Precision targeting of mutant PI3Kα

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***PIK3CA*, which encodes the p110α catalytic subunit of PI 3-kinase alpha (PI3Kα), is one of the most frequently genetically-activated kinases in solid tumors. In two back-to-back papers, Varkaris *et al.* report on the development of a novel allosteric PI3Kα-mutant selective inhibitor and early clinical experience with this compound.**

Developing drugs to target cancer-specific alterations is challenging. This has also been the case for inhibitors of PI3Kα, a heterodimer of the p110α catalytic subunit and a p85 regulatory subunit (**Figure 1A**), and one of the most frequently oncogenically-activated kinases in solid tumours. These activating mutations most commonly occur in so-called hotspot regions of p110α - either in the helical domain (E542K, E545K) or the kinase domain (H1047R, H1047L). Mutations in p85 that activate the PI3Kα complex are also common in cancer. The structural mechanisms of how these mutations activate the PI3Kα complex are well-understood[1].

Alpelisib (NVP-BYL-719), which targets both wild-type (WT) and mutant PI3Kα[2], is the only FDA-approved PI3Kα-selective inhibitor to date, for use in combination with the estrogen receptor degrader fulvestrant for hormone-positive HER2-negative breast cancer. Alpelisib suffers from on-target toxicity, especially metabolic side effects, given the key role that PI3Kα plays in normal organismal metabolism[3].

Ongoing ways to increasing PI3K pathway inhibitor tolerability include intermittent dosing (ongoing studies with capivasertib (AKT inhibitor; AstraZeneca); bimiralisib (PI3K/mTOR inhibitor; TORQUR)) or intravenous administration to avoid gut toxicity (gedatolisib PI3K/mTOR inhibitor; Cellcuity).

Another approach is to generate PI3Kα-mutant selective inhibitors that only target the mutation-bearing cancer cells (**Figure 1A**). In addition to the recently reported STX-478 (Ref.[4]), two papers by Varkaris *et al.* in the current issue of *Cancer Discovery* report on the development of RLY-2608, the first PI3Kα-mutant selective inhibitor to enter human trials[5,6].

On aggregate, these studies strengthen the case that it is possible to generate PI3Kα-mutant selective inhibitors, by targeting an allosteric pocket outside of the ATP-binding site of p110α (**Figure 1A,B**). Such inhibitors show anti-tumour activity in mouse xenografts, with highly reduced metabolic side effects compared to non-mutant-selective PI3Kα inhibitors. While data on human studies with STX-478 have not been reported, Varkaris *et al.* describe two case studies of patients with advanced hormone receptor-positive breast cancer with *PIK3CA* helical or kinase domain mutations in which RLY-2608 elicited objective tumor responses, with no observed toxicities associated with PI3Kα-WT.

This is a hugely important observation, indicating that PI3Kα-mutant selective inhibitors are likely to widen the therapeutic index of targeting PI3Kα, facilitating higher and longer drug dose exposure as well as combination with other therapies.

Earlier studies on an very large cancer cell line panels revealed that *PIK3CA* mutation is the foremost positive predictor of sensitivity to the non-mutant-selective PI3Kα inhibitor alpelisib[2], although this correlation is not absolute, most likely reflecting the multiple ways the PI3K pathway can be oncogenically activated in cancer cells beyond through *PIK3CA* mutation. From the Varkaris *et al*. data[5], it is difficult to conclude whether RLY-2608 inhibits cell proliferation in *PIK3CA*-H1047R tumor cell lines beyond what can be achieved by a non-mutant-selective PI3Kα inhibitor. Engineered paired cell lines without or with mutant *PIK3CA* often show higher sensitivity in the latter to PI3Kα inhibition (either mutant- or non-mutant-selective), especially when monitoring short-term signalling responses such as generation of pAKT. In general, it remains unclear to what extent *PIK3CA* mutated cancer cells are specifically 'addicted' to this mutation, and therefore would be expected to show higher anti-proliferative sensitivity to mutant-selective PI3Kα inhibitors as compared to non-selective PI3Kα inhibitors. It is also important to mention that most cancer cell lines (and tumors) are heterozygous for mutant *PIK3CA* and may depend on both WT and mutant PI3Kα.

Below, we first describe in more detail the reported structural mechanism of action of RLY-2608, followed by the early clinical observations with this compound.

Current PI3Kα-selective inhibitors can be classified into ATP-competitive or allosteric inhibitors, the latter being p110α mutant-selective (**Figure 1A**). ATP-competitive inhibitors interact with p110α non-covalently or covalently, the latter by engaging a cysteine close to the ATP-binding site that is only found in PI3Kα and not in other PI3K isoforms. Non-covalent ATP-competitive inhibitors have been further divided in PI3Kα-non-degrading or -degrading.

The ATP-binding site is highly conserved across PI3Kα-related PI3K isoforms (the so-called class I PI3K isoforms: PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ). Alpelisib achieves selectivity for PI3Kα over other class I PI3Ks by making two H-bonding interactions with the PI3Kα-specific residue Q859. However, due to similarity of the active site between WT and mutant PI3Kα, it has proved extremely difficult to achieve PI3Kα mutant-selectivity with ATP-competitive inhibitors.

In their first manuscript, Varkaris *et al*.[5] present their journey of discovering and developing PI3Kα mutant-selective small molecule inhibitors, focusing on the H1047R-hotspot mutation in the C-terminal tail of p110α. They start off with a combination of cryoEM, molecular dynamics simulations, and follow-up X-ray crystallography to provide additional insight into mutations in the C-terminal tail of PI3Kα that favor kinase activation.

They next exploited the identified conformational differences between PI3Kα-WT and -H1047R along the allosteric network connecting the site of mutation (C-terminal tail) to the catalytic loop HRD motif and activation loop. These differences were then used to perform DNA-encoded library screening, to select for inhibitors with selectivity for PI3Kα-H1047R over PI3Kα-WT. This was followed by a detailed biochemical and biophysical analysis, and X-ray crystallography of their lead compound called RLY-2608. These studies revealed that this compound binds in an allosteric cryptic pocket formed by residues L812, L911, I999, I1019, and I1022 of p110α. Upon opening of the pocket and compound binding, the N-terminal region of the activation loop is displaced, leading to perturbation of key residues and motifs involved in substrate binding, and therefore a loss of catalytic activity.

These studies also defined a rationale for the selectivity of this compound for PI3Kα-H1047R over PI3Kα-WT. In PI3Kα-WT, the C-terminal tail in the kinase domain is buried and constrains the

conformations of key residues leading to stabilization of a ligand-inaccessible cryptic site conformation. However, the H1047X mutations favor the disengaged state of the C-terminal tail, this alters the conformations of these key residues, therefore increasing accessibility of the ligand cryptic site. Indeed, when the C-terminal tail of PI3Kα-WT is truncated, the compound affinity and level of inhibition is non-distinguishable between PI3Kα-WT and -H1047R (Ref.[5]).

Varkaris *et al*.[5] further report that RLY-2608 is also selective for the E542K and E545K helical domain hotspot mutants over PI3Kα-WT. This is particularly interesting because the helical domain mutants have a very different mechanism of PI3Kα activation compared to the C-terminal tail H1047R mutant[1]. The charge swap in E542K and E545K disrupts the inhibitory contacts between the nSH2 domain of p85α and the helical domain of p110α, thereby activating the enzyme. H1047R on the other hand, is known to rearrange the C-terminal tail of PI3Kα leading to increased membrane binding capability. This rearrangement of the C-terminal tail is also linked to movement of the activation loop and an increase in catalytic activity. The fact that RLY-2608 selectively inhibits both kinase and helical domain mutants suggest that the allosteric network identified (C-terminal tail, HRD-motif and activation loop), could be important in regulating the activity of both the kinase and helical domain mutants. Therefore, restoration of this allosteric network by RLY-2608 conveys mutant selectivity. RLY-2608 has no activity on other PI3K isoforms or kinases tested in an *in vitro* kinome screen, as would be expected from allosteric inhibitors.

It is also interesting to compare the binding pocket of RLY-2608 to that of two other recently-reported allosteric modulators of PI3Kα, namely the aforemented PI3Kα mutant-selective inhibitor STX-478 (Ref.[4]) and UCL-TRO-1938, the PI3Kα-selective small molecule activator that we recently reported[7] (**Figure 1B**).

RLY-2608 binds at the same allosteric pocket as STX-478, a H1047X-selective inhibitor that is only mildly (~2-fold) selective for PI3Kα-E545K over PI3Kα-WT (Ref.[4]). Therefore, it was initially surprising that RLY-2608 is 12-fold more selective for both the kinase and helical domain PI3Kα-mutants over PI3Kα-WT. Varkaris *et al*.[5] showed that the selectivity of RLY-2608 for both PI3Kα-E545K and PI3Kα-H1047R mutants greatly increased with increasing duration of compound pre-incubation. It turns out that RLY-2608 has a slow onset (shown by biochemical and microscale thermophoresis data (the latter unpublished, Randy Kipp, Relay Therapeutics, *personal communication*)). The fact that PI3Kα-mutant selectivity increases over time leads to the hypothesis that the sampling rate of conformations that favour H1047R-binding is slow, and even slower for PI3Kα-E545K. And although STX-478 and RLY-2608 bind to the same pocket, this increase in selectivity over time was not observed with STX-478 (Randy Kipp, Relay Therapeutics, *personal communication*). These interesting data provide early indications that just like how different ligand interactions at the ATP-binding site have been reported to have different effects on PI3K inhibitor isoform selectivity and potency, the ligand interactions and local conformational changes in this allosteric pocket may also differentially affect selectivity and potency of mutant-selective PI3Kα inhibitors. This can then be exploited to improve our understanding of how future pan-PI3Kα-mutant selective inhibitors can be developed.

Surprisingly, the binding site of RLY-2608 is in close proximity to the binding site of the PI3Kα-selective small activator UCL-TRO-1938[7] (**Figure 1B**). RLY-2608 binds on the other side of the so-called kinase-activator interface (residues 1002-1016 in p110αand PIKK regulatory domain-like helix (residues 1016-1026 in p110α). UCL-TRO-1938 is proposed to activate PI3Kα by altering the conformation of the activation loop and disrupting the inhibitory contacts between p110α and p85α. It is fascinating how two small molecules that bind to nearby allosteric pockets, and both proposed to affect the activation loop, can have completely opposite effects on enzyme catalytic activity.

The second manuscript of Varkaris *et al*.[6] focuses on genetic mechanisms of clinically-acquired resistance to PI3Kα inhibitors in breast cancer, and potential application of RLY-2608 in this context. Challenges for PI3K-based cancer therapy, like for any targeted therapy, include intrinsic and acquired drug resistance, as well as signalling feedback loops that neutralize PI3K inhibition[3]. A previously published cell-based study identified 63 putative resistance genes to alpelisib[8]. A clinical study from Dejan Juric, the senior author of the current manuscript[6] showed convergent loss of PTEN in different metastases in clinical resistance in a patient with a *PIK3CA*-mutated breast cancer treated with alpelisib[9].

In the current study, Varkaris *et al.* performed serial liquid biopsies and rapid autopsies in patients with advanced breast cancer who developed acquired on-target resistance to two different PI3Kα-selective ATP-competitive inhibitors (alpelisib and inavolisib/GDC-0077), and report that 50% of patients acquire genomic alterations within the PI3K pathway, including the previously-reported PTEN loss and activating AKT1 mutations. Interestingly, they also found 3 secondary mutations in the p110α catalytic pocket that provide resistance to these PI3Kα inhibitors: Q859K, Q859H and W780R. Ligand interactions with Q859 have been shown to be critical for both selectivity and potency of A66, an analogue of alpelisib[10]. This was confirmed by Varkaris *et al.* who showed that the IC_{50} for alpelisib significantly increased in $T47D^{H1047R}$ and $MCF7^{E545K}$ breast cancer cell lines when Q859H/K mutations were engineered in, with no effect on the potency of RLY-2608. On the other hand, the W780R mutation increased the IC_{50} of both PI3Kα-selective and pan-PI3K inhibitors.

Importantly, *in vitro* cellular resistance upon expression of the *PIK3CA* mutations induced by PI3Kα-therapy could be overcome by pan-class I PI3K inhibitors, AKT inhibitors or RLY-2608. This can be explained by the fact that pan-class I PI3K inhibitors interact with slightly different regions in the ATP-binding site of PI3Kα, with a reduced or no need of these inhibitors to interact with Q859. Given that RYL-2608 binds outside of the active site, this means that its activity is unaffected by these secondary active-site mutations that convey PI3Kα resistance to ATP-competitive inhibitors like alpelisib.

The development of mutant-selective inhibitors has proved challenging but is now coming to fruition. The discovery of PI3Kα-mutant heralds a new and exciting era in the quest to target one of the most commonly-activated pathways in cancer, and we very much look forward to seeing future developments of these inhibitors in the clinic. Such compounds could also be of therapeutic benefit to people with *PIK3CA*-related overgrowth syndrome (PROS), a rare non-cancerous overgrowth condition due to mosaic *PIK3CA* mutations early in life.

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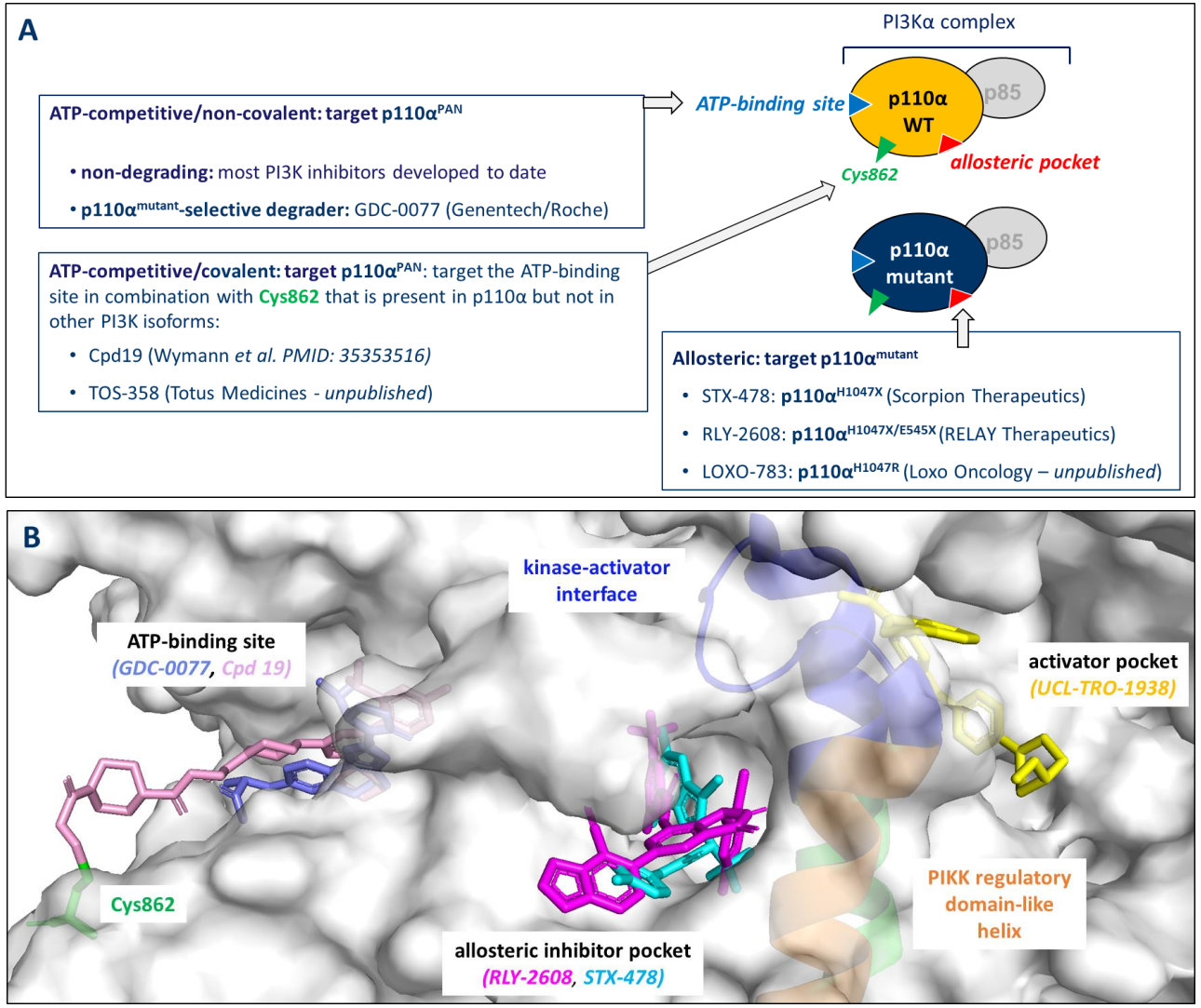
Figure Legend: A. Different approaches used to pharmacologically inhibit PI3Kα. **B.** Crystal structure of RLY-2608 with PI3Kα-WT (magenta, PDB: 8TSD), overlaid with STX-478 (cyan, PDB: 8TDU), GDC-0077 (slate, PDB: 8TDU), Compound 19 (pink, PDB: 7R9V) and UCL-TRO-1938 (yellow, PDB: 8OW2). p110α-WT is shown as a semi-transparent surface, the p85 regulatory subunit is not shown. The 'kinase activator interface' is shown in blue and the 'PIKK regulatory domain-like helix' in orange. Cys862, a p110α-specific residue targeted by covalent modifiers is shown as green sticks.

Figure 1


A
PI3Kα complex
ATP-competitive/non-covalent: target p110α^PAN
• non-degrading: most PI3K inhibitors developed to date
• p110α^mutant-selective degrader: GDC-0077 (Genentech/Roche)
ATP-competitive/covalent: target p110α^PAN: target the ATP-binding site in combination with Cys862 that is present in p110α but not in other PI3K isoforms:
• Cpd19 (Wymann et al. PMID: 35353516)
• TOS-358 (Totus Medicines - unpublished)
ATP-binding site
p110α WT
p85
Cys862
allosteric pocket
p110α mutant
p85
Allosteric: target p110α^mutant
• STX-478: p110α^H1047X (Scorpion Therapeutics)
• RLY-2608: p110α^H1047X/E545X (RELAY Therapeutics)
• LOXO-783: p110α^H1047R (Loxo Oncology – unpublished)
B
kinase-activator interface
ATP-binding site
(GDC-0077, Cpd 19)
activator pocket
(UCL-TRO-1938)
Cys862
PIKK regulatory domain-like helix
allosteric inhibitor pocket
(RLY-2608, STX-478)