a cytokine that recruits monocytes to sites of injury (Daly and Rollins, 2003). Together, these results suggest that pan-PI3K inhibitors reduce some parameters associated with inflammation but exhibit general cytotoxicity and decreased HUVEC proliferation. These effects may significantly limit their utility for chronic conditions requiring frequent administration.

Comparison of Selective PI3Kδ and Selective PI3Kδ/ PI3Kγ Inhibition

In contrast to the effects of pan-PI3K inhibition (PIK90), specific inhibitors of PI3Kδ (SW30) or PI3Kδ/γ (SW14) had effects on inflammatory markers but little antiproliferative activity. SW30 and SW14 showed little effect on HUVECs stimulated with inflammatory cytokines (Figure 4B; 3C, 4H), but were active in suppressing markers of inflammation in cocultures of HUVEC and PBMCs stimulated with LPS, the bacterial cell wall component that binds Toll-like receptor 4, or superantigens, another bacterial product which universally engages the T cell receptor (TCR) (Figure 4B; LPS, SAg). The principal effect of the PI3Kδ selective compound SW30 occurred under superantigen stimulated conditions (reduction of E-selectin expression (Figure 4C) and PBMC proliferation) likely because the TCR, engaged by superantigens, is coupled to PI3Kδ (Okkenhaug et al., 2002; Okkenhaug and Vanhaesebroeck, 2003). PBMC proliferation decreased under the same conditions in response to SW30 and SW14, but they did not exhibit a toxic effect as read out by Alamar blue reduction (PBMC cytotoxicity, Figure 4B), indicating that they can inhibit PBMC growth without inducing cell death. Together, these results suggest that PI3Kδ inhibition is more selective in exerting an anti-inflammatory profile than a pan-PI3K inhibitor and may be particularly suited to treatment of disorders with a dominant T cell component.

PI3Kδ/γ inhibition (SW14) was largely inactive in the HUVEC-only culture conditions, only slightly decreasing proliferation at the highest dose (10 μM; Figures 4B, 4C). In the LPS-stimulated HUVEC/PBMC coculture, PI3Kδ/γ inhibition led to decrease of TNFα production, more so than either PI3Kδ inhibition (SW30) or pan-PI3K inhibition (PIK90) (Figure 4C). When the HUVEC/PBMC coculture was stimulated with superantigen, SW14 decreased proliferation and CCL2/MCP-1, CD38, and E-selectin expression. An enhanced effect of PI3Kδ/γ inhibition was observed: decreased monocyte TNFα production (under LPS stimulation) and more efficacious (magnitude) inhibition of T cell activation (E-selectin, PBMC proliferation; under superantigen stimulation) (Figure 4C). Importantly, although SW14 inhibits PI3Kβ at below 1 μM (Figure 2B), related analogs (SW18, 19) with 3-fold and 10-fold less PI3Kβ inhibition, respectively (Figure 2B; Figure S1) exhibited similar enhanced activity. The profile of SW19 (Supplemental Information) also provides evidence that the effects on TNFα are truly due to additional PI3Kγ activity, and not simply more potent PI3Kδ inhibition. SW19, though less potent on PI3Kδ than SW30 (Figure 2B), displays moderate activity against PI3Kγ, which is enough to exhibit the characteristic reduction of TNFα levels and cluster it with SW14 and SW18, other PI3Kδ/γ dual inhibitors (Supplemental Information). The stronger monocyte and T cell inhibition seen with PI3Kδ/γ inhibition may make these inhibitors particularly effective in the treatment of inflammatory disorders driven by TNFα.

Biological Activity of PI3K Inhibitors Relative to Other Agents

Having defined a potential role for PI3Kδ (SW30) in treatment of T cell-mediated inflammatory diseases and PI3Kδ/γ (SW14) in treatment of TNFα-mediated inflammatory disease, we sought to put these agents in the broader context of approved anti-inflammatory agents. We compared PI3K inhibitors with different isoform selectivities to a panel of clinical and experimental compounds with anti-inflammatory (cyclosporin A, prednisolone) and anticancer activities. Included were CDK inhibitors (kenpaullone, olomoucine, roscovitine), microtubule disruptors (vincristine, colchicine), estrogens (17-β-estradiol, 2-methoxyestradiol), HSP90 inhibitors (17-AAG, geldanamycin, radicicol), taxanes (epithilone B, paclitaxel), p38 inhibitors (BIRB-796, VX-745), IKK inhibitors (Ro106-9920, SC-514), JNK inhibitors (AS602801), and mTOR inhibitors (everolimus, rapamycin) (Figure 5). The profiles for each compound were analyzed using a Pearson correlation metric and visualized in two dimensions through a multidimensional scaling algorithm (Kunkel et al., 2004; Plavec et al., 2004).

Compounds targeting PI3Kγ and PI3Kδ (SW14, SW18, SW30) occupied a distinct space in this map. The more PI3Kδ selective compounds (SW30 and IC87114) clustered with lower doses of the PI3Kδ/γ compounds, confirming our observations on their specificity. Strikingly, compounds with significant activity against PI3Kγ (SW14, SW18) were functionally linked to the glucocorticoid prednisolone, the active metabolite of prednisone. This result is supported by direct comparison of the

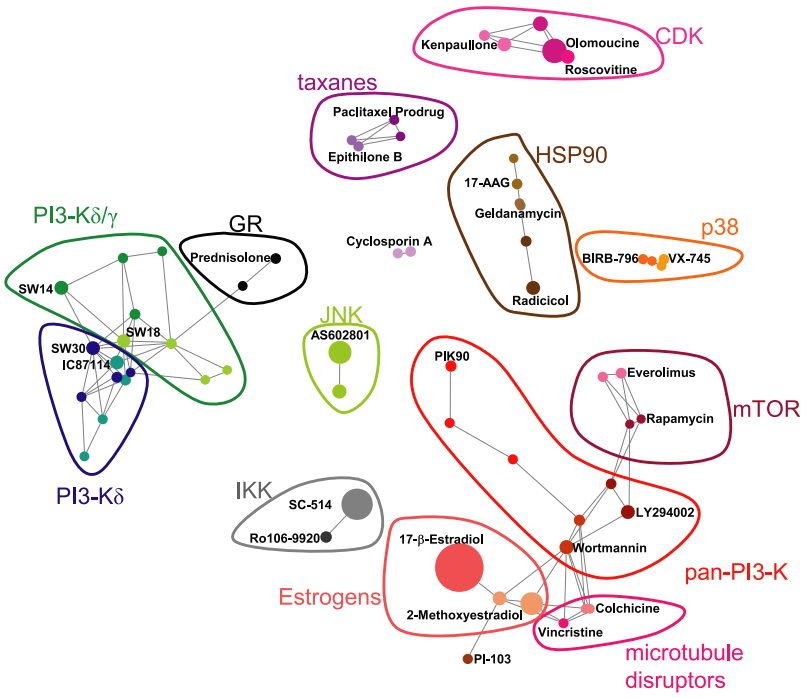


Figure 5. Function Similarity Map of PI3K Inhibitors and Other Compounds

A function similarity map of compounds from the SW series with other anti-inflammatories, PI3K inhibitors, and kinase inhibitors. This map is generated by subjecting the pairwise correlation data of BioMAP profiles to multidimensional scaling. Significant correlations are shown by gray lines. The distance between the compounds is inversely related to the similarity of their profiles. Compounds are color coded, grouped by target class, and the area of the circle is proportional to the dose.

profiles of SW14, SW18, and prednisolone, which showed significant similarities, especially with regard to their effect on TNFα production in the LPS-stimulated HUVEC/PBMC coculture (Figure S3). Conversely, the pan-PI3K inhibitor (PIK-90), grouped with other pan-PI3K inhibitors in a different region of the similarity map (Figure 5) with inhibitors of their downstream target mTOR, microtubule modulators, another downstream target of PI3K (Onishi et al., 2007), and estrogens, which have been linked to PI3Ks (Calippe et al., 2008; Simoncini et al., 2000).

A comparison of compounds that target the PI3K pathway (Figure S4) showed that agents with significant PI3Kα activity, including AS605240, clustered with inhibitors of mTOR. AS605240, often described as a PI3Kγ directed inhibitor, showed significant similarity to the multitargeted inhibitors and clustered with them, likely due to its activity against PI3Kα (Figure S2; Supplemental Information).

DISCUSSION

We describe the design and synthesis of highly selective PI3K inhibitors based on a quinazolinone scaffold that engages the methionine switch observed in both PI3Kγ (Knight et al., 2006) and PI3Kδ (Berndt et al., 2010). Elaboration of this scaffold with aryl and alkyne affinity elements afforded compounds with a wide range of activity against PI3Kδ and PI3Kγ but importantly did not significantly inhibit PI3Kα, PI3Kβ, or any of the 219 protein kinases evaluated. These compounds were used in conjunction with AS605240, a PI3Kγ-directed literature inhibitor, and the pan-PI3K inhibitor PIK90 in order to evaluate the effects of isoform-selective inhibition in single and multicellular contexts.

Several PI3K inhibitors with different isoform selectivities were tested in primary human cell models of inflammatory signaling. Pan-PI3K inhibition (PIK90) was highly antiproliferative to both HUVECs and PMBCs, whereas PI3Kδ inhibition (SW30) or PI3Kδ/γ inhibition (SW14) resulted in selective T cell inhibition. Selective PI3Kδ inhibition (SW30) blocked T cell cytokine production, resulting in decreased HUVEC E-selectin expression and revealing the potential to elicit a potent anti-inflammatory response without a direct effect on endothelial cells. Interestingly, SW30 and other selective PI3Kδ inhibitors such as IC87114 (Supplemental Information) did not inhibit E-selectin expression in HUVECs directly stimulated with IFNγ, TNFα, and IL1β (3C; Figure 4B), but only did so in the superantigen-stimulated HUVEC/PMBC coculture conditions (SAg; Figure 4B; Supplemental Information). This result is consistent with these compounds *indirectly* blocking E-selectin expression in HUVECs as a result of lymphocyte TNFα production inhibition (TNFα is known to rapidly upregulate E-selectin expression in HUVECs [Schindler and Baichwal, 1994]), and these data clearly illustrate the ability of these assays to capture parts of the intercellular communication integral to inflammatory responses.

PI3Kδ/γ inhibition (SW14) was not cytotoxic to HUVECs and displayed more significant anti-inflammatory effects in the HUVEC/PBMC cocultures. Dual targeting of PI3Kδ/γ resulted in enhanced inhibition of LPS-induced TNFα production (Figure 4C) and overall T cell activation. The inhibitory effects observed with inhibition of PI3Kδ/γ in these assays led us to wonder whether they were due to synergistic inhibition of PI3Kδ/γ or whether PI3Kγ inhibition might be sufficient. The most commonly used PI3Kγ compound in the literature and only PI3Kγ selective molecule we were able to synthesize, AS605240, inhibits all PI3K isoforms at 300 nM (Camps et al., 2005), and primary human cell assays displayed a profile similar to pan-PI3K compounds (Figure S4; Supplemental Information).

Our studies using both new and benchmark compounds show the most effective anti-inflammatory PI3K inhibitor is one that inhibits both PI3Kδ and PI3Kγ. Pan-PI3K inhibitors, on the other hand, demonstrated limited effects on anti-inflammatory markers in T cell and monocyte-driven environments with concurrent antiproliferative effects. Nevertheless, it remains possible that selectively inhibiting other combinations of PI3K isoforms, such as PI3Kα/β, PI3Kβ/γ, or PI3Kβ/δ, may also show synergistic anti-inflammatory activity.

Interestingly, additional inhibition of PI3Kα and β (PIK90) did not further suppress inflammatory markers (Figure 4B; LPS, SAg). In fact, when comparing the effects on E-selectin expression (SAg), selective inhibition of PI3Kδ alone was better than

pan-PI3K inhibition, but inhibition of PI3Kδ/γ was the most effective (Figure 4C). The most effective isoform combination at inhibition of LPS-induced TNFα production was also PI3Kδ/γ (Figure 4C). Additional PI3Kα/β inhibition (PIK90), in addition to displaying undesirable antiproliferative properties, did not significantly decrease TNFα expression and was essentially identical to selective PI3Kδ inhibition (Figure 4C). These results together suggest a possible role for PI3Kγ in TNFα production.

Both kinase-dependent and kinase-independent routes exist through which PI3Kγ could regulate TNFα production. PI3Kγ interacts with phosphodiesterase 3B (PDE3B), regulating cyclic AMP (cAMP) levels and activation of protein kinase A (PKA), leading to increased heart contractility (Patrucco et al., 2004) and was also linked to PDE4 (Kerfant et al., 2007). PDE4 is the main cAMP hydrolyzing enzyme in immune cells, and has been pursued as a target for generating novel anti-inflammatories (Teixeira et al., 1997) that suppress LPS-induced TNFα production in monocytes (Souness et al., 1996). Because PI3Kγ is required for PDE4 activity (Kerfant et al., 2007), PI3Kγ inhibition may lead to diminished PDE4 activity and explain the TNFα suppression. However, the first link between PDEs and PI3Kγ involved a scaffolding, non-catalytic effect (Patrucco et al., 2004) and the PDE4-PI3Kγ link was discovered in a PI3Kγ KO mouse (Kerfant et al., 2007), which has no functional PI3Kγ suggesting that this route may not involve the catalytic activity of PI3Kγ.

TNFα is involved in several autocrine signaling loops (Lisby et al., 2007) and it is possible that PI3Kγ is involved in amplifying such signals. TNFα activates PI3Kγ in endothelial cells leading to oxidant generation and NF-κB activation (Frey et al., 2006). NF-κB regulates production of TNFα (Li and Stark, 2002) so TNFα may activate its own production by NF-κB through PI3Kγ. TNFα can upregulate its own mRNA synthesis in keratinocytes (Lisby et al., 2007), and if this happens in PBMCs, it provides an attractive model for PI3Kγ involvement in TNFα production and may explain why PI3Kγ inhibition lowers TNFα levels.

Our data suggest that inhibition of PI3Kγ's catalytic activity is sufficient to regulate TNFα levels, but they do not necessarily rule out other mechanisms. It is possible that application of BioMAP analysis to this question may resolve the conflict. If the combination of a PDE4 inhibitor and a PI3Kγ inhibitor did not alter TNFα levels more than either inhibitor alone, this would suggest that PI3Kγ is signaling through PDE4; if, however, combined inhibition of PDE4 and PI3Kγ further decreased TNFα levels, this might suggest that they work through independent pathways (as mentioned above). Results obtained by treating with more than one compound may be unreliable because of potential off-target effects, thus a combination of a specific inhibitor with siRNA in a BioMAP system might better resolve the dilemma.

In broad terms, PI3K signaling can be viewed into two divisions, a survival pathway (α/β) and inflammatory PI3K (δ/γ) pathway, yet inhibitors that target both units can lead to functional antagonism. Essentially, inhibition of class I PI3-Ks is not necessarily additive, and inhibition of PI3-Kα and PI3-Kβ can actually antagonize the effects of more isoform selective inhibition. For example, inhibition of PI3Kδ/γ by SW14 results in suppression of E-selectin or TNFα but additional inhibition of PI3Kα/β using PIK90 actually results in a smaller degree of suppression (Figure 4B).

The actual mechanisms by which PI3Kα/β inhibition may antagonize PI3Kδ/γ inhibition are not perfectly clear. In the case of TNFα suppression, pan-PI3K inhibition with wortmannin and LY294002 has been shown to reverse amlodipine-induced inhibition of TNFα production in LPS-stimulated rat cardiomyocytes (Li et al., 2009), and of more relevance, treatment of PBMCs and THP-1 monocytes with the same compounds increases LPS induced TNFα expression (Guha and Mackman, 2002). Both wortmannin and LY294002 have more activity outside of the class PI3Ks than PIK90, (Knight et al., 2006; Knight and Shokat, 2005), which may explain why PIK90 did not *increase* TNFα levels, but our results with pan-PI3K inhibition are not necessarily surprising. Studies with pan-PI3K inhibitors cannot identify the actual isoforms responsible for the increase in TNFα levels, but a recent study showed that p110α deficient THP-1 monocytes display increased TNFα production when stimulated by LPS, suggesting that PI3Kα inhibition is responsible for the increase in TNFα production (Lee et al., 2007). Together this evidence suggests that PI3Kα inhibition increases TNFα production, while PI3Kδ/γ inhibition decreases TNFα production, and when all isoforms are inhibited, both activities are balanced, leaving TNFα levels unchanged, but if PI3Kα is not inhibited (in the case of our PI3Kδ/γ inhibitors) then TNFα levels will decrease.

This work illustrates how targeted chemical inhibition can access information not available from genetic inactivation of one or more PI3K isoforms, which is difficult to obtain because these animals often suffer significant defects during development. Furthermore, results obtained using targeted inhibitors can be different from those obtained in animals with sustained genetic inactivation (Knight and Shokat, 2007). PI3Kδ/PI3Kγ knockout mice exhibited a more severe immune phenotype than mice lacking either isoform alone, (Swat et al., 2006; Webb et al., 2005) but the severity of that phenotype is likely due to sustained absence of both PI3Kδ and PI3Kγ and may not be duplicated with pharmacological treatment.

Many clinical anti-inflammatory agents function through different targets yet all have the property of inhibiting immune cell function while leaving nonimmune cells relatively unaffected. We asked if the PI3Kδ/γ inhibitors exhibited this property and which current anti-inflammatory agents they might resemble. The most closely related profile was that of the glucocorticoid receptor (GR) agonist prednisolone. It is interesting that a particular multicellular profile can be achieved through two distinct mechanisms (kinase inhibition versus nuclear hormone receptor activation). Although prednisolone is an effective anti-inflammatory agent, there have been efforts to identify "dissociating GR agonists" that separate anti-inflammatory from other GR effects (bone loss, cardiovascular disease) that limit their long-term use (Schaecke et al., 2007). The discovery that PI3Kδ/γ inhibition can functionally mimic several anti-inflammatory features of prednisolone opens a new way to improve upon a proven class of anti-inflammatories (GR agonists) while targeting completely different enzymes, and could only have been realized through analysis of this compound series on primary human cells.

Despite the related responses of prednisolone and PI3Kδ/γ inhibitors, the similarities may be limited to the cell types we analyzed. Cell types that have documented GR agonist responses (macrophages, coronary artery cells) are not included in our assays. The exceptionally broad cellular effects of GR agonists would likely be distinguished from PI3Kδ/γ inhibitors if

more cell types were analyzed. Despite these caveats, the use of primary human cells provides a powerful early assessment of differential inhibition of important signaling nodes (PI3Ks, nuclear receptors, JNKs, calcineurin, IKK).

With the new availability of small molecules capable of inhibiting the inflammatory PI3Ks (δ/γ) without inhibition of the ubiquitous growth-linked PI3Ks, we are poised to begin to resolve the opposing effects of pan-PI3K inhibition and selective inflammatory PI3K inhibition and to begin further validation of PI3Kδ/γ as a target for the treatment of inflammatory disorders.

SIGNIFICANCE

The work describes the discovery of small-molecule inhibitors of unusual potency and selectivity for immune cell-expressed PI3Ks. Lipid kinases are emerging as critical drug targets for a number of disease states. Although most work has focused on anticancer properties of PI3K inhibitors, here we address the two key challenges, one chemical and one biological, in the development of isoform selective inhibitors of PI3Kγ and PI3Kδ as a novel class of anti-inflammatory agents. The first challenge is how to selectively target PI3Kδ and PI3Kγ without inhibiting the ubiquitously expressed PI3Kα and PI3Kβ isoforms, which have nearly identical ATP binding pockets. We address this challenge by exploiting the first conformation specific binders to PI3Kδ and PI3Kγ and develop the first panel of compounds with differing profiles against these critical inflammatory targets. The second challenge is determining which profile of PI3K inhibition is desirable: completely selective PI3Kδ or PI3Kγ inhibition, dual inhibition of PI3Kδ and PI3Kγ, or possibly pan-PI3K inhibition. Although full or conditional knockouts of each isoform have been generated in mice, such model systems only provide a steady state loss-of-function analysis. We therefore evaluated a panel of the newly discovered PI3Kδ/γ inhibitors, along with benchmark pan-selective inhibitors and a representative set of anti-inflammatory agents with broad mechanism of action, in primary human cocultures stimulated with proinflammatory agents. The resulting map of anti-inflammatory drug action reveals an unanticipated similarity between dual-PI3Kδ/γ inhibitors and anti-inflammatory glucocorticoids. Importantly, our studies also show that inhibition of PI3Ks beyond PI3Kδ/γ result in a decrease of some anti-inflammatory effects, suggesting that pan-PI3K inhibitors will be less immunosuppressive. These studies contribute both chemical and biological insights into the emerging area of PI3K drug discovery for the treatment of inflammatory disease.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

All compounds were synthesized from commercially available starting materials, and purified by reverse-phase HPLC (MeCN:H_2O:0.1% TFA). See Supplemental Experimental Procedures for details.

Kinase Assays

IC_{50}s were determined as previously described (Knight et al., 2007). See Supplemental Experimental Procedures for details.

THP-1 Cell Culture

THP-1 monocytes (ATCC) were grown in RPMI-1640 media with 10% fetal bovine serum (FBS), 0.004% beta mercaptoethanol (BME), and under 5% CO_2 at 300,000-500,000 cells/ml.

THP-1 Signaling Assays

THP-1 monocytes were grown in serum-free RPMI-1640 with 0.004% BME under 5% CO_2 at 1,000,000 cells/ml for 4 hr, incubated with inhibitors or DMSO for 10 min, and stimulated with 100 nM MCP-1 (R&D) for 5 min or 50 ng/ml CSF-1 (Peprotec) for 7 min. Cells were fixed with paraformaldehyde; 1 ml cold methanol was added, and cells were stored at −20°C overnight. Two milliliters of PBS was added, and cells were spun, resuspended in 1 ml PBS with 5% FBS (FACS Buffer), and stored at 4°C. Buffer was aspirated, and 10 μl block solution was added. After 10 min, 30 μl Alexa-conjugated antibody in FACS buffer (1/5 dilution) was added to cells. After 30 min, cells were resuspended in 2 ml PBS and spun. PBS was removed; cells were resuspended in 100 μl FACS buffer and read on the FACS.

Cell Culture

HUVEC were pooled from multiple donors, cultured according to standard methods, and plated into microtiter plates at passage 4. PBMC were prepared from buffy coats from normal human donors according to standard methods. Concentrations of agents added to confluent microtiter plates to build each system: cytokines (IL-1β, 1 ng/ml; TNFα, 5 ng/ml; IFN-γ, 20 ng/ml; IL-4, 5 ng/ml), activators (SAg, 20 ng/ml; histamine, 10 μM; or LPS, 2 ng/ml), and leukocytes (PBMC, 75,000 cells/well).

Compounds

Compounds were tested at the indicated concentrations. Compounds were added 1 hr before stimulation of the cells, and were present during the whole 24 hr stimulation period (or longer for proliferation assays).

Readout Measurements

The expression of many readouts was measured by cell-based enzyme-linked immunosorbent assays (ELISAs). For the ELISAs, microtiter plates are treated, blocked, and then incubated with primary antibodies or isotype control antibodies (0.01–0.5 μg/ml) for 1 hr. After washing, plates were incubated with a peroxidase-conjugated anti-mouse IgG secondary antibody or a biotin-conjugated anti-mouse IgG antibody for 1 hr followed by streptavidin-HRP for 30 min. Plates were washed and developed with TMB substrate and the absorbance (OD) was read at 450 nm (subtracting the background absorbance at 650 nm). Quantitation of TNFα and PGE2 was done using commercially available kits according to the manufacturer's directions. Proliferation of PBMCs (T cells) was quantified by Alamar blue reduction. Proliferation of adherent cell types was quantified by SRB staining.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, Biomap profiles, and Supplemental Experimental Procedures, and can be found with this article online at doi:10.1016/j.chembiol.2010.01.010.

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