

Drugging the PI3 Kinome: From Chemical Tools to Drugs in the Clinic

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Abstract

The phosphatidylinositide 3-kinase (PI3K) pathway is very commonly activated in a wide range of human cancers and is a major driving force in oncogenesis. One of the class I lipid kinase members of the PI3K family, p110 α , is probably the most commonly mutated kinase in the human genome. Alongside genetic, molecular biological, and biochemical studies, chemical inhibitors have been extremely helpful tools in understanding the role of PI3K enzymes in signal transduction and downstream physiological and pathological processes, and also in validating PI3Ks as therapeutic targets. Although they have been valuable in the past, the early and still frequently employed inhibitors, wortmannin and LY294002, have significant limitations as chemical tools. Here, we discuss the case history of the discovery and properties of an increasingly used chemical probe, the pan-class I PI3K and mammalian target of rapamycin (mTOR) inhibitor PI-103 (a pyridofuroprymidine), and its very recent evolution into the thienopyrimidine drug GDC-0941, which exhibits excellent oral anticancer activity in preclinical models and is now undergoing phase I clinical trials in cancer patients. We also illustrate the impact of structural biology on the design of PI3K inhibitors and on the interpretation of their effects. The challenges and outlook for drugging the PI3 kinome are discussed in the more general context of the role of structural biology and chemical biology in innovative drug discovery. *Cancer Res*; 70(6): 2146–57. ©2010 AACR.

Introduction: Points of Activation and Intervention

Phosphatidylinositide 3-kinases (PI3K) are critical elements in a signal transduction pathway that plays a key role in regulating many features of cell behavior, including growth, survival, metabolism, and various specialized functions. They belong to a family of lipid kinases that phosphorylate the 3'-hydroxy position of the inositol ring of phosphatidylinositides, yielding products of which the best characterized is phosphatidylinositol-3,4,5-trisphosphate (PIP₃), the second messenger that recruits protein kinase B (AKT) to the cell membrane (1, 2). PIP₃ is generated by the class I PI3Ks, which comprise p110 α , p110 β , and p110 δ (class IA) and p110 γ (class IB), proteins that are activated to varying extents by receptor tyrosine kinases and G-protein coupled receptors. In addition to the class I PI3Ks, of note in the

present context are the class II and III lipid kinases and also the class IV PI3K-related protein kinases (PIKK), including mammalian target of rapamycin (mTOR), which is downstream on the PI3K pathway, and DNA-PK, ATM, and ATR, which are pivotal in DNA repair (1, 3).

In terms of its importance in cancer, the PI3K signaling cascade is more appropriately referred to as a super-highway than a pathway. It is hijacked in multiple ways in many types of human malignancy (4, 5). *PIK3CA*, which encodes the p110 α catalytic subunit of PI3K, is probably the most commonly mutated kinase in the human genome (15% of all cancers) and is also amplified in some tumors, whereas *PTEN*, which encodes the opposing phosphatase to PI3K, is the second most commonly affected tumor suppressor gene after *p53* (6, 7).⁴ Activation of PI3K signaling in cancer also occurs at the level of mutated or overexpressed receptor tyrosine kinases, AKT and RAS (4, 5). The frequent genetic and epigenetic activation by a range of different molecular mechanisms strongly suggests that activation of the PI3K pathway is very likely to be a critical step in human oncogenesis. The overwhelming degree of genetic validation for PI3K signaling as a therapeutic target in cancer is supported by multiple lines of functional credentialing, including genetically engineered mouse models (5, 8).

The optimal point of therapeutic intervention in the PI3K pathway remains unclear and will likely depend on the particular molecular pathology driving a given cancer (4, 5). Furthermore, emerging evidence shows that various

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⁴ <http://www.sanger.ac.uk/genetics/CGP/cosmic/>

abnormalities in the pathway can have different effects (4, 5). Nevertheless, all class I PI3Ks are compelling targets for therapeutic intervention because p110 α is mutated and amplified in cancer and all four isoforms can generate PIP₃ and are oncogenic in model systems (9). On the other hand, recent data indicate that the preferred class I PI3K target may be dependent on the molecular context, for example, mutation of p110 α , loss of PTEN, or overexpression of p110 α , p110 β , or p110 δ ; thus fueling the ongoing debate on the optimal selectivity profile of PI3K drugs for cancer treatment (5, 9). This important issue of selectivity, upon which PI3K inhibitors are shedding invaluable light, is discussed further below.

The Emergence of Chemical Tools

Following on from the success with protein kinase inhibitors in cancer treatment (10) and in the general context of “drugging the cancer genome” (11), the subsequent therapeutic targeting of PI3K enzymes has been termed “drugging the PI3 kinome” (12).

Alongside genetic, molecular biological, and biochemical studies, chemical inhibitors have been enormously valuable as tools in PI3K research (13–16). They have been used to help understand the role of PI3K enzymes in signal transduction and downstream physiological and pathological processes, as well as to assist validation of PI3Ks preclinically as therapeutic targets (13). The earliest and still widely employed inhibitors were wortmannin and LY294002 (Fig. 1). Wortmannin is a fungal natural product originally identified in 1987 as a potent inhibitor of the respiratory burst in neutrophils and monocytes (17). It was subsequently found to inhibit PI3K (18) by covalent attack on the ATP site Lys 802 (human p110 α numbering; ref. 19). The synthetic flavone LY294002, based on the natural product, broad spectrum protein kinase inhibitor quercetin, was first reported as an inhibitor of PI3K in 1994 (20), at a time when relatively few selective inhibitors of any kinase had been discovered (21). Although these two agents have been extremely useful and are still widely employed as research probes (e.g., refs. 13–15), they both have very significant limitations. Wortmannin is a reactive electrophilic and therefore unstable compound whereas LY294002 is a weak inhibitor with only micromolar potency, and both agents display significant off-target effects on other kinases (9–13, 15). Because of those properties, they are not ideal as probes for specific PI3Ks. Nevertheless, important proof of concept studies with these chemical tools and their analogs in animal models, together with the emerging biology of PI3Ks and their role in a range of disease pathologies, including not only cancer but also inflammation, autoimmune, and cardiovascular disease, has led to an explosion of interest in the discovery of PI3K inhibitors (reviewed in refs. 9, 18–21).

Early drug discovery efforts used wortmannin and LY294002 as chemical starting points for inhibitor design and sought to overcome their respective drawbacks in potency, selectivity, and pharmaceutical properties (22–25). The inspiration that these early inhibitors provided is reflected in the fact that among the privileged chemical scaffolds com-

monly found in PI3K inhibitors, the most frequent is the aryl morpholine present in LY294002 (Fig. 1). The oxygen atom of the morpholine moiety forms a key hydrogen bond interaction with the hinge region of the kinase, as well as making additional hydrophobic interactions in the ATP pocket (ref. 26 and see later). In addition to inhibitors based on wortmannin and LY294002, high-throughput screening (HTS) of large diverse compound collections coupled to medicinal chemistry efforts, frequently supported by structural biology studies, has led to more than 15 chemical classes of PI3K inhibitors (13, 22–25) and has considerably enriched the available chemical equity.

The Discovery of PI-103: An Improved Chemical Tool

In 2006 to 2007, Hayakawa and colleagues published a series of four articles (27–30) that described the use of HTS, based on a scintillation proximity assay, to discover a range of chemically interesting PI3K hits with micromolar inhibitory potency, which were then optimized in the same work by medicinal chemistry to yield single digit nanomolar leads (Fig. 2). One of those lead compounds was the pyridofuropyr-imidine PI-103 (Figs. 1 and 2; ref. 29), which in the initial and later work was shown to exhibit IC₅₀ values of 2 to 3 nM (at 1 μ M ATP) on the class IA α , β , and δ isoforms of p110 PI3K and a slightly higher though still very potent IC₅₀ of 15 nM on the class IB isoform p110 γ (29, 31–33). Antitumor activity was initially reported against four human cancer cell lines *in vitro* and also against HeLa human cervical cancer xenografts in athymic mice (29).

Following the publication of the design, synthesis, and structure-activity relationships for PI-103 (29), the same collaborative team provided a detailed pharmacological characterization and mechanistic evaluation of the new agent (33). PI-103 exhibited advantages over wortmannin and LY294002, with excellent potency and selectivity for class I PI3Ks versus a panel of 70 other kinases, including class II and III PI3Ks and numerous representative protein kinases, as well as having improved stability. However, it was recognized that this agent still had some liabilities with respect to its use in animal models, specifically limited aqueous solubility and extensive metabolism (33). Nevertheless, the compound gave sufficient pharmacokinetic exposure in plasma and tumor tissue of treated mice to allow the demonstration of proof of concept for therapeutic activity against eight human tumor xenograft models representing various cancer types, including several with *PI3KCA* mutations and mutated or silenced *PTEN*, which are thus more likely to be “addicted” to the PI3K pathway (33). PI-103 showed evidence of effects on invasion, angiogenesis, and metastasis, as well as direct antiproliferative activity, mainly resulting from a G1 arrest and involving reduced expression of cyclin D1 and increased p27 (33, 34).

The antitumor activity of PI-103 was associated with effects on pharmacodynamic biomarkers, which were fully consistent with the therapeutic mechanism being PI3K inhibition, including reduced phosphorylation of AKT and other

pathway substrates; PI-103 also induced a gene expression profile consistent with PI3K pathway modulation (33, 34).

PI-103 was included, along with a diverse array of other cell permeable, relatively drug-like PI3K inhibitors selected from the patent literature, in an innovative chemical biology study that revealed fascinating cryptic homologies across PI3K targets and chemotypes and showed clear trends in selectivity across the class I PI3K and PIKKs that were

not predictable from the amino acid sequence (35). In one important exemplification of the approach, a matrix of PI3K-inhibitory chemical tools, including PI-103 and other, more p110 α -specific inhibitors, was used to identify p110 α as the critical PI3K acting downstream of the insulin receptor (35), a result that was consistent with the mouse knock-in studies (36). These findings emphasized the concern at that time that p110 α -specific PI3K inhibitors could

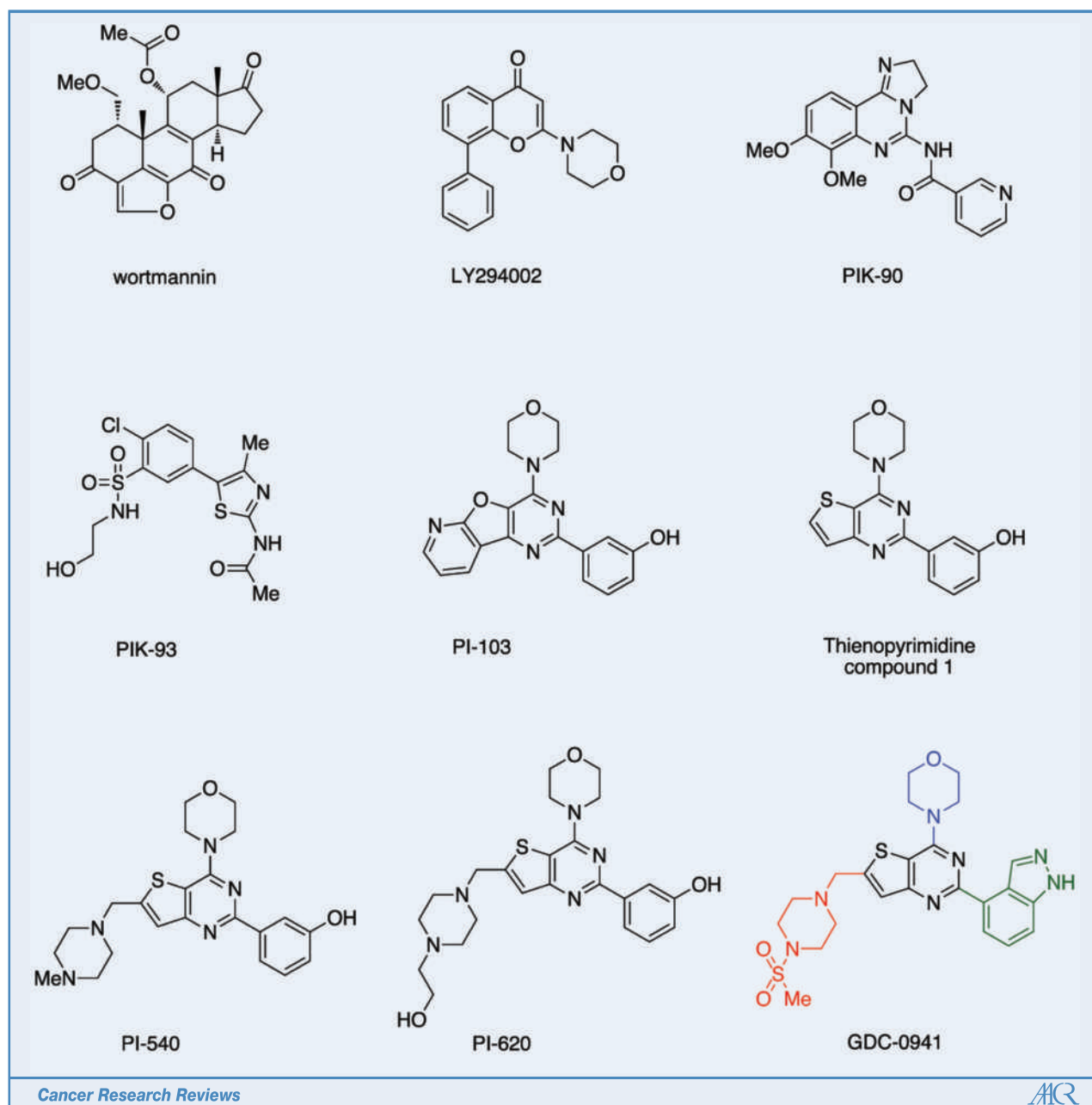
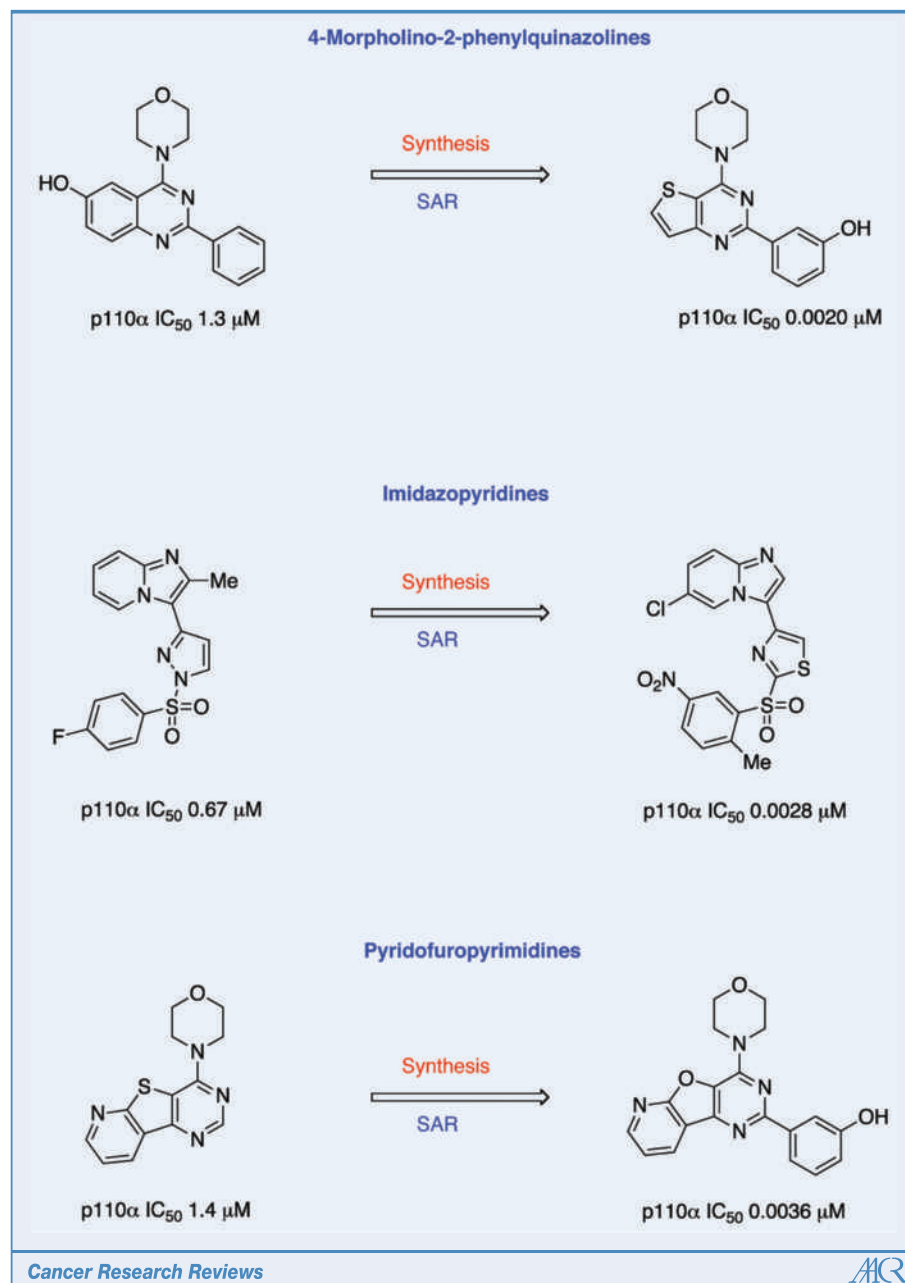


Figure 1. Chemical structures of the PI3K inhibitors wortmannin, LY294002, PIK-90, PIK-93, PI-103, thienopyrimidine compound 1, PI-540, PI-620, and GDC-0941. In the chemical structure of GDC-0941 the indazole moiety is highlighted in green, the sulfonylpiperazine in red, and the morpholino group in blue, whereas the thienopyrimidine core is shown in black.

Figure 2. Three PI3K inhibitory hit compounds identified by HTS against p110 α and their optimization into nanomolar leads. From top to bottom, the 4-morpholino-2-phenylquinazolines optimized to the thienopyrimidine compound 1, the imidazopyridines, and the pyridofuopyrimidines resulting in PI-103. For further details see references 27–30.



potentially cause insulin resistance and associated diabetogenic side-effects.

A PubMed search⁵ revealed that since its initial discovery (29, 31–33), PI-103 has been employed as a chemical tool in at least 30 published studies. In one of the earliest of these (37), PI-103 was shown to have potent activity against human glioma models, also seen in other reports (31–34). Through comparison with a small array of PI3K inhibitors with differing PI3K and PIKK selectivities, this was linked to the com-

⁵ <http://www.ncbi.nlm.nih.gov/sites/entrez>; December 1, 2009.

binatorial inhibition of p110 α and mTOR by PI-103 (37). Indeed the gene expression profile induced by PI-103 in glioma cells shared common elements with changes caused by the mTOR inhibitor rapamycin, amino acid deprivation, and modulation of insulin/IGFR signaling (34). However, this combinatorial effect may not, in fact, be essential for antitumor activity and we will return later to the requirement for mTOR inhibition.

In a recent update (16) of the earlier compendium (14, 15) of selectivity patterns of kinase inhibitors, revealed by profiling large, diverse kinase panels representative of the broader kinome, the suitability of these agents as chemical tools for

use in cellular studies was determined. On the basis of this comparative assessment, the replacement of LY294002 by PI-103 was recommended alongside wortmannin as the two most suitable chemical probes to be used in tandem to determine the involvement of PI3K signaling in cellular phenotypes.

Despite its value as a chemical tool (16, 35) and its utility in showing the therapeutic potential of this series of PI3K inhibitors (33, 34, 37), PI-103 was recognized from the outset to exhibit serious drawbacks in terms of its drug-like properties and thus its suitability for clinical development (33). In particular, the planar tricyclic structure (Fig. 3) results in limited aqueous solubility, and the phenolic hydroxyl group is rapidly glucuronidated. These adverse features needed to be fixed in order to produce a clinical development candidate with suitable formulation and pharmacokinetic and pharmacodynamic properties, while retaining potency and selectivity against PI3K.

From PI-103 to GDC-0941: A Candidate for Clinical Development

Designing a drug candidate suitable for clinical evaluation is like solving Rubik's cube in that all of the essential elements need to be aligned simultaneously in the same molecule. Almost inevitably the eventual clinical agent is to some degree a compromise, because many of the different properties may be dependent on the same structural features within the drug molecule. Solubility and pharmacokinetic and pharmacodynamic behavior are very important alongside target potency and selectivity (10). This is as true for PI3K inhibitors as it is for other drugs.

The multiparameter lead optimization program focused on improving pharmaceutical, pharmacokinetic, and pharmacodynamic properties while maintaining the key inhibitory activity on class I PI3K enzymes and the related antiproliferative activity against cancer cells (38, 39). The two chemical starting points considered were: 1) the tricyclic pyridofuropyrimidine PI-103 (29); and 2) the bicyclic thienopyrimidine "compound 1," also optimized from an HTS hit (Figs. 1 and 2; ref. 27). The latter, although it exhibited even faster clearance than PI-103 in mice, offered greater potential for optimization because of its lower molecular weight and capacity for key chemical substitutions to be made. From among the large number of chemical analogs designed, synthesized, and tested in an iterative fashion (38), the more advanced lead compounds PI-520 and PI-620 illustrate the evolution to the clinical candidate GDC-0941 (Fig. 1; ref. 39).

Both PI-540 and PI-620 are bicyclic thienopyrimidines (Fig. 1) with a solubilizing piperazine-based functionality at the 6-position of the thienopyrimidine ring (left-hand side), which was predicted by computer modeling to extend out of the ATP-pocket into the solvent. This modeling was done using PI-103, the bicyclic thienopyrimidine lead, and the p110 γ structure (38). At the same time, PI-540 and PI-620 keep the phenol moiety (right-hand side) that was thought to be required for binding in the affinity pocket (refs. 35, 38, and see later). PI-540 and PI-620 retained the ≤ 10 -nM potency against p110 α and p110 δ seen with PI-103; although

potency against p110 β and p110 δ was lower by an order of magnitude, these compounds are still potent pan-class I inhibitors (38, 39). Submicromolar antiproliferative activity was maintained against a panel of cancer cell lines, consistent with comparable inhibition of AKT phosphorylation to that seen with PI-103 (39). PI-540 and PI-620 exhibited much improved aqueous solubility and reduced *in vitro* microsomal

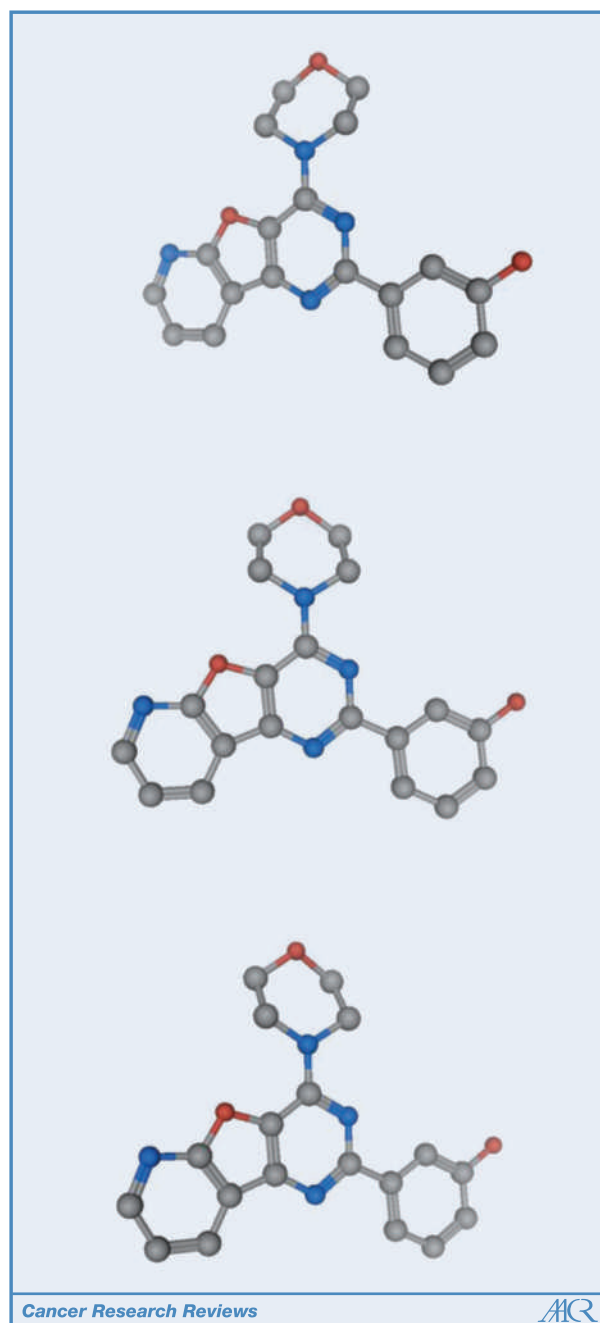


Figure 3. Ball and stick representations of PI-103 to illustrate the planar nature of the tricyclic pyridofuropyrimidine, which limits the aqueous solubility of the compound.

metabolism. Clearance was still quite fast, owing to remaining glucuronidation of the phenolic hydroxyl and extensive tissue uptake, but high tissue-to-plasma ratios led to improved concentrations and better PI3K pathway modulation in human tumor xenografts and resulted in correspondingly greater antitumor activity, as compared with PI-103 (39). Indazoles were investigated as phenol replacements that might markedly reduce glucuronidation while maintaining the necessary target interaction, specifically the hydrogen bond donor properties in the affinity pocket of the ATP site (see later). This strategy paid off and GDC-0941 was designed (38) by combining: 1) a 4-indazole substitution on the right-hand side of the thienopyrimidine ring; with 2) an optimized sulfonylpiperazine solubilizing group on the left-hand side; while at the same time 3) retaining the essential morpholine (top of structure; Fig. 1).

Solving the Rubik's cube conundrum in this way resulted in a range of interesting and attractive features being exhibited by GDC-0941 (38, 39). The drug retains the 3 nM IC₅₀ against wild-type p110 α and has equal potency against both of the common activated oncogenic hotspot p110 α mutants that were tested, namely the helical domain E545K mutant and the C-terminal kinase domain H1047R mutant. Inhibition of p110 α was ATP-competitive with a K_i value of 10 nM. The same 3 nM IC₅₀ seen for p110 α was also exhibited against the class IA PI3K p110 δ , although the IC₅₀ values for the class IA p110 β and the class IB p110 γ are 10-fold and 25-fold higher at 33 nM and 75 nM, respectively. Because all these IC₅₀ values are <75 nM, the overall profile is that of a potent pan-class I PI3K inhibitor with relatively greater selectivity for the p110 α and p110 δ isoforms. A very high degree of selectivity was observed against the class III PI3K C2 β (IC₅₀ 670 nM) and the class III PI3K Vps34 (1,000 nM). In addition, and in contrast to PI-103, GDC-0941 has a very weak potency against the class IV PIKKs DNA-PK (1,230 nM) and mTOR (580 nM). Furthermore, when tested in a panel of 228 protein kinases at a concentration of 1 μ M, GDC-0941 was shown to inhibit only minor activity on Flt3 and TrkA A (50% and 61% inhibition, respectively), further confirming it to be a very selective class I PI3K inhibitor (38).

Despite some differences in selectivity compared with PI-103, especially the markedly reduced potency against DNA-PK and mTOR, GDC-0941 displayed comparable antiproliferative potency against a panel of human cancer cell lines and human vascular endothelial cells (HUVEC), suggesting that a high potency against the class IV PIKKs was not necessary to inhibit tumor cell and HUVEC proliferation (39). Furthermore, GDC-0941, PI-103, PI-540, and PI-620 showed comparable inhibition of phosphorylation of protein substrates on the PI3K pathway in cancer cells; for example, EC₅₀ values were in the range 10 to 36 nM for AKT phosphorylation (39), as measured by quantitative electrochemoluminescent ELISA assays (33, 34, 40). Results indicated that ~90% inhibition of AKT phosphorylation for several hours is needed to inhibit cancer cell proliferation, information that was valuable during lead optimization and identification of GDC-0941 as the clinical candidate (38, 39) and which should also inform clinical studies. In addition, this group of com-

pounds inhibited translocation of the FKHR forkhead transcription factor with EC₅₀ values of 30 to 81 nM, consistent with PI3K inhibition (38, 39).

In vivo animal model testing showed that the major reduction in glucuronidation of GDC-0941 afforded by the indazole substitution resulted in markedly slower systemic clearance compared with PI-103, PI-540, and PI-640 and, moreover, led to 78% oral bioavailability in mice, a major advantage over the other analogs (38, 39). This in turn led to profound and prolonged inhibition of PI3 kinase pathway biomarkers in human cancer xenografts, consistent with the sustained tumor drug exposure (39). Thus tumor levels were well above antiproliferative GI50 concentrations for at least 6 hours, and decreased phosphorylation of AKT, GSK3 β , and P70S6K was maintained for at least 8 hours (39). As a result of these enhanced pharmacokinetic and pharmacodynamic properties, GDC-0941 exhibited excellent dose-dependent therapeutic activity in the PTEN null, PI3K pathway-addicted U87MG human glioblastoma xenograft model in athymic mice, with up to 98% growth inhibition and significant regressions seen in this model (39).

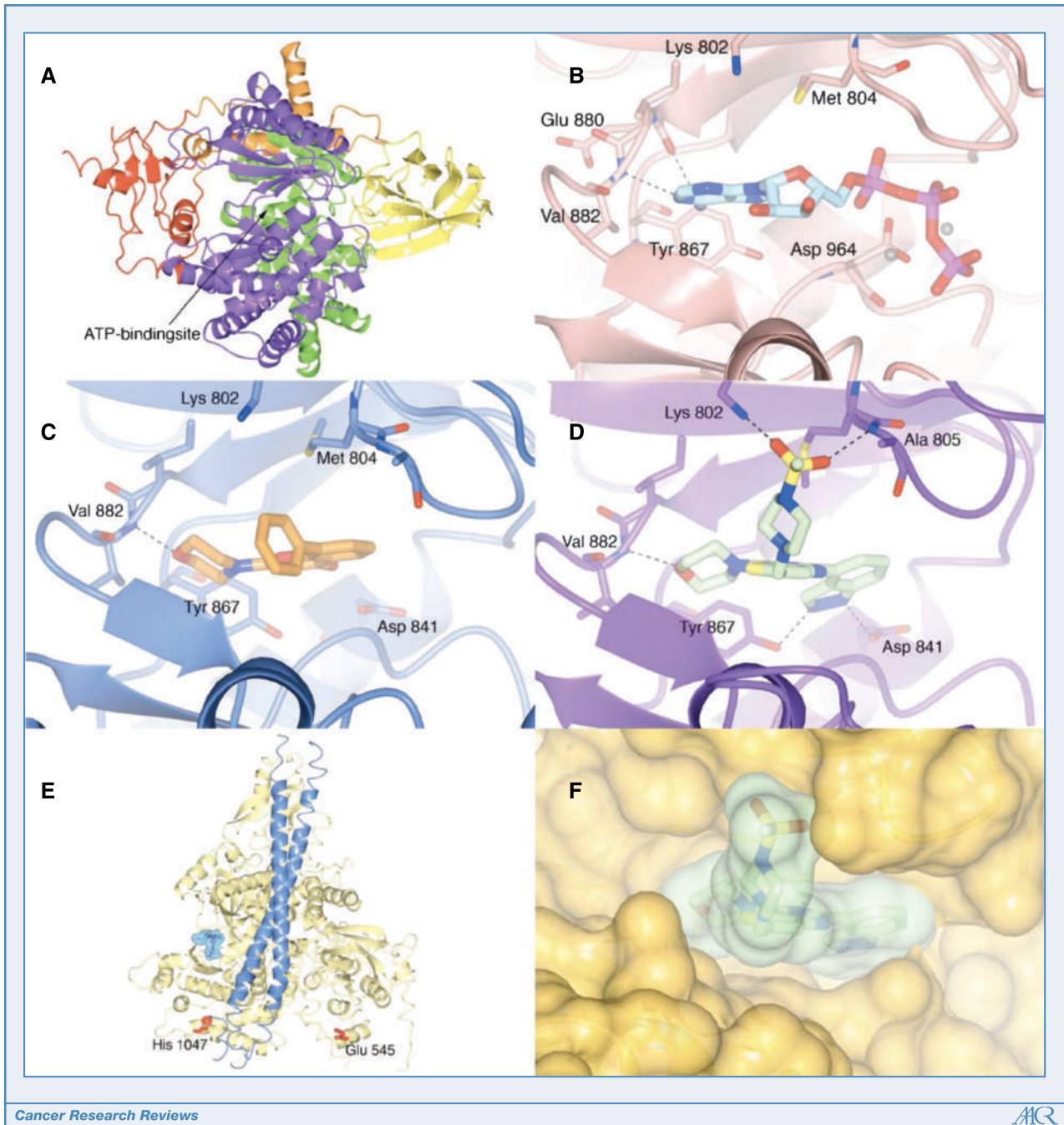
Efficacy was confirmed in the PI3K-addicted IGROV-1 human ovarian cancer xenograft model, which harbors a hetT319F deletion and frameshift in *PTEN* as well as a p85 binding domain hetR38C mutation in p110 α (39). Therapeutic activity was shown in additional human tumor xenografts (41). The linkage of pharmacokinetic exposure, pharmacodynamic biomarker changes in the PI3K pathway, and therapeutic tumor response to the drug provided a convincing pharmacologic audit trail (42, 43), which forms a good platform for subsequent clinical studies. On the basis of its very attractive molecular, pharmacologic, and therapeutic profile, including minimal effects on cytochrome P450s and the hERG channel (38), GDC-0941 was selected for clinical development.

Structural Studies on the PI3 Kinome: Invaluable Insights and Unfinished Business

The design of GDC-0941 was guided by structural data on PI3Ks and their interaction with small molecule inhibitors, a situation that is almost taken for granted in many current drug discovery programs on soluble drug targets. However, it was only a decade ago that the first light was shed on the three-dimensional structure of PI3Ks with the elucidation of the apo- and ATP-bound crystal structures of porcine p110 γ (44). These studies revealed a five-domain structure, which includes an *N*-terminal adaptor binding domain (ABD-domain), a Ras-binding domain (RBD-domain), a C2 domain that has been proposed to be involved in membrane binding, a helical domain, and a catalytic domain (Fig. 4A). The fold of the catalytic domain of the lipid kinase turned out to be very similar to the archetypical protein kinase fold, and comprises an *N*-terminal and a C-terminal lobe connected by a flexible peptide linker called the hinge region. Similar to the protein kinases, the PI3K ATP binding site is located in the groove in between the two components of the catalytic domain.

However, in contrast to the abundance of protein kinase structures, the structural studies on PI3Ks have long been restricted to porcine and human p110 γ . Nevertheless, crystal structures of porcine p110 γ complexed with ATP and early non-specific PI3K inhibitors such as LY294002, wortmannin, and the broad spectrum protein kinase inhibitors quercetin, myricetin, and staurosporine have yielded exciting insight into the molecular determinants of ligand binding in the ATP site of PI3Ks (26). For example, the structures show that the inhibitors fill the space occupied by the adenine moiety of

ATP (Fig. 4B) and show that the overlapping ring systems bind in the same plane as the adenine group. They further mimic protein-ATP interactions through the formation of a hydrogen bond with the backbone nitrogen of the hinge residue Val 882 (porcine p110 γ numbering), which also interacts with the N1 atom in ATP, analogous to the hinge-binding observed for protein kinase inhibitors (Fig. 4B and C). In addition, the five inhibitors studied extend to some degree into the affinity pocket, located at the back of the ATP site, which is not occupied by ATP in the ATP-bound



structure (26). This pocket is defined by Lys 833, Asp 836, Leu 838, Asp 841, Asp 861, Tyr 867, Ile 879, and Asp 964 and is crucial in controlling inhibitor potency (35).

Indeed, the crystal structures of p110 γ bound to two potent PI3K inhibitors, the imidazoquinazoline PIK-90 and the phenylthiazole PIK-93, show that both exploit the affinity pocket, PIK-90 through its pyridine moiety and PIK-93 using a chlorine atom (35). In addition, PIK-90 forms the hinge hydrogen bond with the backbone nitrogen of Val 882, whereas PIK-93 forms two hydrogen bonds with this residue, one with its backbone amide and one with its carbonyl group. On the basis of these protein-inhibitor structures, a model of the binding of PI-103 suggests that the morpholino group of PI-103 forms the hydrogen bond with the hinge and that its phenol moiety binds in the affinity pocket (35).

A crystal structure of the tridentate GDC-0941 bound to human p110 γ shows that it is very efficiently anchored in the ATP-binding site, which may explain its high potency (Fig. 4D and Supplementary Movie S1; ref. 38). Similar to the early PI3K inhibitor LY294002, GDC-0941 uses an essential morpholino group to form the key hydrogen bond with the hinge. In addition, the indazole moiety fits snugly deep in the affinity pocket with the two indazole nitrogen atoms forming hydrogen bonds with the hydroxyl group of Tyr 867 and the carboxylate group of Asp 841, further strengthening the interactions in this pocket. Furthermore, the 4-methanesulfonyl-piperazin-1-ylmethyl group of GDC-0941 points toward the solvent, with the piperazine ring lying against the side chain of Met 804 and the oxygen atoms of the sulfonyl group forming hydrogen bonds with the backbone amide of Ala 805 and the side-chain nitrogen atom of Lys 802. Finally, the thienopyrimidine scaffold of GDC-0941 is effectively sandwiched between, on the one hand, the side chains of Met 953 and Ile 963, which form the floor of the ATP-binding site, and, on the other hand, the side chains of Met 804, Trp 812, and Ile 831, which form its ceiling.

As mentioned, so far all crystallographic investigations into the binding interactions of PI3K inhibitors, from the first inhibitors to advanced clinical candidates, have been carried out using porcine and human p110 γ . However, as discussed earlier, it is p110 α that is most frequently mutated and amplified in human cancers (6, 7). The recent elucidation of the crystal structure of the human p110 α /p85 α complex (45) yielded a detailed structural understanding of the effects of oncogenic mutations in PI3K α . For example, the p110 α /p85 α structure with the poorly visible nSH2 domain of p85 modeled in is consistent with a biochemically identified interaction between the frequently mutated Glu 542, Glu 545, and Gln 546 with residues in the p85 nSH2 domain and with the conclusion that mutation of these residues disrupts the inhibition of the catalytic subunit by this interaction (45). In addition, the structure shows that another oncogenic mutation hotspot His 1047 is located close to the C-terminal end of the activation loop, suggesting that His 1047 mutations could influence the conformation of the activation loop and therefore affect the binding of phosphoinositide substrates (45). However, both Glu 545 and His 1047 are quite far away from the ATP-binding site in PI3K (Fig. 4E), and it is therefore not surprising that the oncogenic E545K and H1047R mutations have no effect on the potency of GDC-0941.

Although the structure elucidation of the p110 α /p85 α complex is an important milestone in the structural characterization of the PI3K family, the quest for potent and isoform-specific next generation PI3K inhibitors would greatly benefit from the availability of protein-inhibitor structures of this and other isoforms. Unfortunately, the p110 α /p85 α structure shows that the crystals used to obtain it are not suitable for crystallographic soaking experiments, as a loop from the RBD domain of a neighboring molecule protrudes into the ATP-binding site, thereby blocking the binding of nucleotides or small molecule PI3K inhibitors in these crystals.

Figure 4. Structural biology of PI3K and inhibitors. A, ribbon diagram of human p110 γ (PDB code 3dbs) illustrating the five-domain structure of the enzyme. The RBD domain is shown in red, the C2 domain in yellow, the helical domain in green, and the bilobal catalytic domain in purple. The N-terminal ABD domain preceding the RBD-domain is shown in orange. The ATP-binding site is located in between the N- and C-terminal lobes of the catalytic domain, as indicated by the arrow. All figures were made with CCP4MG (67). B, binding of ATP to porcine p110 γ (PDB code 1e8x), showing the hydrogen bond interactions of ATP with the hinge region. The protein is shown in pink, ATP is displayed in light blue. The hydrogen bond interactions with the amide group of Val 882 and with the carbonyl group of Glu 880 are shown as dotted lines. Shown in gray are the two metal ions coordinating the phosphate groups of ATP. C, binding of LY294002 to porcine p110 γ (PDB code 1e7v) illustrates the crucial hydrogen bonding interaction between the oxygen of morpholino group of the weakly potent and relatively unselective inhibitor and Val 882 of the PI3K hinge region (left-hand side). The protein is shown in blue with key PI3K residues displayed and labeled. LY294002 is shown in orange. The critical hydrogen bond is shown as a dotted line. Although LY294002 extends into the affinity pocket (right-hand side) it does not fill the pocket as efficiently as the indazole moiety of GDC-0941 (see D). D, binding of GDC-0941 to human p110 γ (PDB code 3dbs) showing the hydrogen bond interactions that anchor the inhibitor in the ATP-site. p110 γ is shown in purple and GDC-0941 is shown in light green. The oxygen of the morpholino group in GDC-0941 forms a hydrogen bond with the amide of the hinge residue Val 882 (left-hand side) whereas the indazole moiety (right-hand side) binds deep in the affinity pocket with its two nitrogen atoms forming hydrogen bonds with the side chains of Tyr 867 and Asp 841, respectively. The oxygen atoms of the sulfonyl group (projecting out of the plane, center) interact with the side chain of Lys 802 and the amide group of Ala 805 at the mouth of the ATP pocket. E, ribbon diagram of the p110 α /p85 α structure, showing p110 α in yellow and the p85 nSH2 domain blue (PDB code 2rd0). To indicate the location of the ATP site, the ATP from the ATP-bound p110 γ structure (PDB code 1e8x) superimposed on the p110 α /p85 α complex is shown. The ATP is displayed in cylinder representation with its semitransparent molecular surface superimposed in blue. Highlighted in red are the oncogenic mutation hotspots Glu 545 and His1047. F, superposition of the GDC-0941-bound p110 γ structure and the human p110 α /p85 α structure (PDB code 2rd0). The superposition was done using the CCP4 program suite (68). p110 α is shown with its solvent-accessible surface in yellow and GDC-0941 with its (semitransparent) surface in light green. Based on this superposition, the overall fit of GDC-0941 in the p110 α ATP site is very good with only a minor clash of the indazole ring with the wall of the ATP site (right-hand side). It seems likely that GDC-0941 will be able to form hydrogen bond interactions with the hinge valine (Val 851 human p110 α numbering left-hand side) and with the tyrosine and aspartate side chains (Tyr 836 and Asp 810, p110 α numbering; right hand side) in the affinity pocket, as observed in the GDC-0941 p110 γ structure. Differences could occur in the interaction of the sulfonyl oxygens of GDC-0941 (center) with the protein, because the equivalent of Lys 802 in p110 γ is an arginine residue in p110 α and the equivalent residue of Ala 805 in p110 γ has a different backbone conformation in p110 α .

Nevertheless, some insight into inhibitor binding to PI3K α can be obtained from superposition of the p110 α /p85 α structure with that of p110 γ -inhibitor complexes. Such overlays show that inhibitors like the potent pan-class I-selective imidazoquinazoline PIK-90, phenylthiazole PIK-93, and GDC-0941 itself (Fig. 4F) would fit into the p110 α ATP site with only relatively minor clashes, consistent with their pan-specific PI3K inhibitor profile. However, the sequence and structural similarities of the p110 α and p110 γ ATP sites illustrate the challenges faced in the design of highly specific PI3K inhibitors.

Despite these challenges, inhibitors are emerging with surprising selectivity profiles, even differentiating between the class I PI3K isoforms (35), and as a result we are beginning to uncover the structural determinants that govern selectivity across the PI3Ks. For example, the crystal structure of p110 γ bound to the quinazolinone purine PIK-39 (IC87114), one of the most isoform-specific (p110 δ -selective) PI3K inhibitors to date, suggested that differences in plasticity in PI3Ks coupled to a conformational rearrangement of Met 804 (porcine p110 γ numbering) could provide a mechanism to obtain isoform selectivity (35). In addition, recent results have illustrated the role of nonconserved residues at the entrance of the ATP-pocket with respect to inhibitors that are surprisingly p110 β -selective (46). Future design efforts could greatly benefit from the availability of structures of the other class I isoforms PI3K β , PI3K δ , and also class IV protein kinases such as mTOR, ATM, and ATR. It is therefore exciting to see the first structural insights provided by the crystal structures of p110 δ in complex with a series of pan- and PI3K δ -specific inhibitors that were published at the time of writing this review (47). These structures reveal a slightly different binding of GDC-0941 to p110 δ compared with its binding to p110 γ . Importantly, in contrast to GDC-0941 and other predominantly flat pan-specific inhibitors, the new structures also show that the PI3K δ -specific inhibitors prefer a propeller shape, which allows them to efficiently exploit the greater plasticity of PI3K δ , by accessing the so-called specificity pocket that was first observed in the p110 γ -PIK-39 complex.

Understanding the mechanistic basis underlying selectivity for class I PI3Ks versus PI3Ks such as mTOR remains an important goal. As discussed earlier GDC-0941 has much greater selectivity for class I PI3Ks versus mTOR than does PI-103. Very recent results indicate that when comparing two compounds related to GDC-0941—but in which the indazole moiety is replaced by an aminopyridine group as the substituent binding in the affinity pocket—the addition of a single methyl group resulted in a major decrease in mTOR activity without affecting potency against class I PI3Ks (69). PI3K γ -ligand crystal structures showed that in contrast to the unsubstituted aminopyridine, the 4-methyl group twists the aminopyridine ring out of plane of the thienopyrimidine scaffold while steric clashes cause the 4-methyl group to point away from Tyr867 and towards the ceiling of the ATP binding pocket. This region and the sequence around Tyr867 show differences in hydrophobic character and conformation/flexibility respectively that may be hypothesized to account for the remarkable disparities in pan-class I PI3K and mTOR

selectivity between the two compounds. Interestingly, the selectivity differences did not affect antiproliferative potency against cancer cells, indicating once again that class I PI3K activity dictates the therapeutic effect (69).

In addition to the increased understanding of selectivity of inhibitors within the PI3K family, a recent attempt to identify hotspots for resistance mutations revealed that, in contrast to the protein kinases, nonconservative mutations in the PI3K gatekeeper residue Ile 848 (p110 α numbering) are not well tolerated, suggesting that this residue is an unlikely hotspot for resistance mutations (48). Indeed, the study showed that resistance mutants overall are likely to be less common than with many protein kinases. Intriguingly, mutations of Ile 800 to leucine and methionine were shown to confer some resistance to a variety of PI3K inhibitors including PIK-90 and PIK-93, but the I800L mutation was sensitized to the inhibitors PW-12 and BEZ-235. From the available crystal structures it can be seen that Ile 800 and its equivalent in p110 γ are located in the ceiling of the ATP-binding site and interact with a variety of inhibitors through hydrophobic contacts. It therefore seems reasonable to speculate that mutations of this residue, in particular to a large methionine residue, could sterically hinder efficient binding of various classes of inhibitors. It will be exciting to see the emergence of crystal structures of these resistant mutants complexed with a variety of inhibitors, which will undoubtedly aid the design of new PI3K agents.

Context, Progress, Issues, and Outlook

The case history discussed in this article highlights a number of issues that are of more general significance and interest.

Tools to drugs. Since the availability of the human genome sequence,⁶ there has been an increasingly urgent need to develop not only genetic tools [such as RNA interference (RNAi)], but also small molecule probes to interrogate the function of all genes in normal biology and disease states, and to identify and exploit druggable targets for clinical benefit. Chemical probes can often give different phenotypes from RNAi knockdown, for example providing an immediate inhibitory effect that is usually confined to the catalytic activity, whereas RNAi gives a slower effect and removes the entire protein from the cell. Developments in the biology of PI3Ks and the discovery of inhibitors have illustrated the value of the combined small molecule and genetic approach very well and the field has moved forward aggressively over the last few years. The progression of PI3K inhibitors from bioactive chemical biology tools, used to probe mechanisms and show feasibility and consequences of pathway inhibition, into inhibitors with improved, more drug-like properties that provide preclinical proof of concept and candidates for clinical evaluation is a pattern that is seen with other molecular targets (10, 49).

To be useful as chemical tools, small molecule inhibitors need to have at least a minimum level of performance. On the basis of continuously increasing experience, useful

⁶ http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml

criteria are emerging for chemical tools. For example in the initial pilot phase of the US National Institutes of Health Molecular Libraries and Imaging Initiative (MLI), the chemical probes to be generated were envisaged as having “adequate potency and solubility for *in vitro* (cell-based) experimentation” but requiring further “chemical modifications ... to confer the selectivity, pharmacokinetic, and metabolic properties required for *in vivo* use” (50). Increasingly, the focus is not only on potency (for MLI < 100 nM), but also on selectivity (for MLI > 10 fold over related targets) and on aqueous solubility, together with demonstrable improvements over existing probes for the designated target (51).⁷ There is now an urgent need for chemical tools to be usable *in vivo*, for example in animal models of cancer, and for this the pharmaceutical, pharmacokinetic, and pharmacodynamic demands are usually much greater, requiring extensive medicinal chemistry efforts. There is in reality a continuum in terms of the somewhat overlapping criteria for initial chemical hits; improved chemical tools, some of which may evolve into lead compounds for drug discovery; and eventual clinical candidates. The latter are usually differentiated by virtue of fully optimized drug-like properties such as oral bioavailability and freedom from effects on anti-targets such as hERG channels and cytochrome P450s, as exemplified by GDC-0941.

The early chemical tools for PI3K inhibition, particularly wortmannin and LY294002, were extremely valuable but clearly had limitations. The case history reviewed herein illustrates how various micromolar hits were improved to a potent and selective chemical tool inhibiting class I PI3Ks/mTOR (PI-103), then into more advanced leads and potential preclinical drug candidates (PI-540 and P-620), and finally into the eventual pan-class I PI3K drug GDC-0941 that is now in phase I clinical trials. In addition to the lessons learned when morphing from a chemical biology tool to a clinical candidate, another take-home message is the value of structure-based strategies now used in all stages in the design of molecular cancer therapeutics against a wide variety of targets (52). A structure-based approach was also used in another example, the imidazoquinoline clinical agent BEZ-235, which is, like PI-103, a dual class I PI3K/mTOR inhibitor; BEZ-235 evolved by target-hopping from a PDK1 inhibitor lead (53). There are now at least nine PI3K inhibitors in the clinic with various isoform selectivity profiles (23, 24, 54).⁸

Isoform selectivity, biomarkers, efficacy, and tolerability. A key challenge for the development of inhibitors of p110 α isoforms and indeed other PI3K pathway inhibitors in cancer is to determine the optimal selectivity profile, or more likely profiles, and to identify those patients in which a particular profile will be most effective (4, 5, 9, 23, 54). Intriguing new results suggest that genetic context will be key. For example, whereas tumor cells harboring activating p110 α are dependent on this isoform, consistent with addiction to it, PTEN-deficient cancer cells are, in contrast, dependent on p110 β (55). Further confusing the issue is

the finding that p110 β seems to have a role that is not dependent on kinase catalytic activity (9, 56). In addition, AKT-independent signaling downstream of oncogenic p110 α has been discovered, involving PDK1/SGK3 (57). The p110 δ isoform is mainly restricted to hemopoietic cells and may be a target in leukemia and lymphoma as well as immune and inflammatory disease (9). Results with chemical inhibitors do not currently give a clear picture about the impact of PI3K pathway and RAS mutations on sensitivity to agents that generally hit the class I PI3Ks, with or without mTOR (reviewed in refs. 39, 54).

In the case of GDC-0941, findings to date suggest that although cancer cells lines with mutations in *PIK3CA* or loss of PTEN expression are often sensitive to this agent, whereas some with *RAS* mutations may be resistant, the drug nevertheless shows broad activity across malignant cell panels and human tumor xenografts (39, 41). One contributory factor is that cancer cells with activated upstream receptor tyrosine kinases may also be sensitive. The situation *in vivo* may be further complicated by the clear anti-angiogenic effects of class I/mTOR inhibitors (33, 53), which may relate to the role of p110 α in endothelial cell migration and vascular development shown by mouse genetic studies (58). In addition, PI3K inhibitors may exert other effects on the tumor microenvironment and immune cells (4). In some ways the above results argue for the broad therapeutic utility of pan-class I inhibitors, assuming that such agents are well tolerated, which seems to be the case from animal model studies and early clinical trials (5, 23). Prediction of sensitivity may require more complex signatures rather than single mutational events. Nevertheless, it is not unreasonable to look for initial clinical activity by enriching trials with patients with 1) oncogenic mutations in *PIK3CA*, 2) loss of PTEN expression, or 3) activated receptor tyrosine kinases, before broadening the studies to a wider patient population. The alternative is to treat a more diverse cohort of patients up front and to collect information on their molecular status.

Although inhibition of p110 α was predicted to block insulin signaling and potentially to induce diabetes (35, 36), this does not seem to be a problem with chronic dosing. Experience with protein kinase inhibitors in the clinic, where inhibition of multiple kinases can be advantageous (10), would support the view that at least limited polypharmacology with PI3K inhibitors may be a good thing. Inhibitors of specific isoforms may be valuable in particular settings (see above). Drugs targeting particular isoforms could avoid particular side-effects (5). For example, p110 α/β inhibitors that do not hit p110 γ and p110 δ would likely spare immune cells. p110 δ -selective inhibitors may be advantageous in some leukemias and lymphomas and avoid possible metabolic effects on p110 α/β . Preferential activity on p110 α may be desirable in cancers with mutant p110 α , whereas p110 β selectivity may be valuable in PTEN-deficient malignancies. There are no reports of inhibitors selective to mutant versus wild-type p110 α , which could have advantages in relevant cancers addicted to that mutant kinase. Ongoing use of chemical biology and structure-based design approaches should continue to deliver a variety of isoform-specific or pan-selective profiles. Unexpected cancer

⁷ <http://grants.nih.gov/grants/guide/rfa-files/RFA-RM-08-005.html>

⁸ <http://clinicaltrials.gov>

dependencies and synthetic lethality may emerge, as shown by the preferential activity of PI3K pathway inhibitors in mismatch repair-deficient colorectal cell lines (59).

Combinations. Experience with modern targeted molecular cancer therapeutics (10) suggests that the presence of multiple genetic abnormalities and the induction of feedback loops and other resistance mechanisms is likely to mean that PI3K inhibitors will need to be given in combination with other agents (4, 5). In support of this approach, recent studies with GDC-0941 have shown promising results by combining this agent with the HER2-directed antibody trastuzumab, including treatment of trastuzumab-resistant cancer models (60, 61). The combination of GDC-0941 with a BRAF inhibitor was beneficial in cancer models with oncogenic *BRAF* and *PTEN* deficiency (62), and dual blockade involving GDC-0941 with a MEK inhibitor was advantageous in *PTEN* wild-type or *PTEN* null basal-like breast cancer models (63). “Horizontal” inhibition of parallel pathways as well as “vertical” blockade at more than one point within the PI3K pathway are of great interest, while also recognizing that these pathways likely operate in complex networks in which the outcomes of pharmacologic modulation may be difficult to predict.

Concluding remarks. The 20-plus-year journey from chemical tools to PI3K drugs has been an exciting and productive one. We have interesting times ahead as the first generation of PI3K inhibitors progress through early clinical trials. Initial reports from phase I clinical trials show GDC-0941 to be well tolerated with appropriate pharmacokinetics, biomarker evidence of target modulation, and some signs of biologic activity (64, 65). A number of other inhibitors of PI3K are now in the clinic (22, 23). With suitable pharmacodynamic biomarkers in hand to show proof of mechanism, the key challenges are now to identify predictive biomarkers so that

PI3K inhibitors can be directed to the most appropriate patients, to sort out the preferred inhibitor selectivity profiles for patients with various pathway abnormalities (66), and to develop rational mechanism-based combinatorial treatments for optimal therapeutic efficacy.

Disclosure of Potential Conflicts of Interest

The authors are employees of the Institute of Cancer Research, which has a commercial interest in the development of PI3K inhibitors, including GDC-941, and operates a rewards-to-inventors scheme. Paul Workman, Paul Clarke, and Florence Raynaud have been involved in a commercial collaboration with Yamanouchi (now Astellas Pharma) and with Piramed Pharma and intellectual property arising from the program has been licensed to Genentech. Paul Workman was a founder of, consultant to, and Scientific Advisory Board member of Piramed Pharma (acquired by Roche); is a founder of, consultant to and Scientific Advisory Board and Main Board member of Chroma Therapeutics; and was formerly an employee of AstraZeneca. Florence I. Raynaud is a consultant for ELARA Pharmaceuticals. Rob van Montfort is a former employee of Astex Therapeutics.

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