# Acetylation of the catalytic lysine inhibits kinase activity in PI3Kδ

Julie C. L. Fournier[†,‡], John P. Evans[ƨ], Francesca Zappacosta[§], Daniel A. Thomas[ƨ], Vipulkumar K. Patel[†,*], Gemma V. White[†], Sebastien Campos[‖], Nicholas C. O. Tomkinson[‡]

[†]GSK Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK
[‡]Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK
[ƨ]Arctoris, 120E Olympic Avenue, Milton Park, Oxford, OX14 4SA
[§]GSK, South Collegeville Road, Collegeville, PA 19426, USA
[‖]Pharmaron, West Hill Innovation Park, Hertford Road, Hoddesdon, Hertfordshire EN11 9FH, UK

*Abstract:*

Covalent inhibition is a powerful strategy to develop potent and selective small molecule kinase inhibitors. Targeting the conserved catalytic lysine is an attractive method for selective kinase inactivation. We have developed novel, selective inhibitors of phosphoinositide 3-kinase δ (PI3Kδ) which acylate the catalytic lysine, Lys779, using activated esters as the reactive electrophiles. The acylating agents were prepared by adding the activated ester motif to a known selective dihydroisobenzofuran PI3Kδ inhibitor. Three esters were designed, including an acetate ester which was the smallest lysine modification evaluated in this work. Covalent binding to the enzyme was characterised by intact protein mass spectrometry of the PI3Kδ-ester adducts. Enzymatic digest coupled with tandem mass spectrometry identified Lys779 as the covalent binding site, and a biochemical activity assay confirmed that PI3Kδ inhibition was a direct result of covalent lysine acylation. These results indicate that a simple chemical modification such as lysine acetylation is sufficient to inhibit kinase activity. The selectivity of the compounds was evaluated against lipid kinases in cell lysates using a chemoproteomic binding assay. Due to the conserved nature of the catalytic lysine across the kinome, we believe the covalent inhibition strategy presented here could be applicable to a broad range of clinically relevant targets.

*Graphical abstract:*

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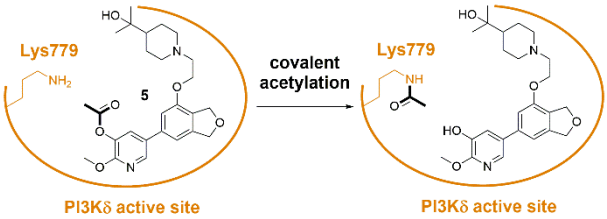
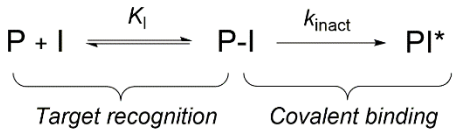


Covalent drugs are a subclass of small molecule therapeutics which induce a physiological response by covalently binding to their biological targets.[1] They have mostly been exploited as secondary drug discovery strategies for chronic indications, because of a perceived risk of increased toxicity arising from unselective covalent binding.[2, 3] Despite these concerns, several widely-prescribed covalent drugs including aspirin, β-lactam antibiotics and clopidogrel demonstrate that toxicity issues can be managed to provide safe and effective therapeutics.[4] The risk of promiscuous covalent binding is minimized by fine-tuning the reactivity of the small molecules to the desired targets. Accordingly, the preferred reactive centers for covalent drugs are mildly electrophilic functional groups.[5] The choice of electrophile depends on the targeted nucleophile: usually cysteines, lysines, serines or other nucleophilic amino acids.[6, 7] Covalent drugs bind to their protein targets in a two-step process (Scheme 1).[8] Non-covalent interactions initially lead to the formation of a reversible protein-drug complex (P–I), characterised by a thermodynamic binding constant $K_I$. The increased effective concentrations of both reactive partners create forcing conditions to promote covalent binding, which can be described by a kinetic inactivation constant $k_{inact}$.[9] As a result, the selectivity profile of a covalent drug depends on multiple factors, including its binding affinity to the desired target, the intrinsic chemical reactivity of the electrophile and its spatial orientation with respect to the nucleophilic residue.[10]




Scheme 1 – Two-step binding of covalent drugs. Equilibrium-based interactions first form the reversible complex P–I, characterised by a thermodynamic binding constant $K_I$. Subsequent covalent bond formation affords the covalent adduct PI*, characterised by a kinetic inactivation constant $k_{inact}$.

Rational design strategies have been emerging in the literature, highlighting a growing interest in covalent drug development.[6, 11, 12] Common methods to identify hit molecules involve screening covalent fragment libraries,[13, 14] virtual screens,[15] or adding electrophilic motifs to known reversible ligands.[16] Our work contributes to an on-going effort focused on developing methodologies for covalent drug design.

This renewed interest in developing covalent drugs has uncovered unique advantages over non-covalent alternatives, including increased potencies and longer duration of action.[17] These characteristics have been successfully exploited in the field of kinase inhibition. Dysregulation of kinase activity has been linked to various pathologies, highlighting them as pharmaceutical targets of interest.[18, 19] As of 2020, 52 kinase inhibitors had been approved by the FDA;[20]

however, developing these drugs presents a number of difficulties.[18] A key challenge is achieving selectivity for the desired target: most kinase inhibitors interact with the ATP-binding site, which is highly conserved across the human kinome (>500 kinases).[21, 22] Furthermore, reversible ATP-competitive inhibitors require high binding affinities to maintain target engagement in cellular environments with elevated ATP concentrations.[23] Covalent inhibition can overcome this challenge by preventing displacement of the inhibitor by endogenous ATP.[8, 24]

Early efforts in covalent kinase inhibition identified acrylamide electrophiles which targeted non-conserved cysteines close to the ATP pocket.[24] This led to the approval of tyrosine kinase inhibitors ibrutinib, afatinib and osimertinib for oncology indications (Figure 1A).[25, 26] Cysteines offer good opportunities for selectivity, because of their low occurrence (2.3%) in the human proteome.[27] Consequently, this strategy is limited to kinases bearing a reactive cysteine in the vicinity of a binding pocket.[28] Furthermore, the development of resistance mechanisms through cysteine mutations were observed in patients treated with osimertinib and ibrutinib, considerably decreasing drug efficiency.[29-31] These limitations warrant the need for complementary methods to expand the toolbox for covalent kinase inhibition.[32]

Inspiration for alternative covalent inhibition strategies can be found in natural product chemistry. Wortmannin (**1**), for example, is a broad-spectrum covalent inhibitor of the phosphoinositide 3-kinase (PI3K) family (Figure 1B).[33] The Michael acceptor motif in **1** binds to the kinase catalytic lysine in two closely related isoforms: PI3Kα and γ.[34, 35] The catalytic lysine is a conserved residue across the kinome which is crucial for kinase activity. The primary amine of the lysine side-chain interacts with the α and β-phosphates of ATP, participating in the correct positioning of the γ-phosphate for substrate phosphorylation.[36, 37] Wortmannin **1** displays $IC_{50}$ values in the low nanomolar range against PI3K isoforms (at 50 μM ATP),[38] which suggests that covalently blocking the catalytic lysine is an efficient method of kinase inactivation. However, wortmannin **1** exhibits toxicity as a result of poor kinase selectivity, and is chemically unstable due to facile hydrolytic opening of the furan ring.[39]

Recent work on targeting the kinase catalytic lysine from Dalton *et al*. reported activated esters as covalent PI3Kδ inhibitors.[40] Despite the conserved nature of the catalytic lysine (Lys779 in PI3Kδ sequence), compound **2** (Figure 1B) was found to selectively inhibit PI3Kδ in a live cell chemoproteomics assay. This work demonstrated that it was possible to exploit the conserved catalytic lysine for covalent kinase inhibition while minimizing off-target activity.

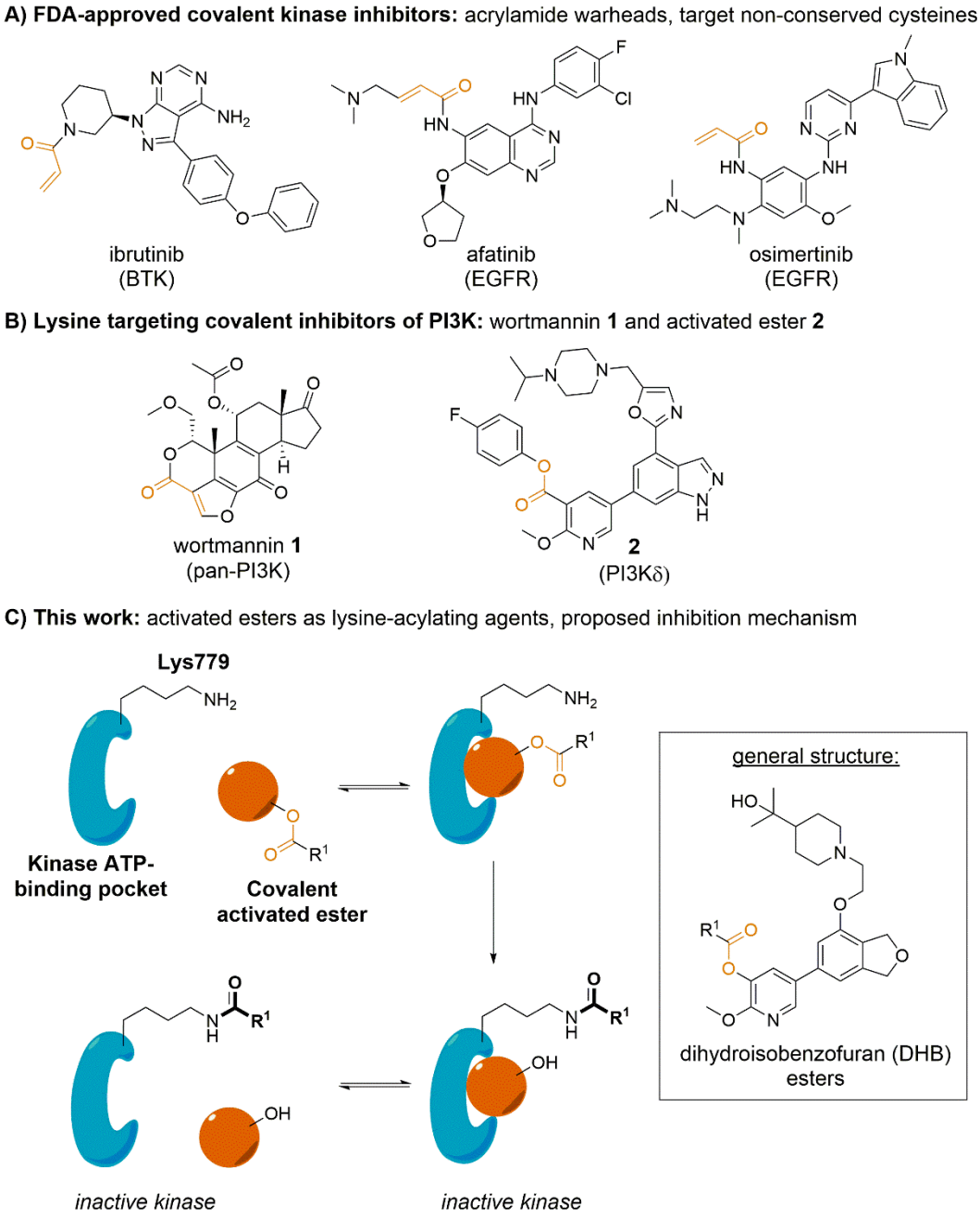



Figure 1 – Covalent kinase inhibition strategies. A) FDA-approved covalent inhibitors ibrutinib (Bruton's Tyrosine Kinase, BTK), afatinib (Epidermal Growth Factor Receptor tyrosine kinase, EGFR) and osimertinib (EGFR). The 3 inhibitors contain an acrylamide warhead which covalently bind to non-conserved cysteines; B) Lysine targeting covalent inhibitors of PI3K: wortmannin **1** (pan-PI3K) and activated ester **2** (PI3Kδ); C) Scope of this work: exploring lysine acylation with activated esters as a covalent inhibition method. The dihydroisobenzofuran (DHB) core drives selective reversible binding to PI3Kδ, bringing the ester close to Lys779.

Building on these results, we further investigated the impact of Lys779 modification on PI3Kδ activity. A known PI3Kδ ligand was repurposed to design covalent acylating agents of Lys779, using activated ester warheads (Figure 1C). Similarly to well-established methods for the regioselective labelling of native proteins,[41] our covalent probes would rely on group transfer

chemistry to induce a controlled modification of a specific residue in the enzyme active site. We were specifically interested in exploring lysine acetylation as a covalent inactivation mechanism. This idea was supported by previous work on acetyltransferase mediated processes, demonstrating that kinase activity could be endogenously modulated by acetylation of active site lysines.[42-44]

Lysine acetylation is a naturally occurring post-translational modification (PTM) used to regulate protein function in cells.[45] By contrast, most covalent drugs induce exogenous structural modifications of proteins. Irreversible covalent bonds can subsist after proteolysis, generating unnatural peptide fragments which could be recognized as "non-self" by immune cells.[46] This is believed to be one of the mechanisms responsible for immune mediated adverse drug reactions.[47] The risk of triggering an immune response would theoretically be minimized if the protein modification induced by the covalent inhibitor was similar to naturally occurring PTMs. We therefore sought to exploit small molecule mediated lysine acetylation as a novel mechanism for covalent kinase inhibition, which could provide a solution to existing risks of immune mediated adverse drug reactions.

Within this manuscript we present the design of novel covalent PI3Kδ inhibitors, as well as the biochemical assays used to evaluate covalent binding to the protein and inactivation of kinase activity. A range of protein mass spectrometry experiments were used to characterize binding to PI3Kδ and elucidate the covalent labelling site. The inhibitory activity of the compounds was evaluated in kinase activity assays, which allowed the determination of their mode of action. Finally, the kinase selectivity profile of the inhibitors was assessed in a cell lysate chemoproteomic kinobead binding assay against a panel of lipid and atypical kinases.

## RESULTS AND DISCUSSION

**Design of PI3Kδ covalent inhibitors.** Starting from crystal structures of known PI3Kδ ligands, we carried out computational docking to identify appropriate chemical vectors for the incorporation of the activated ester motif. It was decided that the Lys779-targeting electrophiles should not disrupt any reversible interactions which were critical to PI3Kδ-selectivity. We initially examined the indazole template described by Dalton *et al.*, which selectively inhibited PI3Kδ and formed a covalent bond with Lys779.[40] The electrophile was adapted for covalent lysine acetylation, by reversing the phenolic ester of compound **2** to acetate ester **3** (Figure 2A). Docking of ester **3** in PI3Kδ suggested that the acetate group could adopt a favorable position

for covalent binding to Lys779. However, ester **3** showed poor chemical stability and hydrolyzed rapidly upon storage (Supplementary discussion and Figures S3/S4).

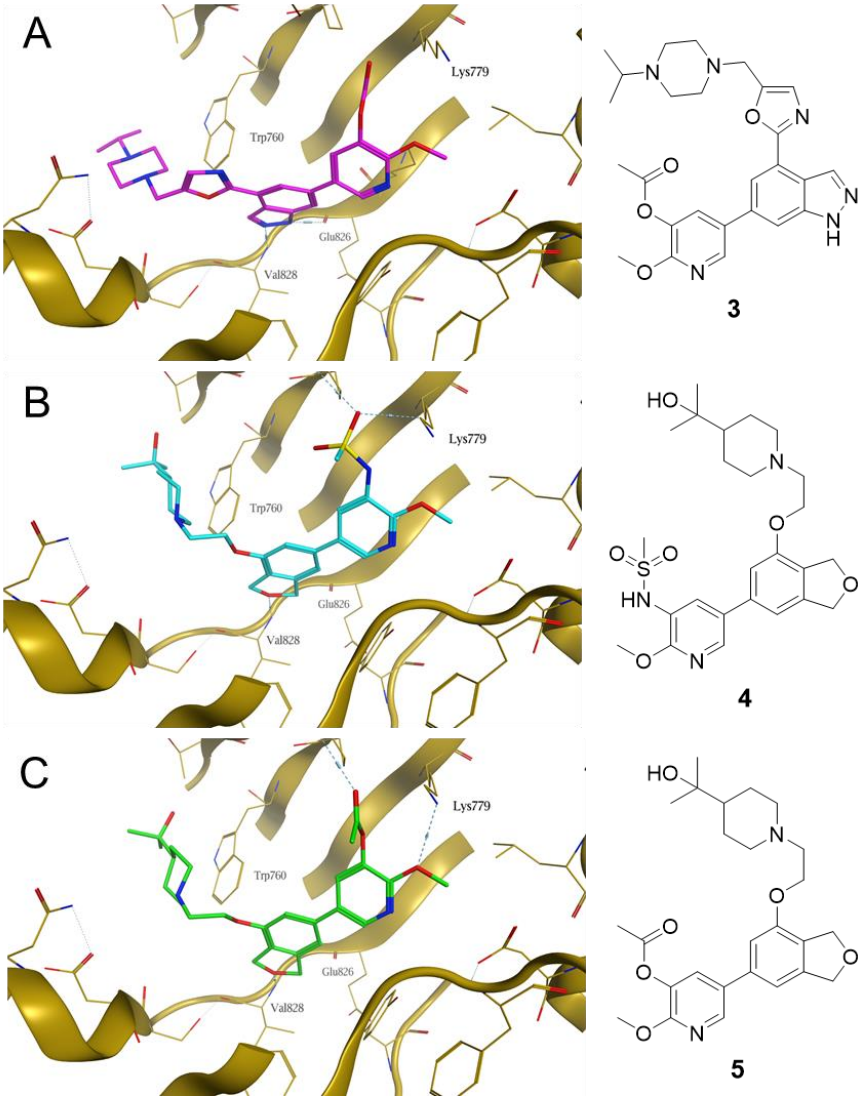
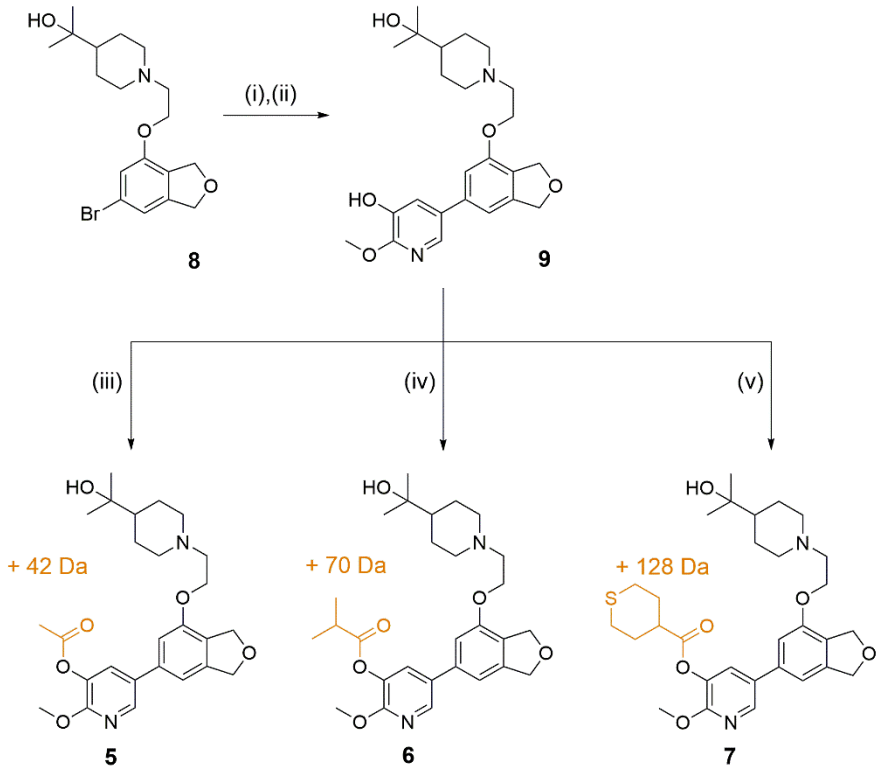



Figure 2 – Binding of compounds **3**, **4** and **5** in PI3Kδ active site. A) Docking model of indazole ester **3**. The acetate ester is positioned close to Lys779; B) Crystal structure of DHB sulfonamide **4**. The piperidine ring extends onto the tryptophan shelf (Trp760), and the DHB oxygen forms a hinge-binding interaction with Val828. The sulfonamide is close to Lys779; C) Docking model of DHB ester **5**. Key interactions (Trp760 and Val828) are maintained and the acetate ester is positioned close to Lys779.

This prompted the investigation of a second chemical template, based on the dihydroisobenzofuran (DHB) core of sulfonamide **4** (Figure 2B). The DHB template originated from a series of orally bioavailable PI3Kδ inhibitors;[48] the core scaffold adopted a similar binding mode to the indazole template in the kinase active site and was deemed a suitable replacement for our covalent inhibitor design. Both the indazole and DHB series contain a methoxy-substituted pyridine ring, which confers PI3Kδ-selectivity by occupying a lipid kinase affinity pocket.[48] Other key interactions retained from sulfonamide **4** were the DHB

hinge-binding motif and the decorated piperidine which extends onto the tryptophan shelf of PI3Kδ.[48] Similar to the design of **3**, we anticipated that positioning the acetate ester as a substituent of the pyridine ring in **5** could maintain reversible binding to the kinase, as well as provide a good geometry for covalent modification. Docking of **5** in PI3Kδ (Figure 2C) suggested that the acetate ester could adopt a favorable position for Lys779 acetylation.




Scheme 2 – Synthesis of the DHB esters. (i) $B_2pin_2$, KOAc, $PdCl_2(dppf)$, 1,4-dioxane, 90 °C; (ii) 5-bromo-2-methoxypyridin-3-ol, $Na_2CO_3$, $PdCl_2(dppf)$, 1,4-dioxane:water (4:1), 90 °C, 74% (2 steps); (iii) acetic anhydride, $NEt_3$, $CH_2Cl_2$, r.t., 29%; (iv) isobutyryl chloride, $NEt_3$, DMAP, $CH_2Cl_2$, r.t., 29%; (v) tetrahydro-2*H*-thiopyran-4-carboxylic acid, DMAP, DCC, $CH_2Cl_2$, 0 °C to r.t., 57%. The acylating moieties of esters **5**, **6** and **7** are highlighted in orange, along with the annotated expected protein mass shift arising from lysine acylation.

Our initial intent was to study the impact of Lys779 acetylation on kinase activity with acetate ester **5**, due to the prevalence of lysine acetylation as an endogenous PTM. In order to investigate the effect of steric hindrance on the covalent reaction rate and activity of the compounds, the set of ester warheads was expanded to include bulkier acylating groups with isobutyrate ester **6** and tetrahydro-2*H*-thiopyran-4-carboxylate ester **7** (Scheme 2). The increased size of the esters allowed examination of the space available in this region of the kinase active site. Additionally, the incorporation of a sulfur atom in **7** provided a good substrate for possible X-ray crystallography studies of the PI3Kδ-ester adducts, due to the improved phase refinement resulting from the anomalous dispersion effect of sulfur.[49]

Miyaura borylation of advanced brominated intermediate **8** (Supplementary Scheme S1),[48] followed by Suzuki-Miyaura cross-coupling of the resulting pinacol boronic ester with 5-bromo-2-methoxypyridin-3-ol yielded the key phenol intermediate **9**. Acylation of **9** was then carried out with acetic anhydride, isobutyryl chloride or tetrahydro-2*H*-thiopyran-4-carboxylic acid to afford the desired DHB esters **5**, **6** and **7** (Scheme 2).

**DHB compounds are potent inhibitors of PI3Kδ.** The activity of the DHB compounds against the four class I PI3K isoforms (PI3Kα, β, γ and δ) was first evaluated in a biochemical TR-FRET assay (Table 1). Compounds **9**, **5**, **6** and **7** all achieved submicromolar potencies ($pIC_{50} > 6$) against PI3Kδ under the assay conditions. As expected, the DHB ligands exhibited selectivity for PI3Kδ compared to the related PI3K isoforms; between 60-fold (PI3Kδ over PI3Kβ for **5**) and 500-fold (PI3Kδ over PI3Kγ for **5**). The compounds with the smallest pyridine substitution, phenol **9** and acetate ester **5**, were the most active across all isoforms, showing $pIC_{50}$ values of up to 8.1 against PI3Kδ. A 10-fold drop in potency was observed with the larger isobutyrate ester **6** and tetrahydro-2*H*-thiopyran-4-carboxylate ester **7**.

Table 1 – $pIC_{50}$ values of compounds **9**, **5**, **6** and **7** against PI3K isoforms

| Compound | PI3Kδ $pIC_{50}$ | PI3Kα $pIC_{50}$ | PI3Kβ $pIC_{50}$ | PI3Kγ $pIC_{50}$ |
|---|---|---|---|---|
| **9** | 7.9 | 5.5 | 5.8 | 5.3 |
| **5** | 8.1 | 6.0 | 6.3 | 5.4 |
| **6** | 7.2 | 5.2 | 5.3 | 5.0 |
| **7** | 7.0 | 4.7 | 4.7 | 5.0 |

$pIC_{50}$ values for inhibitors **9**, **5**, **6** and **7** were derived from a biochemical TR-FRET assay; the values shown in the table are the mean results from 3 independent replicates.

**Protein LC-MS demonstrates covalent binding to PI3Kδ.** The extent of covalent labelling after incubation with the DHB esters was probed by LC-MS analysis of the intact protein. Recombinant PI3Kδ (1 μM) was incubated with DHB esters **5**, **6** and **7** (10 μM; 5–7.5 h) and analyzed by LC-MS. The deconvoluted mass spectra were compared with unmodified PI3Kδ reference samples. Precursor phenol **9** was also examined in this assay, as a reversible ligand control: without the activated ester motif, no shift in protein mass was detected (Figure 3A). Pleasingly, incubation with esters **5**, **6** and **7** resulted in the expected mass shifts from each acyl group (Figure 3B–D), indicating covalent protein modification.

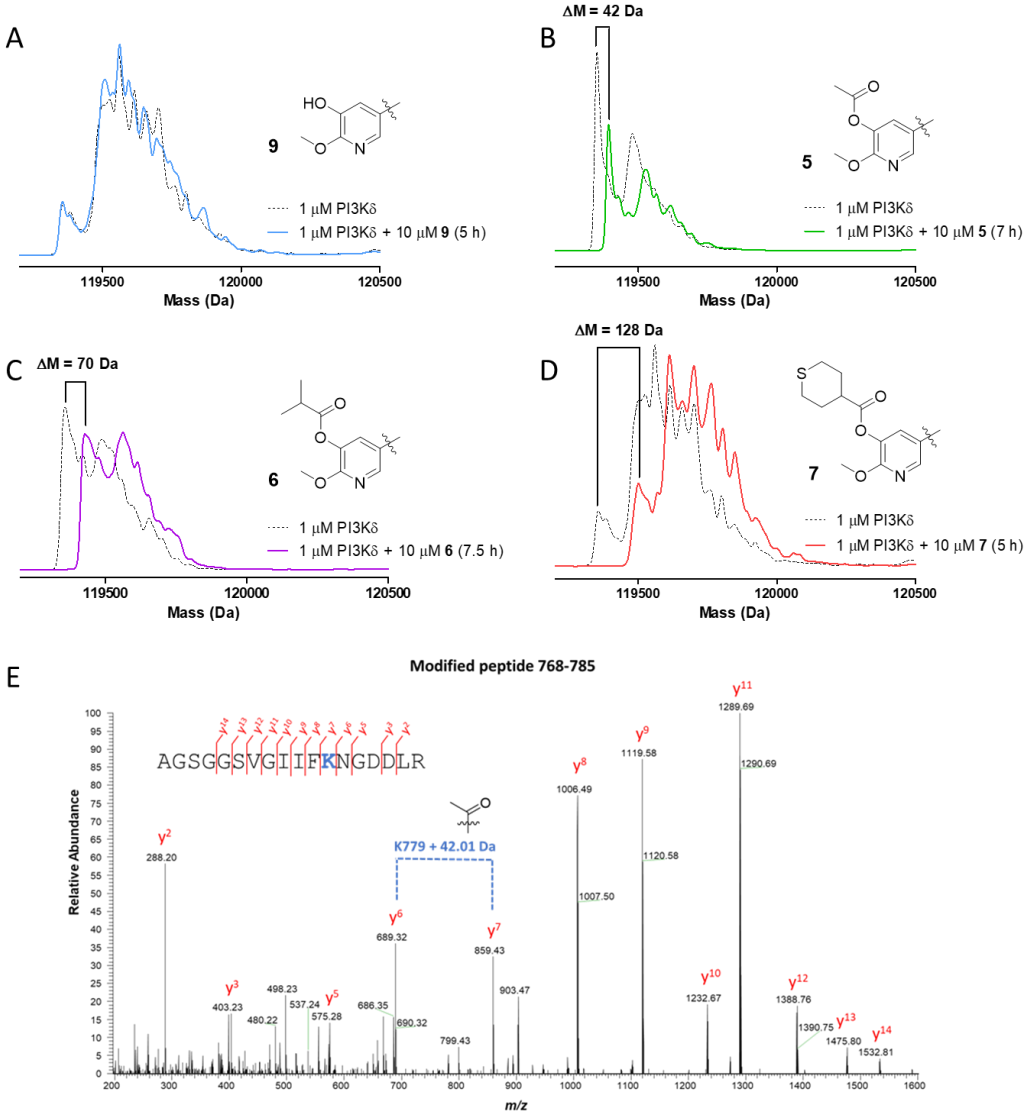
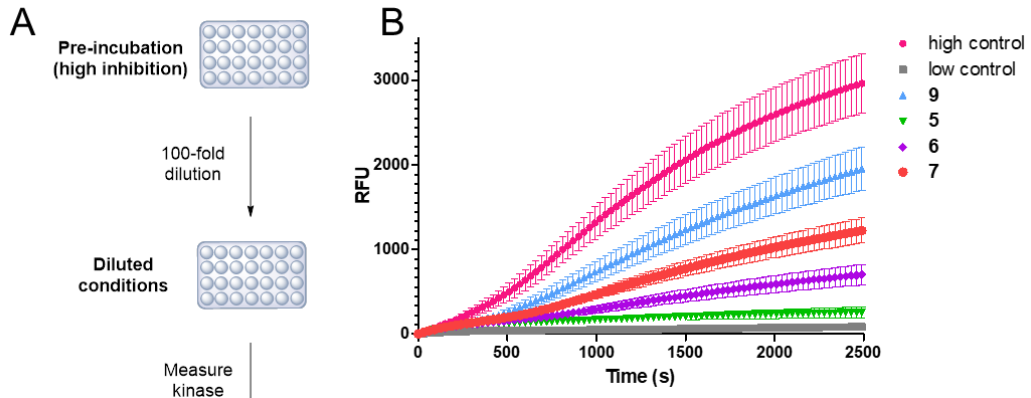



Figure 3 – LC-MS analysis of PI3Kδ-ester adducts. A–D) Recombinant PI3Kδ samples after incubation with 10-fold excess of the DHB ligands. The mass range reported (119,200–120,500 Da) shows the p110δ subunit. For each ligand, the deconvoluted spectra (solid line) is overlaid with a reference of the intact protein (dashed line), and mass shifts (ΔM) observed with esters **5**, **6** and **7** are highlighted. A) PI3Kδ + phenol **9**, reversible ligand, no mass shift; B) PI3Kδ + acetate ester **5**, ΔM = 42 Da; C) PI3Kδ + isobutyrate ester **6**, ΔM = 70 Da; D) PI3Kδ + tetrahydro-2*H*-thiopyran-4-carboxylate ester **7**, ΔM = 128 Da; E) MS/MS spectrum of modified peptide 768-785. LC-MS/MS analysis of the trypsin followed by GluC digest of PI3Kδ (5 μM) incubated with acetate ester **5** (10 μM – 4 h) identified 768-785 as the modified peptide. Acetylation (+ 42.01 Da) was confirmed on Lys779.

Endogenous PI3Kδ is a heterodimeric protein composed of a catalytic p110δ subunit, which contains the kinase domain, and a p85-like regulatory subunit.[50] LC-MS analysis showed covalent acylation only of p110δ, and no apparent shift in mass of p85 (Supplementary Figure S5). This suggested that the DHB esters were binding to the active site of PI3Kδ, however, further experiments were required to confirm the precise site of covalent modification.

**Acetate ester 5 selectively acetylates Lys779.** A competitive protein binding assay using wortmannin **1**, a known covalent inhibitor of the PI3K family,[51] confirmed that the DHB ligands were interacting with the kinase active site as intended (Supplementary Figure S6).
We then used tandem mass spectrometry (LC-MS/MS) following enzymatic digest of the modified protein to determine which PI3Kδ amino acid residues were covalently labelled by the activated esters. This technique relies on cleavage of the protein with sequence-specific proteases,[52] and subsequent LC-MS/MS analysis of the resulting peptides to identify modified amino acids. In this experiment, recombinant PI3Kδ (5 μM) was incubated with a two-fold excess of acetate ester **5** (10 μM – 4 h), prior to separation of the p85 and p110δ subunits by SDS-PAGE. The band corresponding to p110δ was excised and subjected to trypsin digestion followed by GluC digestion, before LC-MS/MS analysis. The results indicated that, under these experimental conditions, acetate ester **5** was selectively acetylating Lys779 (Figure 3E). A similar analysis of the p85 subunit after trypsin digestion confirmed that incubation with acetate ester **5** did not induce covalent acetylation of the regulatory subunit.

**Jump-dilution determines the inhibitors' mechanism.** The results from the TR-FRET assay (Table 1) had shown that the DHB compounds were active kinase inhibitors with apparent selectivity for PI3Kδ. However, the $pIC_{50}$ values did not reflect the time-dependent inhibition which is characteristic of a covalent mode of action.[5] Consequently, these results were not sufficient to determine whether kinase activity was inhibited in a reversible or covalent manner. We expected that PI3Kδ acylation with DHB esters **5**, **6** and **7** would liberate the parent phenol **9** in the kinase active site. Because **9** is also a potent PI3Kδ inhibitor (Table 1), there are two possible modes of inhibition once the kinase is acylated: (i) covalent inhibition due to Lys779 acylation, (ii) non-covalent inhibition through binding of **9**. A second biochemical assay was required to determine if kinase inactivation was a direct consequence of Lys779 acylation.
Evidence of a covalent inhibition mechanism could be obtained from a biochemical jump-dilution assay (Figure 4A). During the jump-dilution, the enzyme was pre-incubated with high concentrations of the inhibitors. After 25 min, the solutions were diluted 100-fold to achieve non-inhibitory concentrations of the compounds. Upon dilution, kinase activity was measured with DiscoverX ADP Quest detection reagents to provide a continuous fluorescence read-out. As reversible inhibition is equilibrium-based, recovery of kinase activity is expected after dilution due to the displacement of the protein-ligand equilibrium in favor of ATP. If the measured activity after dilution remains consistent with the pre-incubation conditions, then inhibition is no longer equilibrium-based, indicating a covalent mode of action.




C

| | $\%A_{pre\text{-}inc}$ | %A | $\%A_{max}$ |
|---|---|---|---|
| **9** | 1 | 63 | 34 |
| **5** | 1 | 4 | 39 |
| **6** | 3 | 19 | 74 |
| **7** | 2 | 38 | 64 |

D

| | $k_{inact}$ ($s^{-1}$) | $K_I$ (M) | $k_{inact}/K_I$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|
| **5** | 0.00234 ± 0.00012 | $6.0 \pm 1.0 \times 10^{-9}$ | $4.00 \pm 0.50 \times 10^{5}$ |
| **6** | 0.00054 ± 0.00004 | $5.7 \pm 0.4 \times 10^{-8}$ | $9.53 \pm 0.05 \times 10^{3}$ |
| **7** | 0.00029 ± 0.00008 | $3.6 \pm 0.9 \times 10^{-8}$ | $9.23 \pm 4.66 \times 10^{3}$ |

Figure 4 – Jump-dilution assay. A) General workflow: the enzyme was pre-incubated with a high concentration of inhibitor, for which enzyme activity is expected to be low. The media was then diluted 100-fold. Upon dilution, the enzyme activity was measured over time to examine recovery of activity; B) PI3Kδ activity in the jump-dilution assay with compounds **9**, **5**, **6** and **7**. High control: non-inhibited enzyme. Low control: no enzyme. Kinase activity was related to a fluorescence read-out (RFU: Relative Fluorescence Units). Graph shows mean results (and standard deviation) from 3 independent replicates; C) For each compound, the table shows predicted pre-incubation kinase activity ($\%A_{pre\text{-}inc}$), measured activity (%A) and predicted maximum recovered activity for a fully reversible inhibitor ($\%A_{max}$); D) Table of $k_{inact}$, $K_I$, and $k_{inact}/K_I$ values (average ± standard deviation between independent replicates). Kinetic measurements were performed in triplicate (**5**) or duplicate (**6** and **7**).

Results from the jump dilution with compounds **9**, **5**, **6** and **7** against PI3Kδ are shown in Figure 4B/C. From each plot (Figure 4B), we calculated an experimental value of percentage kinase activity (%A), relative to the non-inhibited high control. This value was derived from the slope of a linear fit applied to the curves (between 300–1980 s; Supplementary Table S1). A theoretical model was used to predict kinase activity in the pre-incubation ($\%A_{pre\text{-}inc}$) and diluted conditions ($\%A_{max}$) (Supplementary Table S1); with $\%A_{max}$ representing an expected maximum recovery of activity after dilution for a reversible inhibitor. Experimental %A values were then compared with predicted $\%A_{pre\text{-}inc}$ and $\%A_{max}$ to determine if the compounds were acting as reversible or covalent inhibitors of PI3Kδ (Figure 4C).

The measured kinase activity from reversible phenol **9** was greater than the model predictions: 63% and 34% respectively. We believed this was due to an inaccuracy in the model leading to

an underestimation of the maximum possible recovery after dilution. Phenol **9** was an essential control to validate the jump dilution assay: the high recovery of kinase activity was consistent with reversible inhibition. This showed that phenol **9** was not a tight-binding inhibitor of PI3Kδ, since it had been displaced from the kinase active site by dilution. As a by-product of the kinase acylation reaction, a tight-binding phenol **9** could have generated false positive results for covalent inhibition. For isobutyrate ester **6** and tetrahydro-2*H*-thiopyran-4-carboxylate ester **7**, %A values were intermediate between $\%A_{pre\text{-}inc}$ and $\%A_{max}$. This could be explained by partial covalent inhibition of PI3Kδ if the acylation reaction did not reach completion during the 25 min pre-incubation. Pleasingly, the measured activity from acetate ester **5** was consistent with $\%A_{pre\text{-}inc}$ (4% and 1%, respectively). This suggested that acetylation of Lys779 was directly responsible for PI3Kδ inactivation.

Time-dependent kinase activity measurements were also used to determine kinetic inactivation constants $k_{inact}$ and thermodynamic binding constants $K_I$ to characterize the covalent inactivation of PI3Kδ (Figure 4D), according to the method described by Krippendorff *et al.*[53] Our findings correlated well with previous observations on rates of covalent PI3Kδ inhibition, with acetate ester **5** showing faster covalent bond formation than the bulkier analogues **6** and **7**.

**Inhibitor selectivity is assessed against lipid kinases.** The selectivity of the DHB inhibitors across a range of PI3Kδ-related kinases was assessed in a dose-dependent chemoproteomic binding assay.[54] This assay evaluated binding of the inhibitors to lipid and atypical kinases in cell lysates in the presence of kinobeads, a competing affinity matrix consisting of promiscuous kinase inhibitors immobilized on sepharose beads. Preliminary stability studies of DHB esters **5**, **6** and **7** in the assay buffer (50 mM Tris-HCl pH 8.0, 5% *v/v* glycerol, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM $Na_3VO_4$, 25 mM NaF, 1 mM DTT) showed rapid hydrolysis of acetate ester **5** to the precursor phenol (> 30% within 1 h; Supplementary Figure S11). Compound **5** could not be assessed in the chemoproteomic kinobead assay due to poor hydrolytic stability under the experimental conditions. The bulkier analogues **6** and **7** showed improved stability in the assay buffer, with ester hydrolysis not exceeding 10% within 1 h. This was deemed suitable for the chemoproteomic assay which involved a 45 min incubation period of the inhibitors in cell lysates. We therefore relied on results from phenol **9**, and esters **6** and **7** to evaluate trends in selectivity of the DHB inhibitors (Figure 5).

A mixture of lysates from HeLa, Jurkat and K-562 cells were treated with the DHB compounds over a range of concentrations (80 pM – 30 μM). The depletion of kinase binding to the

kinobeads was measured as a function of DHB inhibitor concentration by LC-MS/MS. Apparent dissociation constants p$K_d^{app}$ were derived from this data, which allowed to evaluate the strength of binding of the inhibitors to each kinase target.

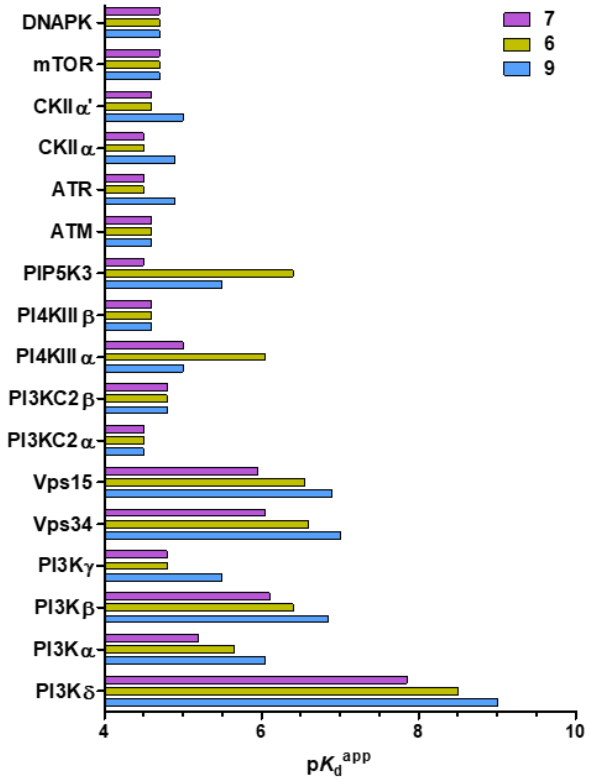
Against a panel of 17 lipid and atypical kinases, DHB inhibitors **6**, **7** and **9** showed the desired PI3Kδ-selectivity profile. This was consistent with the kinase profile of the related DHB inhibitor: 10 μM of sulfonamide **4** had shown <50% inhibition against 348 kinases.[48]

The reversible inhibitor control, phenol **9**, showed the greatest selectivity for PI3Kδ in our assay. The closest off-target kinases were other members of the PI3K family, detected with 100-fold selectivity: PI3Kβ, Vps34, and Vps15. PI3Kβ and PI3Kδ are closely related class I PI3K isoforms, and Vps34 is a class III PI3K isoform which forms heterotetrametric complexes with Vps15 and other binding partners.[55] Vps34 had previously been identified as an off-target of the related DHB sulfonamide **4**.[48]

Both DHB esters presented similar trends in binding affinity: isobutyrate **6** showed 100-fold selectivity over PI3Kβ and 80-fold over the Vps kinases, and **7** showed 60-fold selectivity over PI3Kβ and the Vps kinases. The visible drop in selectivity and lower p$K_d^{app}$ values observed with the esters compared to phenol **9** could be the consequence of a covalent mode of action.

All DHB inhibitors showed excellent selectivity against the other class I isoforms, PI3Kα and PI3Kγ (Supplementary discussion and Figure S7). In addition, greater than 100-fold selectivity was observed against all other lipid and atypical kinases examined in this assay. Encouragingly, the activated esters maintained a PI3Kδ-selective profile despite the potential for covalent modification of multiple kinases. Further investigation would be required to confirm that covalent lysine acylation occurs in cells.

We anticipate that the progression of our PI3Kδ covalent inhibitors in cells would involve further optimization of the ester moiety, as supported by the observed hydrolysis of acetate **5** in the kinobead assay buffer. It is possible that the activated ester warheads could undergo esterase-mediated hydrolysis in cellular environments, or react with competing nucleophiles such as glutathione, preventing covalent lysine acylation. Cellular studies would therefore require in-depth understanding of ester reactivity to ensure the compounds are stable under native physiological conditions and able to selectively modify the desired target.




| Targets | 9 | 6 | 7 |
|---|---|---|---|
| PI3Kδ | 9 | 8.5 | 7.9 |
| PI3Kα | 6.1 | 5.7 | <5.2 |
| PI3Kβ | 6.9 | 6.4 | 6.1 |
| PI3Kγ | 5.5 | <4.8 | <4.8 |
| Vps34 | 7.0 | 6.6 | 6.1 |
| Vps15 | 6.9 | 6.6 | 6 |
| PI3KC2α | <4.5 | <4.5 | <4.5 |
| PI3KC2β | <4.8 | <4.8 | <4.8 |
| PI4KIIIα | <5 | 6.1 | <5 |
| PI4KIIIβ | <4.6 | <4.6 | <4.6 |
| PIP5K3 | 5.5 | 6.4 | <4.5 |
| ATM | <4.6 | <4.6 | <4.6 |
| ATR | 4.9 | <4.5 | <4.5 |
| CKIIα | 4.9 | <4.5 | <4.5 |
| CKIIα' | 5 | <4.6 | <4.6 |
| mTOR | <4.7 | <4.7 | <4.7 |
| DNAPK | <4.7 | <4.7 | <4.7 |

Figure 5 – Chemoproteomic binding assay. The selectivity of DHB inhibitors **6**, **7** and **9** was assessed against 17 lipid and atypical kinases. The bar chart and table show average p$K_d^{app}$ values derived from 2 independent replicates of the assay.

## CONCLUSIONS

We have demonstrated that targeting the conserved catalytic lysine with PTM-resembling modifications can result in the selective covalent inhibition of PI3Kδ. This work allowed rapid access to novel PI3Kδ covalent inhibitors by taking advantage of pre-existing structural knowledge from the selective DHB chemical series. Covalent labelling of the enzyme with the activated phenolic esters (**5**, **6** and **7**) was confirmed by intact protein mass spectrometry experiments. The site of covalent modification was elucidated by LC-MS/MS, indicating that acetate ester **5** could selectively acetylate the PI3Kδ catalytic lysine, Lys779. Biochemical activity assays demonstrated that the DHB esters were potent inhibitors of PI3Kδ, and that lysine acetylation resulted in kinase inactivation. Although acetate ester **5** was not stable enough for kinase profiling in the kinobead assay, we were able to show that the bulkier esters (**6** and **7**) were selectively binding to PI3Kδ over related kinases in cell lysates during the course of the study.

Covalent inhibition strategies which target the conserved catalytic lysine have the potential to be broadly applicable across the kinome. There is evidence to suggest that the catalytic lysine

would be less prone to mutation than cysteine residues, based on studies of "kinase-dead" mutants.[56] Our lysine-targeting approach could lower the risk of drug-resistance mechanisms associated with cysteine-reactive kinase inhibitors, thus offering a complementary strategy to expand the scope of covalent kinase inhibition. The activated ester method we have described here allows to induce PTM-resembling modifications of protein sequences, which could reduce the risk of immune mediated adverse drug reactions. A natural continuation of the work we have presented here would include progressing our activated esters in cells to evaluate kinase engagement in a physiologically relevant environment. This effort would constitute part of a future research project, as it would potentially require further optimization of the molecules.

We envisage that our work will expand the growing interest in modulation of enzyme activity through small molecule induced lysine acetylation. This strategy builds on a known mode of action of aspirin, which is believed to inhibit the biosynthesis of prostaglandins primarily through acetylation of a serine in the active sites of cyclooxygenase enzymes COX-1 and 2.[57-59] Although the effects of aspirin-mediated acetylation have been accepted for a long time, there are only a few reports examining covalent acetylation as a drug development strategy.[60, 61] Our work aimed to adapt small molecule mediated acetylation to covalent kinase inhibitors.

The supporting information contains supplementary discussions and figures, computational modelling, synthesis and characterization of compounds, procedures for biochemical assays (LC-MS, LC-MS/MS, TR-FRET and jump-dilution) and chemoproteomic assay. This material is available free of charge *via* the internet at https://urldefense.com/v3/__http://pubs.acs.org__;!!AoaiBx6H!jDDlrkzoMxTCcdbZ1cbY_h6-nfoAMmHSIMpB0tgRVrAypx7eWktVk70dHAPguEZPxw$.

## AUTHOR INFORMATION

**Julie C. L. Fournier –** julie.8.fournier@gsk.com – GSK Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK; julie.fournier@strath.ac.uk – Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK
**John P. Evans –** john.evans@arctoris.com – Arctoris, 120E Olympic Avenue, Milton Park, Oxford, OX14 4SA
**Francesca Zappacosta –** francesca.zappacosta@gsk.com – GSK, South Collegeville Road, Collegeville, PA 19426, USA
**Daniel A. Thomas –** daniel.thomas@arctoris.com – Arctoris, 120E Olympic Avenue, Milton Park, Oxford, OX14 4SA
**Vipulkumar K. Patel –** vipul.k.patel@gsk.com – GSK Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK
**Gemma V. White –** gemma.v.white@gsk.com – GSK Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK

**Sebastien Campos** – sebastien.campos@pharmaron-uk.com – Pharmaron, West Hill Innovation Park, Hertford Road, Hoddesdon, Hertfordshire EN11 9FH, UK
**Nicholas C. O. Tomkinson** – nicholas.tomkinson@strath.ac.uk – Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK

**Funding:** The authors thank the GSK/University of Strathclyde Collaborative PhD programme for funding this work, as well as the EPSRC for funding via Prosperity Partnership EP/S035990/1.

## ACKNOWLEDGMENTS

We would like to thank S. Lynn for his assistance with NMR spectroscopy, J. Rowedder for performing the routine PI3K TR-FRET assays, E. Grant for her help with protein LC-MS, B. Dümpelfeld and the Omics team (Functional Genomics department at Cellzome, a GSK company) for performing the kinobead chemoproteomics assay, as well as J. Bush, M. Convery and D. Thomson for their valuable expertise which contributed to the progression of this work.

## ABBREVIATIONS

ATM: ataxia-telangiectasia mutated protein kinase; ATP: adenosine triphosphate; ATR: ataxia-telangiectasia and Rad3-related protein kinase; BTK: Bruton's Tyrosine Kinase; CK: casein kinase; DHB: dihydroisobenzofuran; DNAPK: DNA-dependent protein kinase; EGFR: Epidermal Growth Factor Receptor; FDA: Food and Drug Administration; LC-MS: Liquid Chromatography-Mass Spectrometry; LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; PI4K: phosphatidylinositol 4-kinase; PIP5K3: phosphatidylinositol phosphate 5-kinase; PTM: post-translational modification; RFU: Relative Fluorescence Units; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TR-FRET: Time Resolved Förster Resonance Energy Transfer; Vps: vacuolar protein-sorting.

## REFERENCES

[1] Tuley, A., and Fast, W. (2018) The Taxonomy of Covalent Inhibitors, *Biochemistry 57*, 3326-3337.
[2] Ghosh, A. K., Samanta, I., Mondal, A., and Liu, W. R. (2019) Covalent Inhibition in Drug Discovery, *ChemMedChem 14*, 889–906.
[3] Johnson, D. S., Weerapana, E., and Cravatt, B. F. (2010) Strategies for discovering and derisking covalent, irreversible enzyme inhibitors, *Future Med. Chem. 2*, 949–964.
[4] Bauer, R. A. (2015) Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies, *Drug Discov. Today 20*, 1061–1073.
[5] Singh, J., Petter, R. C., Baillie, T. A., and Whitty, A. (2011) The resurgence of covalent drugs, *Nat. Rev. Drug Disc. 10*, 307–317.
[6] Abranyi-Balogh, P., Petri, L., Imre, T., Szijj, P., Scarpino, A., Hrast, M., Mitrovic, A., Fonovic, U. P., *et al.* (2018) A road map for prioritizing warheads for cysteine targeting covalent inhibitors, *Eur. J. Med. Chem. 160*, 94–107.
[7] Mukherjee, H., and Grimster, N. P. (2018) Beyond cysteine: recent developments in the area of targeted covalent inhibition, *Curr. Opin. Chem. Biol. 44*, 30–38.
[8] Strelow, J. M. (2017) A Perspective on the Kinetics of Covalent and Irreversible Inhibition, *SLAS Discov. 22*, 3–20.
[9] Craven, G. B., Affron, D. P., Kosel, T., Wong, T. L. M., Jukes, Z. H., Liu, C. T., Morgan, R. M. L., Armstrong, A., *et al.* (2020) Multiparameter Kinetic Analysis for Covalent Fragment

Optimization by Using Quantitative Irreversible Tethering (qIT), *ChemBioChem 21*, 3417–3422.
[10] De Cesco, S., Kurian, J., Dufresne, C., Mittermaier, A. K., and Moitessier, N. (2017) Covalent inhibitors design and discovery, *Eur. J. Med. Chem. 138*, 96–114.
[11] Lonsdale, R., and Ward, R. A. (2018) Structure-based design of targeted covalent inhibitors, *Chem. Soc. Rev. 47*, 3816–3830.
[12] Mah, R., Thomas, J. R., and Shafer, C. M. (2014) Drug discovery considerations in the development of covalent inhibitors, *Bioorg. Med. Chem. Lett. 24*, 33–39.
[13] Keeley, A., Abranyi-Balogh, P., and Keseru, G. M. (2019) Design and characterization of a heterocyclic electrophilic fragment library for the discovery of cysteine-targeted covalent inhibitors, *Med. Chem. Commun. 10*, 263–267.
[14] Resnick, E., Bradley, A., Gan, J., Douangamath, A., Krojer, T., Sethi, R., Geurink, P. P., Aimon, A*., et al.* (2019) Rapid Covalent-Probe Discovery by Electrophile-Fragment Screening, *J. Am. Chem. Soc. 141*, 8951–8968.
[15] London, N., Miller, R. M., Krishnan, S., Uchida, K., Irwin, J. J., Eidam, O., Gibold, L., Cimermancic, P*., et al.* (2014) Covalent docking of large libraries for the discovery of chemical probes, *Nat. Chem. Biol. 10*, 1066–1072.
[16] Akçay G., Belmonte M. A., Aquila B., Chuaqui C., Hird A. W., Lamb M. L., Rawlins P. B., Su N*., et al.* (2016) Inhibition of Mcl-1 through covalent modification of a noncatalytic lysine side chain, *Nat. Chem. Biol. 12*, 931–936.
[17] Baillie, T. A. (2016) Targeted Covalent Inhibitors for Drug Design, *Angew. Chem. Int. Ed. 55*, 13408–13421.
[18] Ferguson, F. M., and Gray, N. S. (2018) Kinase inhibitors: the road ahead, *Nat. Rev. Drug Disc. 17*, 353–377.
[19] Muller, S., Chaikuad, A., Gray, N. S., and Knapp, S. (2015) The ins and outs of selective kinase inhibitor development, *Nat. Chem. Biol. 11*, 818–821.
[20] Roskoski Jr., R. (2020) Properties of FDA-approved small molecule protein kinase inhibitors: A 2020 update, *Pharmacol. Res. 152*, 104609.
[21] Fabbro, D. (2015) 25 years of small molecular weight kinase inhibitors: potentials and limitations, *Mol. Pharmacol. 87*, 766–775.
[22] Klaeger, S., Heinzlmeir, S., Wilhelm, M., Polzer, H., Vick, B., Koenig, P. A., Reinecke, M., Ruprecht, B*., et al.* (2017) The target landscape of clinical kinase drugs, *Science 358*.
[23] Knight, Z. A., and Shokat, K. M. (2005) Features of selective kinase inhibitors, *Chem. Biol. 12*, 621–637.
[24] Singh, J., Petter, R. C., and Kluge, A. F. (2010) Targeted covalent drugs of the kinase family, *Curr. Opin. Chem. Biol. 14*, 475–480.
[25] Dias, A. L., and Jain, D. (2013) Ibrutinib: A New Frontier in the Treatment of Chronic Lymphocytic Leukemia by Bruton's Tyrosine Kinase Inhibition, *Cardiovasc. Hematol. Agents Med. Chem. 11*, 265–271.
[26] Fischer, P. M. (2017) Approved and Experimental Small-Molecule Oncology Kinase Inhibitor Drugs: A Mid-2016 Overview, *Med. Res. Rev. 37*, 314–367.
[27] Miseta, A., and Csutora, P. (2000) Relationship Between the Occurrence of Cysteine in Proteins and the Complexity of Organisms, *Mol. Biol. Evol. 17*, 1232–1239.
[28] Zhang, J., Yang, P. L., and Gray, N. S. (2009) Targeting cancer with small molecule kinase inhibitors, *Nat. Rev. Cancer 9*, 28–39.
[29] Engel, J., Lategahn, J., and Rauh, D. (2016) Hope and Disappointment: Covalent Inhibitors to Overcome Drug Resistance in Non-Small Cell Lung Cancer, *ACS Med. Chem. Lett. 7*, 2–5.
[30] Hamasy, A., Wang, Q., Blomberg, K. E., Mohammad, D. K., Yu, L., Vihinen, M., Berglof, A., and Smith, C. I. (2017) Substitution scanning identifies a novel, catalytically active ibrutinib-resistant BTK cysteine 481 to threonine (C481T) variant, *Leukemia 31*, 177–185.

[31] Woyach, J. A., Furman, R. R., Liu, T. M., Ozer, H. G., Zapatka, M., Ruppert, A. S., Xue, L., Li, D. H., *et al.* (2014) Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib, *N. Engl. J. Med. 370*, 2286–2294.
[32] Abdeldayem, A., Raouf, Y. S., Constantinescu, S. N., Moriggl, R., and Gunning, P. T. (2020) Advances in covalent kinase inhibitors, *Chem. Soc. Rev. 49*, 2617–2687.
[33] Marone, R., Cmiljanovic, V., Giese, B., and Wymann, M. P. (2008) Targeting phosphoinositide 3-kinase: moving towards therapy, *Biochim. Biophys. Acta 1784*, 159–185.
[34] Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., and Williams, R. L. (2000) Structural Determinants of Phosphoinositide 3-Kinase Inhibition by Wortmannin, LY294002, Quercetin, Myricetin and Staurosporine, *Mol. Cell 6*, 909–919.
[35] Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) Wortmannin Inactivates Phosphoinositide 3-Kinase by Covalent Modification of Lys802, a Residue Involved in the Phosphate Transfer Reaction, *Mol. Cell. Biol. 16*, 1722–1733.
[36] Cheek, S., Ginalski, K., Zhang, H., and Grishin, N. V. (2005) A comprehensive update of the sequence and structure classification of kinases, *BMC Struct. Biol. 5*, 6.
[37] Iyer, G. H., Moore, M. J., and Taylor, S. S. (2005) Consequences of lysine 72 mutation on the phosphorylation and activation state of cAMP-dependent kinase, *J. Biol. Chem. 280*, 8800–8807.
[38] Ihle, N. T., Paine-Murrieta, G., Berggren, M. I., Baker, A., Tate, W. R., Wipf, P., Abraham, R. T., Kirkpatrick, D. L., *et al.* (2005) The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non-small cell lung cancer xenografts, *Mol. Cancer Ther. 4*, 1349–1357.
[39] Wipf, P., and Halter, R. J. (2005) Chemistry and biology of wortmannin, *Org. Biomol. Chem. 3*, 2053–2061.
[40] Dalton, S. E., Dittus, L., Thomas, D. A., Convery, M. A., Nunes, J., Bush, J. T., Evans, J. P., Werner, T., *et al.* (2018) Selectively Targeting the Kinome-Conserved Lysine of PI3Kdelta as a General Approach to Covalent Kinase Inhibition, *J. Am. Chem. Soc. 140*, 932–939.
[41] Tamura, T., and Hamachi, I. (2019) Chemistry for Covalent Modification of Endogenous/Native Proteins: From Test Tubes to Complex Biological Systems, *J. Am. Chem. Soc. 141*, 2782–2799.
[42] Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions, *Science 325*, 834–840.
[43] Pillai, V. B., Sundaresan, N. R., Samant, S. A., Wolfgeher, D., Trivedi, C. M., and Gupta, M. P. (2011) Acetylation of a conserved lysine residue in the ATP binding pocket of p38 augments its kinase activity during hypertrophy of cardiomyocytes, *Mol. Cell. Biol. 31*, 2349–2363.
[44] Sabò, A., Lusic, M., Cereseto, A., and Giacca, M. (2008) Acetylation of conserved lysines in the catalytic core of cyclin-dependent kinase 9 inhibits kinase activity and regulates transcription, *Mol. Cell. Biol. 28*, 2201–2212.
[45] Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016) The world of protein acetylation, *Biochim. Biophys. Acta 1864*, 1372–1401.
[46] Mosedale, M., and Watkins, P. B. (2020) Understanding Idiosyncratic Toxicity: Lessons Learned from Drug-Induced Liver Injury, *J. Med. Chem. 63*, 6436–6461.
[47] Cho, T., and Uetrecht, J. (2017) How Reactive Metabolites Induce an Immune Response That Sometimes Leads to an Idiosyncratic Drug Reaction, *Chem. Res. Toxicol. 30*, 295–314.
[48] Henley, Z. A., Amour, A., Barton, N., Bantscheff, M., Bergamini, G., Bertrand, S. M., Convery, M., Down, K., *et al.* (2020) Optimization of Orally Bioavailable PI3Kdelta Inhibitors and Identification of Vps34 as a Key Selectivity Target, *J. Med. Chem. 63*, 638–655.
[49] Dauter, Z., Dauter, M., de La Fortelle, E., Bricogne, G., and Sheldrick, G. M. (1999) Can Anomalous Signal of Sulfur Become a Tool for Solving Protein Crystal Structures?, *J. Mol. Biol. 289*, 83–92.

[50] Burke, J. E., and Williams, R. L. (2015) Synergy in activating class I PI3Ks, *Trends Biochem. Sci. 40*, 88–100.
[51] Liu, P., Cheng, H., Roberts, T. M., and Zhao, J. J. (2009) Targeting the phosphoinositide 3-kinase pathway in cancer, *Nat. Rev. Drug Disc. 8*, 627–644.
[52] Steen, H., and Mann, M. (2004) The ABC's (and XYZ's) of peptide sequencing, *Nat. Rev. Mol. Cell Biol. 5*, 699–711.
[53] Krippendorff, B. F., Neuhaus, R., Lienau, P., Reichel, A., and Huisinga, W. (2009) Mechanism-based inhibition: deriving K(I) and k(inact) directly from time-dependent IC(50) values, *J. Biomol. Screen. 14*, 913–923.
[54] Eberl, H. C., Werner, T., Reinhard, F. B., Lehmann, S., Thomson, D., Chen, P., Zhang, C., Rau, C.*, et al.* (2019) Chemical proteomics reveals target selectivity of clinical Jak inhibitors in human primary cells, *Sci. Rep. 9*, 14159.
[55] Ohashi, Y., Tremel, S., and Williams, R. L. (2019) VPS34 complexes from a structural perspective, *J. Lipid Res. 60*, 229–241.
[56] Iyer, G. H., Garrod, S., Woods, V. L., Jr., and Taylor, S. S. (2005) Catalytic independent functions of a protein kinase as revealed by a kinase-dead mutant: study of the Lys72His mutant of cAMP-dependent kinase, *J. Mol. Biol. 351*, 1110–1122.
[57] Ornelas, A., Zacharias-Millward, N., Menter, D. G., Davis, J. S., Lichtenberger, L., Hawke, D., Hawk, E., Vilar, E.*, et al.* (2017) Beyond COX-1: the effects of aspirin on platelet biology and potential mechanisms of chemoprevention, *Cancer Metastasis Rev. 36*, 289–303.
[58] Vane, J. R., and Botting, R. M. (2003) The mechanism of action of aspirin, *Thromb. Res. 110*, 255–258.
[59] Mahdi, J. G. (2010) Medicinal potential of willow: A chemical perspective of aspirin discovery, *J. Saudi Chem. Soc. 14*, 317–322.
[60] Yang, X., Forster, E. R., Darabedian, N., Kim, A. T., Pratt, M. R., Shen, A., and Hang, H. C. (2020) Translation of Microbiota Short-Chain Fatty Acid Mechanisms Affords Anti-infective Acyl-Salicylic Acid Derivatives, *ACS Chem. Biol. 15*, 1141–1147.
[61] Zhang, X., Jiang, L., Huang, K., Fang, C., Li, J., Yang, J., Li, H., Ruan, X.*, et al.* (2020) Site-Selective Phosphoglycerate Mutase 1 Acetylation by a Small Molecule, *ACS Chem. Biol. 15*, 632–639.