Targeting the gatekeeper residue in phosphoinositide 3-kinases

Peter J. Alaimo, a,† Zachary A. Knight b,† and Kevan M. Shokat a,c,*

a Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143-2280, USA
b Program in Chemistry and Chemical Biology, University of California, San Francisco, CA 94143, USA
c Department of Chemistry, University of California, Berkeley, CA 94720, USA

Received 1 December 2004; accepted 12 February 2005

Abstract—A single residue in the ATP binding pocket of protein kinases—termed the gatekeeper—has been shown to control sensitivity to a wide range of small molecule inhibitors (Chem. Biol. 2004, 11, 691; Chem. Biol. 1999, 6, 671). Kinases that possess a small side chain at this position (Thr, Ala, or Gly) are readily targeted by structurally diverse classes of inhibitors, whereas kinases that possess a larger residue at this position are broadly resistant. Recently, lipid kinases of the phosphoinositide 3-kinase (PI3-K) family have become the focus of intense research interest as potential drug targets (Chem. Biol. 2003, 10, 207; Curr. Opin. Pharmacol. 2003, 3, 426). In this study, we identify the residue that corresponds structurally to the gatekeeper in PI3-Ks, and explore its importance in controlling enzyme activity and small molecule sensitivity. Isoleucine 848 of p110α was mutated to alanine and glycine, but the mutated kinase was found to have severely impaired enzymatic activity. A structural bioinformatic comparison of this kinase with its yeast orthologs identified second site mutations that rescued the enzymatic activity of the I848A kinase. To probe the dimensions of the gatekeeper pocket, a focused panel of analogs of the PI3-K inhibitor LY294002 was synthesized and its activity against gatekeeper mutated and wild-type p110α was assessed.

© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

In the past decade protein kinases have emerged as one of the most important new classes of drug targets. Protein kinases play a central role in many signaling pathways disregulated in disease, and these enzymes can be readily targeted with cell permeable, small molecule inhibitors. These facts have led to the hope that inhibitors of individual protein kinases might be tailored to specific diseases based on an understanding of their molecular etiology. Recently, this concept has been dramatically validated by the clinical success of Gleevec, an inhibitor of the Abl tyrosine kinase, in the treatment of chronic mylogenous leukemia, a disease driven by the activity of the BCR-ABL oncogene.

The search for protein kinase inhibitors has led to the realization that not all kinases are equally amenable to targeting with potent, ATP-competitive small molecules. In this regard, a single residue in the ATP binding pocket (corresponding to threonine 338 in Hck) has been shown to control kinase sensitivity to a wide range of structurally unrelated compounds, including pyridinylimidazoles, pyrazolopyrimidines, purines, quinazolones, phenylaminopyrimidines, and staurosporines. This residue is conserved as a threonine or larger amino acid in the human kinome (no wild-type protein kinases contain an alanine or glycine at this position), and structural analysis has shown that the size of this gatekeeper residue restricts access to a pre-existing cavity within the ATP binding pocket. Kinases that possess a threonine at this position are readily targeted by diverse classes of small molecule inhibitors that can access this natural pocket. Moreover, mutation of the gatekeeper residue to a smaller amino acid, such as alanine or glycine, has been shown to reduce sensitivity to pyrazolopyrimidine inhibitors at low nanomolar concentrations in over 30 protein kinases—even though in many cases the wild-type kinase is completely insensitive to compounds of this class.

The importance of the gatekeeper in controlling inhibitor sensitivity is underscored by the fact that most kinase inhibitors currently in clinical use target kinases that contain a threonine at this position, even though threonine is found in only ~20% of the human kinome.
(e.g., Iressa: EGFR; Gleevec: Abl, PDGFR, and c-Kit; BAY43-9006: Raf). Indeed, analysis of mutations in BCR-Abl that confer drug resistance has shown that mutation of the gatekeeper to a larger amino acid (T315I) is one of the most common mechanisms of resistance to Gleevec.11 Remarkably, second generation BCR-Abl inhibitors designed to target resistant alleles have been shown to effectively inhibit every naturally occurring mutant of this kinase except those mutated at the gatekeeper residue.14,15

Recently, lipid kinases of the phosphoinositide 3-kinase (PI3-K) family have attracted considerable interest as a new class of drug targets.3,4 These enzymes act by generating the lipid second messengers phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃), which in turn activate downstream enzymes in a wide-range of signaling pathways involved in cell growth, survival, differentiation, and motility.16 Activating mutations in the PI3-K isoform p110α have recently been identified at high frequency in several types of cancer,17 and PTEN, the lipid phosphatase that reverses the phosphorylation reaction, has been identified as one of the most commonly inactivated tumor suppressors in the human genome.18 Moreover, the clinical efficacy of recently approved agents that target the epidermal growth factor receptor in breast19 and lung20,21 cancers has been demonstrated to correlate with the dependence of those cancers on aberrant PI3-K signaling and the ability of these agents to suppress that pathway. For these reasons, considerable effort has been directed toward the development of selective inhibitors of these enzymes as potential cancer therapeutics.22

Although PI3-Ks possess very low overall sequence homology to protein kinases, they possess the same overall fold, share several consensus sequences (e.g., the DFG motif that is responsible for coordination of Mg⁺), and are sensitive to two pan-specific protein kinase inhibitors (staurosporine and quercetin).23 Due to the importance of these enzymes as an emerging class of drug targets, we sought to identify the residue in lipid kinases analogous to the gatekeeper residue, and to explore how the size of this residue affects the active site structure and inhibitor sensitivity of this class of enzymes.

2. Results and discussion

2.1. The gatekeeper residue is conserved in lipid kinases

The crystal structure of the PI3-K p110γ bound to ATP has been solved,25 and the catalytic domain was found to share several features with reported protein kinase structures. These similarities include a two-lobed structure consisting of a N-terminal lobe containing a 5–7-stranded β-sheet, a loop connecting two of these strands that interacts with the phosphate groups of ATP, a conserved lysine residue that positions the α and β phosphate groups of ATP for the phosphotransfer reaction, and a primarily α-helical C-terminal lobe that binds the phosphoacceptor. In protein kinases, conserved hydrogen bonds to the N6 and N1 of positions of the adenine ring of ATP are made by the backbone amides of two conserved residues (corresponding to Glu 339 and Met 341 of Hck, respectively). The gatekeeper residue immediately precedes Glu 339, and forms the hydrophobic interior face of the ATP binding pocket, with Cβ of the gatekeeper typically positioned 4–7 Å from the N6 and N7 residues of adenine. Inspection of the ATP binding pocket of p110γ reveals a similar set of contacts. Hydrogen bonds to ATP are made by backbone amides from two residues—Glu 880 and Val 882—and these residues are immediately preceded by a large hydrophobic residue (Ile 879 in p110γ) that is positioned approximately 5 Å from N6 and N7 of adenine (Fig. 1A). To directly compare the orientation of these two residues (Ile 879 in p110γ and Thr 338 in Hck), we superimposed the crystal structures of Hck and p110γ with respect to the adenine ring of ATP using the WebLab Viewer 4.0. This structural alignment indicates that these two residues occupy a largely overlapping, but not identical, space within the interior of the ATP binding pocket, forming the innermost face that contacts the adenine ring of ATP (Fig. 1B). The most important difference between the p110γ and Hck structures in this region is that Cα of Ile 879 in p110γ is shifted slightly perpendicular to the plane of the adenine ring (∼2 Å) relative to Thr 338 in Hck, such that Ile 879 also participates in forming the interior roof of the ATP binding pocket, whereas Thr 338 is more completely fixed within the plane of the adenine ring.

To compare this region of primary sequence between PI3-Ks and protein kinases, a structure-based sequence alignment was generated. This analysis reveals that the

Figure 1. Conservation in the ATP binding pocket of PI3Ks. (A) Schematic illustrating the hydrogen bonding contacts to ATP. (B) Surface representation of the interior face of the ATP binding pocket of Hck (gray) and p110γ (orange), from published crystal structure data. (C) Structure-based sequence analysis of several residues lining the ATP binding pockets of Hck and several PI3Ks.
gatekeeper residue is situated within a small region of sequence homology between lipid and protein kinases (Fig. 1C). As observed for the gatekeeper residue in protein kinases, Ile 879 is conserved as a large hydrophobic residue in all PI3-Ks (isoleucine, leucine, methionine, or valine, although no PI3-Ks contain threonine at this position). The residue immediately following the Ile 879 is conserved in PI3-Ks as a glutamate (83%), and this is also the most common residue at that position in protein kinases (75%). The residues at the −1 and +1/+2 positions relative to Ile 879 in PI3-Ks are conserved as large hydrophobic residues, and the corresponding positions in protein kinases show a similar preference (Fig. 1C). For example, position 337 is either isoleucine, leucine, valine, or methionine in 86% of protein kinases and in 92% of human PI3-Ks. On this basis, we conclude that p110γ residue Ile 879 structurally corresponds to the gatekeeper residue in protein kinases.

As the gatekeeper residue is structurally conserved between PI3-Ks and protein kinases, we asked whether it is also functionally conserved, and in particular whether mutagenesis of this position to a smaller residue might induce inhibitor sensitivity as has been observed for protein kinases. Three representative members of the PI3-K family were selected for this analysis: the yeast PI3-K VPS34, the yeast PI3-K-related protein kinase MEC1, and the prototypical mammalian PI3-K p110α. The gatekeeper residue in each of these kinases was mutated to alanine and glycine, and the effect of this mutation on enzyme activity and inhibitor sensitivity was assessed.

2.2. Effects of gatekeeper mutation on enzymatic activity

Plasmids encoding mutated VPS34 and MEC1 kinases were transformed into S. cerevisiae knockout strains, such that the mutant allele functionally replaces the wild-type enzyme. Although yeast are viable in the absence of these proteins, specific growth conditions can induce a requirement for their catalytic activity, allowing us to examine whether the gatekeeper mutated alleles encode active kinases. The activity of VPS34 is required for growth of yeast at elevated temperature (37 °C) or high salt (1.0 M NaCl), whereas MEC1 activity is required for growth in the presence of agents that alkylate DNA (0.02% methylmethanesulfonate) or inhibit ribonucleotide reductase (60 mM hydroxylurea). Growth of the mutant strains under these conditions showed that the alanine gatekeeper mutant of VPS34 (I670A) as well as the alanine and glycine gatekeeper mutants of MEC1 (L2129A and L2129G) are able to functionally complement for the knockout at a level comparable to the wild-type enzyme (Fig. 2). Importantly, transformation with the empty vector or a vector containing a catalytically inactive, kinase-dead (kd) mutant of each protein did not rescue the knockout phenotype, confirming that the observed complementation is due to the catalytic activity of these proteins (Fig. 2).

For p110α, the gatekeeper residue was mutated to alanine and glycine (I848A and I848G) and the mutant kinases were expressed by transient transfection in cos-1 cells. The myc-tagged mutated kinases were purified by immunoprecipitation and their lipid kinase activity was assessed using a kinase assay in vitro. The enzymatic activity of the I848A and I848G mutants was significantly impaired relative to wild-type p110α, with the gatekeeper mutants displaying approximately one-hundredth (I848A) to one-thousandth (I848G) of the wild-type activity (Fig. 3A). The expression level of the wild-type and I848A proteins was similar by western blotting (Fig. 3B), suggesting that the gatekeeper mutation had disrupted the integrity of the enzyme active site in this mutant without globally destabilizing the protein.

As VPS34 and MEC1 appear to tolerate the gatekeeper mutation, the impaired activity of I848A p110α was surprising, and led us to search for differences in primary sequence between these related kinases that might account for the observed differences in biochemical activity. Based on the crystal structure of p110γ, the
residues that form the core of the ATP binding pocket were identified, and these residues were aligned among 15 members of the PI3-K family (Fig. 4). We then identified residues that are common to VPS34 and MEC1, but different in p110α, that might account for the observed difference in tolerating the gatekeeper mutation. This analysis focused our attention on cysteine 838 in p110α (Position 13, Fig. 4). Among the class I PI3-Ks such as p110α, this residue is conserved as a cysteine, whereas among the class III PI3Ks (e.g., VPS34) and PI3-K-related protein kinases (e.g., MEC1), this residue is conserved as a β-branched residue (valine or isoleucine). Importantly, inspection of the crystal structure of p110γ reveals that C838 is located in β-strand 6, directly adjacent to I848 in β-strand 7 (Fig. 5), suggesting that it may cooperate with the gatekeeper residue to stabilize this region of the protein. As β-branched amino acids have been shown to promote β-sheet formation, we reasoned that the presence of an isoleucine or valine residue at this position might stabilize that region of the protein. As such, this residue at position 838 stabilizing β-strands 6 and 7, rather than a nonspecific consequence of additional hydrophobic surface in the protein core.

2.3. Effects of gatekeeper mutation on inhibitor sensitivity

We next sought to assess how mutation of the gatekeeper residue to a smaller amino acid would affect inhibitor sensitivity. In protein kinases, mutation of the gatekeeper to alanine or glycine has been shown to induce sensitivity to both pyrazolopyrimidine inhibitors based on the Src-family kinase inhibitor PP1 and analogs of the natural product K252a.12 p110γ has been shown to be sensitive to staurosporine (Kd = 0.29 μM),23 which is structurally related to K252a, suggesting that K252a analogs may also target engineered lipid kinases. Although wild-type PI3-Ks are not sensitive to the protein kinase inhibitor PP1, mutation of the gatekeeper residue in protein kinases can induce sensitivity to this class of compounds in kinases that otherwise show no affinity for this scaffold.12 Following this reasoning, we screened a small panel of these compounds in vitro against the wild-type and I848A p110α and in vivo against the wild-type and I670A mutant VPS34 (Supplementary Fig. 1). None of these compounds showed selective inhibition of the I848A allele of p110α at a concentration of 50 μM in vitro or I670A VPS34 at a concentration of 1 mM in a yeast halo assay. These results suggest that the structural differences between protein and lipid kinases may be too significant to bridge with a single inhibitor scaffold, and that new inhibitor analogs may be necessary to explore the gatekeeper pocket in lipid kinases.

To probe more directly the engineered gatekeeper pocket, we prepared a panel of analogs of the PI3-K inhibitor LY294002.30 LY294002 reversibly inhibits PI3-Ks at IC50 values in the low micromolar range, but shows little selectivity among individual family members.31 The crystal structure of LY294002 bound to p110γ has been solved,23 and reveals that the C3 position of LY294002 is located adjacent to the gatekeeper residue at a distance of approximately 4 Å. Molecular modeling based on this structure suggests that analogs of LY294002...
containing extended substituents at C3 would access the nascent pocket created by the gatekeeper mutation (Fig. 5). A series of C3-substituted analogs of LY294002 were designed (lacking the 8-phenyl group of LY294002, as this moiety has been shown to be dispensable for binding to PI3-Ks 30) and a panel of such analogs was prepared. This series of compounds was designed to include analogs that possess C3 substituents similar in size to the space created directly by the isoleucine to alanine mutation (e.g., Et, n-Pr), as well as those that contain much larger substituents (Ph, Bn). These latter compounds were included to probe whether the gatekeeper mutation would allow access to a deeper cavity within the kinase active site; in protein kinases, mutation of the gatekeeper has been shown to facilitate binding of substrate and inhibitor analogs containing bulky substituents much larger than the space created directly by the amino acid change. 12,13

Synthesis was accomplished by addition of an excess of the appropriate Grignard reagent to salicylaldehyde to afford benzylic alcohols 1c–g (Scheme 1). The resulting alcohols were oxidized to the corresponding ketones 2c–g using MnO₂, and the ring closing was performed using a morpholine phosgene salt 30,34 to afford the desired 3-substituted analogs of LY294002 in high purity as white solids.

This panel of LY294002 analogs was tested for inhibition of the wild-type and C838V/I848A mutant of p110α in a PI3-K assay in vitro (Table 1). Both LY294002 and the desphenyl analog LY292223 inhibited wild-type and C838V/I848A p110α at low micromolar concentrations, although inhibition by LY292223 was modestly reduced for the double mutant. As the size of the C3-moiety was increased, IC₅₀ values against both the mutant and wild-type p110α increased by ~10 to >100-fold, and this increase tracked with the size of the C3 substituent, with the weakest inhibition by compounds 3g (Ph) and 3h (Bn). Surprisingly, only compound 3c (Et) showed enhanced binding to mutant p110α relative to wild-type p110α, with modest selectivity for the engineered kinase (~6-fold). This compound contains an ethyl moiety at the C3 position, which is the most similar in size to the space created directly by the amino acid change. 12,13

3. Conclusions

We have explored the role of the gatekeeper residue in PI3-Ks by a convergent engineering approach that combines mutagenesis of the target residue with design of inhibitor analogs to complement this mutation. This study suggests that mutation of the gatekeeper residue in lipid kinases can create a nonnatural pocket, but that this mutation does not provide access to a deeper pocket such as that found in protein kinases. This difference may reflect the different way that protein and lipid kinases utilize their primary sequence to construct the innermost wall of the ATP binding pocket. Crystal structures of protein kinases reveal that residues from...
β-strand 5, which include the gatekeeper, form the deepest face of the ATP binding pocket, and that most of the contacts that this face makes with adenine involve the side chain of the gatekeeper residue directly. By comparison, in the crystal structure of p110α, the gatekeeper is shifted upward and away from the N6 of adenine by ~2 Å. To help fill this space, the side chain of Tyr 867 from β-strand 6 (which is otherwise positioned in a second sphere of residues that do not contact ATP directly) infiltrates the ATP binding pocket to make a direct contact with adenine near N6. The analogous residue in protein kinases (corresponding to Leu 325 in Hck) is positioned within a second sphere that is obstructed from accessing ATP by the gatekeeper. Thus, it appears that in lipid kinases two residues (Tyr 867 and Ile 879) collaborate to fill the space that is otherwise occupied by a single residue in protein kinases. Unfortunately, we have found that mutation of Tyr 836 in p110α (which corresponds to Tyr 867 in p110γ) to any other amino acid tested (glycine, alanine, threonine, aspartic acid, leucine, methionine, or histidine) results in a complete loss of catalytic activity, indicating that this region of the protein is not amenable to structural modification (Z.A.K. and K.M.S., unpublished observations). These results, as well as the nearly perfect conservation of Tyr 867 within the PI3-K family (Fig. 4), suggest that a different set of residues in lipid kinases are likely responsible for controlling sensitivity to small molecule inhibitors. Ultimately, the identification of rules guiding inhibitor sensitivity for this important family of enzymes will require the discovery of new structural classes of PI3-K inhibitors and the broad characterization of their specificity against individual PI3-K isoforms, a process that is currently underway in our laboratory and others.

4. Experimental

4.1. Protein expression

Mutations were introduced by Quikchange (Stratagene), and confirmed using standard dideoxy-based sequencing. Myc-tagged p110α was expressed by transient transfection of cos-1 cells. Cells were lysed in lysis buffer (50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% NaN₃, 1% Triton X-100), protease inhibitors (protease inhibitor cocktail tablets (Roche); sodium orthovanadate (8 mM); PMSF (83 μM)), and 8 mM DTT. The kinase was immunoprecipitated using a Protein-G-9E10 antibody complex, and washed twice with buffer A (PBS, 1 mM EDTA, 1% Triton X-100), twice with buffer B (100 mM Tris (pH 7.4), 500 mM LiCl, 1 mM EDTA), twice with buffer C (50 mM Tris (pH 7.4), 100 mM NaCl), and once with PBS. In control
experiments, no differences in inhibitor sensitivity were observed between wild type p110x protein that was obtained from transfected cos-1 cells, SP9 cells using a baculovirus system, or commercially available recombinant protein (Jena Bioscience).

For SDS-PAGE and Western blot analyses, protein concentrations in cell lysates were determined using a Bradford assay. Proteins were loaded onto a Tris-glycine gel (8–16% gradient; Gradipore) and separated by SDS-PAGE before being transferred onto a nitrocellulose membrane. The blot was treated with blocking reagent (5% dry milk in TBST) for 1 h, then primary antibody (9E10 (anti-Myc; Santa Cruz Biotech), 1:500 in TBST) overnight at 4 °C. The blot was then rinsed (5 min each) with deionized water (1 x 20 mL) and TBST (3 x 20 mL) before treatment with secondary anti-mouse-HRP antibody (1:1000 in TBST) for 30 min at rt.

4.2. PI3-Kinase enzymatic assay

The PI3K assay was performed essentially as described.31 Briefly, a mixture of kinase, inhibitor, buffer (25 mM HEPES (pH 7.4), 10 mM MgCl₂), and freshly sonicated phosphatidylinositol (200 µg/mL) was prepared at 4 °C, and aliquotted into eppendorf tubes. The tubes were allowed to warm to rt over 5 min, and the enzymatic reaction was initiated with the addition of ATP (10 µCi γ-32P-ATP; final [ATP] = 20 nM). Reactions were incubated for 20 min at rt, and quenched by addition of 1 M HCl (105 µL) followed by 1:1 MeOH–CHCl₃ (160 µL). The resulting biphasic mixture was vortexed (ca. 5 s), briefly centrifuged (ca. 5 s), and the organic phase (~100 µL) was transferred to a new tube using a gel-loading tip pre-coated with CHCl₃. This extract was spotted on TLC plates (silica gel 60 F254, 250 µm) and developed for 3–4 h using 65:35 1-propyl alcohol–CHCl₃ (160 µL). The resulting radiolabeled impurities that were kinase-independent (data not shown). In control experiments, no difference was observed in inhibitor sensitivity between performing the assay in the absence or presence (1 µM) of cold ATP.

4.3. Analysis of yeast expressing PI3-K mutant alleles

The VPS34 S. cerevisiae knockout strain 15149 was obtained from the ATCC. The MECl/SML1 S. cerevisiae knockout strain yBRT7-4b was a gift from David Toczyski (UCSF). Knockout strains were transformed with plasmids (pRS416 CEN URA3) encoding wild-type and mutant VPS34 and MECl and transformants were selected on SD-URA media. Cells were streaked onto plates containing SD-URA or the same media supplemented with either 1 M NaCl (VPS34) or 0.02% MMS or 60 mM HU (MECl), grown at 30 or 37 °C for 3 days, and photographed.

4.4. Chemical synthesis

4.4.1. General methods. All reactions were performed under argon in oven- or flame-dried glassware fitted with rubber septa, and were stirred magnetically. Thin layer chromatography was performed on Merck precoated silica gel F-254 plates (0.25 mm). Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh). Proton NMR spectra were recorded at 400 MHz and are reported in δ (ppm) as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad), and are referenced to the residual solvent signal: CDCl₃ (7.26) or C₆D₆ (7.15). Carbon NMR spectra were recorded at 100 MHz and are reported in ppm, and are referenced to the solvent signal: CDCl₃ (77.0), C₆D₆ (128.0). Infrared spectra were recorded on a Nicolet Impact 400 spectrometer using thin films of sample and resonances are reported in wavenumbers (cm⁻¹). Mass spectra were recorded on a VG-70S mass spectrometer and are reported in units of mass/charge (m/z). Unless otherwise noted, all materials were obtained from commercial sources and used without further purification. In experiments involving air- or moisture-sensitive compounds, solvents and reagents were either distilled or purchased as anhydrous grade material. Dichloromethylenemorpholin-4-ium chloride was prepared according to literature procedures.33,34

4.4.2. General procedure for the preparation of alcohols 1c–g. A round bottom flask charged with salicylaldehyde and THF was cooled to −78 °C. To this mixture was added the appropriate Grignard reagent (5–10 equiv) by syringe over 10 min, and the resulting mixture was allowed to stir at −78 °C for 10 min. The stirred reaction mixture was allowed to warm to rt overnight. In air, this mixture was slowly added to a 0 °C saturated NH₄Cl solution. The azeotropic was extracted with ether and the combined organic phases were dried over Na₂SO₄, filtered, and concentrated to give a crude oil. Further purification and characterization of each derivative is described below.

4.4.2.1. 2-(1-Hydroxybutyl)phenol (1c). Synthesis was performed using the general procedure described above using 1.15 g salicylaldehyde (9.4 mmol), 94 mL THF, 94 mmol propylmagnesium chloride (47 mL of 2.0 M solution in ether), saturated NH₄Cl solution (75 mL), and ether (3 x 40 mL). The crude oil was purified by flash column chromatography (SiO₂, 10% EtOAc–hexanes) to give a clear, pale yellow oil (1.226 g, 7.4 mmol, 79%).1H NMR: (CDCl₃) δ 8.16 (s, 1H), 7.12 (t, J = 8 Hz, 1H), 6.91 (d, J = 8 Hz, 1H), 6.82 (m, 2H), 4.76 (t, J = 7 Hz, 1H), 3.35 (s, 1H), 1.83 (m, 1H), 1.74 (m, 1H), 1.43 (m, 1H), 1.30 (m, 1H), 0.91 (t, J = 8 Hz, 3H).13C{1H} NMR: (CDCl₃) δ 155.2, 128.7, 127.8, 127.2, 119.7, 116.9, 75.5, 39.3, 18.9, 13.8. IR: 3321. HRMS: (EI) calcd for C₁₀H₁₄O₂ (M⁺) 166.0994; found
of 2.0 M solution in THF), saturated NH₄Cl (75 mL), and ether (3 × 50 mL). The crude oil was purified by flash column chromatography (SiO₂, 50% EtOAc–hexanes) to give a colorless solid (1.42 g, 6.6 mmol, 75%). ¹H NMR: (CDCl₃) δ 8.01 (s, 1H), 7.34–7.14 (m, 5H), 6.93–6.79 (m, 4H), 4.98 (dt, J = 3 Hz, J = 7 Hz, 1H), 3.10 (d, J = 7 Hz, 2H), 2.81 (d, J = 3 Hz, 1H). Lit. 37 (partial) δ 5.02 (t, J = 7 Hz, 1H), 3.11 (d, J = 7 Hz, 2H). ¹³C{¹H} NMR: (CDCl₃) δ 151.6, 133.6, 125.8, 125.3, 125.0, 125.3, 123.3, 122.8, 116.1, 113.5, 73.0, 40.4. IR: 3327. HRMS: (EI) 214.0993 calc’d for C₁₄H₁₄O₂ (M⁺); found 214.1003.

4.4.3. General procedure for the preparation of ketones 2c–g. A round bottom flask was charged with the appropriate diol 1, MnO₂, and CH₂Cl₂, and stirred for 7 h at rt. The heterogeneous reaction mixture was filtered through a pad of Celite, and the solids were washed with 500 mL CH₂Cl₂. The filtrate was concentrated in vacuo to give a crude oil. The crude oil was purified by flash column chromatography (SiO₂, 10% EtOAc–hexanes) to give a colorless oil. Characterization of each derivative is described below.

4.4.3.1. 1-(2-Hydroxyphenyl)butan-1-one (2c). Synthesis was performed using the general procedure described above using 1g (2.5 g, 15.1 mmol), MnO₂ (11.5 g, 132 mmol), and CH₂Cl₂ (140 mL). Compound 2c was isolated as a colorless oil (1.13 g, 6.9 mmol, 46%). ¹H NMR: (CDCl₃) δ 12.38 (s, 1H), 6.78 (dd, J = 1 Hz, J = 7 Hz, 1H), 7.36 (dt, J = 2 Hz, J = 7 Hz, 1H), 6.90 (dd, J = 1 Hz, J = 7 Hz, 1H), 6.80 (dt, J = 2 Hz, J = 7 Hz, 1H), 2.87 (t, J = 8 Hz, 2H), 1.70 (sextet, J = 8 Hz, 2H), 0.96 (t, J = 8 Hz, 3H). Lit. 40 δ 12.40 (s, 1H), 7.82–6.80 (m, 4H), 2.96 (t, J = 7.4 Hz, 2H), 1.78 (sextet, J = 7.4 Hz, 2H), 1.02 (t, J = 7.4 Hz, 3H). ¹³C{¹H} NMR: (CDCl₃) δ 206.7, 162.5, 136.1, 129.9, 119.3, 118.8, 114.8, 101.1, 17.8, 13.7. Lit. 40 δ 206.79, 162.49, 136.17, 130.00, 119.36, 118.82, 118.48, 40.17, 17.90, 13.38. IR: 1641, 1614, 1581, 1488, 1447, 1265, 1202, 1158, 754. Lit. 40 1640, 1448, 1226, 1203. HRMS: (EI) 164.0837 calc’d for C₇H₁₂O₂ (M⁺); found 164.0840. Lit. 40. 164. Anal. Calc’d for C₁₀H₁₂O₂: C; 73.15; H; 7.37. Found: C; 73.22; H; 7.45.

4.4.3.2. 1-(2-Hydroxyphenyl)pentan-1-one (2d). Synthesis was performed using the general procedure described above using 1d (2.0 g, 11.1 mmol), MnO₂ (9.7 g, 110 mmol), and CH₂Cl₂ (100 mL). Compound 2d was isolated as a colorless oil (796 mg, 4.47 mmol, 40%). ¹H NMR: (CDCl₃) δ 12.38 (s, 1H), 7.71 (dd, J = 1 Hz, J = 8 Hz, 1H), 7.41 (m, 1H), 6.92 (dd, J = 1 Hz, J = 8 Hz, 1H), 6.84 (m, 1H), 2.93 (t, J = 7 Hz, 2H), 1.68 (m, 2H), 1.38 (m, 2H), 0.93 (t, J = 7 Hz, 3H). ¹³C{¹H} NMR: (CDCl₃) δ 208.7, 162.3, 135.9, 129.8, 119.0, 118.5, 118.2, 37.8, 26.3, 22.1, 13.6. IR: 1640, 1582, 1487, 1446, 1353, 1249, 119, 1157, 753. HRMS: (EI) 178.0994 calc’d for C₁₄H₁₄O₂ (M⁺); found 178.0996. Combustion analysis was not obtained for this compound due to its similarity to 2c–g.

4.4.3.3. 1-(2-Hydroxyphenyl)-3-methylbutan-1-one (2e). Synthesis was performed using the general proce-
The reaction mixture was isolated as a colorless oil (650 mg, 3.65 mmol, 33%). 1H NMR: (CDCl₃) δ 2.45 (s, 1H), 7.69 (dd, J = 2 Hz, J = 8 Hz, 1H), 7.38 (dt, J = 2 Hz, J = 8 Hz, 1H), 6.91 (dd, J = 2 Hz, J = 8 Hz, 1H), 6.82 (dt, J = 2 Hz, J = 8 Hz, 1H), 7.27 (d, J = 7 Hz, 2H), 2.24 (nonet, J = 6 Hz, 1H), 0.96 (d, J = 6 Hz, 6H). Lit. δ 12.47 (s, 1H), 7.81–6.82 (m, 4H), 2.85 (d, J = 6.9 Hz, 2H), 1.75 (nonet, J = 6.7 Hz, 1H), 1.02 (d, J = 6.6 Hz, 6H). 13C{1H} NMR: (CDCl₃) δ 206.4, 162.5, 136.0, 130.0, 119.5, 118.6, 118.3, 46.9, 25.3, 22.5. Lit. δ 207.2, 163.1, 136.7, 130.6, 120.1, 119.3, 119.0, 47.6, 26.0, 23.2. IR: 1638, 1488, 1447, 1306, 1202, 1158, 753. Lit. 1639, 1488, 1447, 1158. HRMS: (EI) 178.0994 calcd for C₁₁H₁₂O₂ (M⁺); found 178.0999. Lit. 178. 178.

4.4.3.4. 1-(2-Hydroxyphenyl)-2-phenylethanone (2g). Synthesis was performed using the general procedure described above using 1f (2.0 g, 13.3 mmol), MnO₂ (8.0 g, 92 mmol), and CH₂Cl₂ (110 mL). Compound 2g was isolated as a colorless oil (900 mg, 4.68 mmol, 46%). 1H NMR: (CDCl₃) δ 12.38 (s, 1H), 7.70 (dd, J = 2 Hz, J = 8 Hz, 1H), 7.40 (m, 1H), 6.92 (m, 1H), 6.83 (m, 1H), 2.92 (t, J = 7 Hz, 2H), 1.70 (m, 2H), 1.33 (m, 4H), 0.88 (t, J = 7 Hz, 3H). Lit. δ 12.40 (s, 1H), 7.82–6.80 (m, 4H), 2.98 (t, J = 7.6 Hz, 2H), 1.84–1.25 (m, 6H), 0.91 (t, J = 6.7 Hz, 3H). 13C{1H} NMR: (CDCl₃) δ 206.8, 162.4, 136.0, 129.9, 119.2, 118.7, 118.3, 38.1, 31.3, 24.0, 22.4, 13.8. Lit. 178. 178. 207.18, 162.4, 153.5, 132.2, 125.2, 124.6, 122.8, 116.3, 87.0, 65.8, 44.5. Lit. δ 176.9, 162.4, 153.5, 132.1, 125.3, 124.6, 122.7, 116.1, 87.2, 65.8, 44.4. IR: 1616, 1555, 1418, 1300, 1251, 1117, 985, 766. Lit. 1622, 1559. HRMS: (EI) 231.0895 calcd for C₁₁H₁₀NO₂ (M⁺); found 231.0887. Anal. Calcd for C₁₁H₁₀NO₂: C, 72.57; H, 5.66; N, 6.00. Found: C, 67.59; H, 5.69; N, 5.97.

4.4.4. 2-Morpholin-4-yl-chromen-4-one (LY292223) (3a). Synthesis was performed using the general procedure described above using 2'-hydroxyacetophenone (565 mg, 4.15 mmol), CH₂Cl₂ (11 mL), TiCl₄ (6.5 mL of a 1.0 M CH₂Cl₂ solution, 6.5 mmol), diisopropylmethylamine (2.7 mL, 15.5 mmol), 4-dichloromethylenemorpholin-4-ium chloride (1.2 g, 5.0 mmol), Et₃N (1.0 mL), and MeOH (5 mL). Column chromatography: SiO₂, 40% EtOAc–hexanes + 1% Et₃N to 90% EtOAc–MeOH + 1% Et₃N gradient (344 mg, 1.5 mmol, 36%). 1H NMR: (CDCl₃) δ 7.95, 7.35, 7.14, 7.08, 5.27 (s, 1H), 3.63 (t, J = 5 Hz, 4H), 3.30 (t, J = 5 Hz, 4H). Lit. δ 8.15 (m, 1H), 7.56 (m, 1H), 7.31 (m, 2H), 5.49 (s, 1H), 3.83 (t, J = 4.7 Hz, 4H), 3.50 (t, J = 4.7 Hz, 4H). 13C{1H} NMR: (CDCl₃) δ 176.8, 162.5, 153.5, 132.2, 125.4, 122.8, 116.3, 87.0, 65.8, 44.5. IR: 1616, 1555, 1418, 1300, 1251, 1117, 985, 766. Lit. 1622, 1559. HRMS: (EI) 245.1051 calcd for C₁₃H₁₂NO₂ (M⁺); found 245.1050. Combustion analysis was not obtained for this compound due to its similarity to 3a–i.

4.4.4.3. 3-Ethyl-2-morpholin-4-yl-chromen-4-one (3c). Synthesis was performed using the general procedure described above using 407 mg 2c, 30 mL CH₂Cl₂, 3.71 mmol TiCl₄, 3.2 g i-Pr₂EtN, 759 mg dichloromethylenemorpholin-4-ium chloride, 0.6 mL Et₃N, and 3 mL MeOH. Column chromatography: SiO₂, 30% EtOAc–hexanes + 1% Et₃N (364 mg, 1.40 mmol, 57%). 1H NMR: (C₆D₆) δ 8.29 (d, J = 8 Hz, 1H), 7.12 (m, 1H), 6.95 (m, 2H), 3.40 (t, J = 5 Hz, 4H), 2.83 (t, J = 5 Hz, 4H), 2.48 (q, J = 7 Hz, 2H), 1.19 (t, J = 7 Hz, 3H). 13C{1H} NMR: (C₆D₆) δ 177.8, 161.8,
4.4.4. 3-Benzyl-2-morpholin-4-yl-chromen-4-one (3b). Synthesis was performed using the general procedure described above using 1.0 g 2'-hydroxy-3-phenyl-propiophenone, 12 mL CH₂Cl₂, 6.63 mmol TiCl₄, 2.7 mL i-Pr₂EtN, 1.2 g dichloromethylchromen-4-ium chloride, 0.2 mL Et₃N, and 1.2 mL MeOH. Column chromatography: SiO₂, 35% EtOAc–hexanes + 1% Et₂N (352 mg, 1.80 mmol, 41%). HRMS: (C₆H₁₁NO₃)²⁺ found 287.1521. Combustion analysis was not obtained for this compound due to its similarity to 3a-i.

4.4.4.9. 3-Decyl-2-morpholin-4-yl-chromen-4-one (3i). Synthesis was performed using the general procedure described above for 102 mg 2'-hydroxydodecanophenone, 3.0 mL CH₂Cl₂, 0.551 mmol TiCl₄, 473 mg i-Pr₂EtN, 113 mg dichloromethylchromen-4-ium chloride, 0.06 mL Et₃N, and 0.3 mL MeOH. Column chromatography: SiO₂, 20% EtOAc–hexanes + 1% Et₂N. HRMS: (C₆H₁₁NO₃)²⁺ found 321.5786. Combustion analysis was not obtained for this compound due to its similarity to 3a-i.

Acknowledgements

We thank the members of the Shokat research group, particularly Quincey Justman, Scott Lazzerwitz, Jennifer Paulson, and Daniel Rauh, as well as Professor James Bobbitt (Univ. Connecticut) for helpful discussions. Funding was provided by postdoctoral fellowships from the Susan G. Komen Breast Cancer Foundation and the California Section of the American Cancer Society (P.J.A.), a predoctoral fellowship from the Howard

154.2, 132.3, 126.0, 124.5, 123.5, 116.9, 110.4, 66.6, 49.3, 18.6, 13.3. IR: 1615, 1556, 1467, 1401, 1361, 1264, 1223, 1116, 759. HRMS: (EI) 259.1208 calcd for C₁₇H₁₇NO₃ (M⁺); found 259.1199. Anal. Calcd for C₁₇H₁₇NO₃: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.43; H, 6.60; N, 5.36.

4.4.4.2. 2-Morpholin-4-yl-3-propylchromen-4-one (3d). Synthesis was performed using the general procedure described above using 68 mg 2d, 5 mL CH₂Cl₂, 0.57 mmol TiCl₄, 490 mg i-Pr₂EtN, 117 mg dichloromethylchromen-4-ium chloride, 0.1 mL Et₃N, and 0.5 mL MeOH. Chromatography: SiO₂, 20% EtOAc–hexanes + 1% Et₂N (58 mg, 0.21 mmol, 56%). HRMS: (C₆H₁₁NO₃)²⁺ found 287.1520. Combustion analysis was not obtained for this compound due to its similarity to 3a-i.

4.4.4.3. 2-Morpholin-4-yl-3-phenylchromen-4-one (3g). Synthesis was performed using the general procedure described above using 143 mg 2g, 5 mL CH₂Cl₂, 1.01 mmol TiCl₄, 870 mg i-Pr₂EtN, 206 mg dichloromethylchromen-4-ium chloride, 0.1 mL Et₃N, and 0.5 mL MeOH. Column chromatography: SiO₂, 20% EtOAc–hexanes + 1% Et₂N (144 mg, 0.47 mmol, 70%). HRMS: (C₆H₁₁NO₃)²⁺ found 287.1521. Combustion analysis was not obtained for this compound due to its similarity to 3a-i.
Supplementary data


References and notes


