AKT degradation selectively inhibits the growth of PI3K/PTEN pathway mutant cancers with wild type KRAS and BRAF by destabilizing Aurora kinase B

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Competing interests

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Abstract:

Using a panel of cancer cell lines, we characterized a novel degrader of AKT, MS21. In mutant PI3K/PTEN pathway lines, AKT degradation was superior to AKT kinase inhibition for reducing cell growth and sustaining lower signalling over many days. AKT degradation but not kinase inhibition profoundly lowered Aurora kinase B (AURKB) protein, which is known to be essential for cell division, and induced G2/M arrest and hyperploidy. PI3K activated AKT phosphorylation of AURKB on threonine 73, which protected it from proteasome degradation. A mutant of AURKB (T73E) that mimics phosphorylation and blocks degradation rescued cells from growth inhibition. Degrader resistant lines were associated with low AKT phosphorylation, wild type PI3K/PTEN status, and mutation of KRAS/BRAF. Pan-cancer analysis identified that 19% of cases have PI3K/PTEN pathway mutation without RAS pathway mutation, suggesting that these cancer patients could benefit from AKT degrader therapy that leads to loss of AURKB.

Statement of significance:

MS21 depletes cells of phosphorylated AKT and a newly identified AKT substrate, Aurora kinase B, to inhibit tumor growth in mice. MS21 is superior to prior agents that target PI3K and AKT due to its ability to selectively target active, phospho-AKT and sustain repression of signaling to deplete AURKB.
Introduction:

The serine/threonine kinase AKT (also known as protein kinase B (PKB)) is a key node of the PI3K–PTEN–AKT–mTOR signaling cascade. Driver mutations of the *PIK3CA* and *AKT1* oncogenes and the PTEN tumor suppressor initiate the development of cancers that activate AKT kinase and downstream mTOR kinase complexes (1-3). The PI3K–PTEN–AKT–mTOR signaling pathway is also a key effector arm of receptor tyrosine kinase oncogenes such as EGFR and HER2 as well as RAS family oncogenes (4). Genetic studies in mice have demonstrated that tumors initiated by PTEN mutation are dependent on PI3K and AKT1 (5,6). Pharmacologically targeting of the kinases on the PI3K–PTEN–AKT–mTOR pathway inhibits the growth of tumors driven by any of the above cancer driver genes in multiple genetically engineered mouse model systems (7-9).

Translating these findings into effective cancer therapies for patients is a high priority because the PI3K–PTEN–AKT–mTOR signaling cascade is arguably the most commonly activated driver pathway in human cancer (10). However, this effort has proven to be particularly challenging and contrasts with the successes in the clinic that have occurred for targets like mutant EGFR, BRAF, and BCR-ABL. Rapamycin-derived inhibitors of the TORC1 complex of mTOR show preclinical efficacy for tumors driven by PTEN, PIK3CA or AKT mutation, but activate AKT signaling through feedback signaling mechanisms that blunt their effectiveness (11); moreover, they do not show selectivity for pathway mutant tumors relative to pathway wild type tumors in the clinic and cause meaningful side-effects for many patients (12). PI3K inhibitors demonstrate preclinical benefit in *PTEN* and *PIK3CA* mutant tumors, often in combination with inhibitors of other pathways, but their impact in the clinic has been limited (9,13). *PIK3CA* mutant tumors are dependent on PI3Kα for tumor growth (14), showing mutant-enriched responses in the clinic when treated with a PI3Kα inhibitor in combination with anti-estrogen therapy in breast cancer (15); however, *PIK3CA* mutant tumors can acquire resistance rapidly and managing the toxicity of PI3Kα selective inhibitors has been challenging because the wild type enzyme functions as part of the insulin signaling pathway. In the case of PTEN driver mutations, tumor cells are often...
dependent on PI3Kβ or PI3Kα but inhibition of both is needed for stable growth suppression (16,17). Selective inhibition of PI3Kα or PI3Kβ reciprocally activates the other kinases leading to reduced efficacy of selective inhibitors (18,19). On the other hand, use of a pan-class I PI3K inhibitor that inhibits both PI3Kα and PI3Kβ has been associated with rapid feedback activation of AKT signaling in the tumor and has not shown selectivity for PI3K/PTEN pathway mutant tumors in the lab or in the clinic when compared to other cancer genotypes (13,20,21). Therefore, though PI3Kα inhibitors are useful for PIK3CA mutant cancers, selective targeting of cancers with PTEN alterations for treatment with PI3K inhibitors has been more challenging.

Cell surface receptors activate PI3K to generate the signaling lipid phosphatidylinositol 3,4,5 trisphosphate (PIP3), which is dephosphorylated by PTEN phosphatase. AKT kinases are activated through binding to PIP3 at the plasma membrane followed by PDK1 phosphorylation on threonine 308 in the activation loop and by TORC2 phosphorylation at serine 473 (4). Initial AKT small molecule kinase inhibitors were not able to inhibit PI3K/PTEN pathway mutant tumors relative to tumors that were wild type for the pathway and had side effects in the clinic (22). Later, AKT ATP-pocket competitive inhibitors including GSK2110183, AZD5363 and GDC-0068 that bind the active form of the enzyme for all three isoforms of AKT (AKT1, AKT2, AKT3) and stabilize the phosphorylation of AKT at threonine 308 and serine 473 showed selectivity for inhibiting the growth of cancer cell lines with PIK3CA, PTEN, HER2 or AKT alterations; however, many mutant cell lines remained resistant to treatment (23-26). Some of these inhibitors have been evaluated in Phase II clinical trials and have a tolerable toxicity profile with efficacy that has some selectivity for cancers with alterations of the PI3K/PTEN pathway (27,28).

Proteolysis targeting chimeras (PROTACs) are a powerful targeted protein degradation technology that hijacks the cellular ubiquitin-proteasome system to induce selective polyubiquitination and degradation of the target proteins (29-31). Using this technology, we designed and developed a library of novel small-molecules putative degraders to degrade AKT by recruiting either the cereblon (CRBN) or von Hippel-Lindau (VHL) E3 ligase, which we reported in the patent literature (32), that have
the ability to degrade AKT in cells and lower downstream signalling to various extents. Another group has also developed a CRBN-recruiting AKT degrader and reported that it is able to inhibit the growth of some breast cancer cell lines (33). Here, we describe one of our VHL-recruiting AKT degraders, MS21, and its characterization in a battery of biochemical, cellular, and in vivo assays using a panel of 38 cancer cell lines with wild type VHL and diverse genotypes and tissue origins. Taken together, our results suggest that efficient pharmacologic degradation of AKT phosphorylated on threonine 308 and serine 473 leads to selective inhibition of the growth of tumor cells with alterations of HER2, PIK3CA, PTEN, or AKT1. MS21 inhibits the growth of tumor cells that contain a PI3K/PTEN pathway mutation through its ability to lower the level of Aurora Kinase B, which we find is an AKT substrate protein. Resistance to MS21 was associated with low levels of baseline AKT phosphorylation in cells, which prevented its degradation and downstream signalling changes, as well as mutations of either KRAS or BRAF. In addition, KRAS or BRAF mutations that occur in conjunction with HER2/PIK3CA/PTEN/AKT1 alterations (hereafter referred to as the PI3K/PTEN pathway) were also resistant to MS21 despite effective degradation of AKT. Resistance to MS21 could be overcome—where both pathways or only KRAS was mutated—by the combination treatment of a MEK inhibitor trametinib with MS21, which not only inhibited MEK but also increased AKT phosphorylation and enhanced AKT degradation. Our findings show that virtually all of the PI3K/PTEN pathway altered tumor cell lines that lack a co-occurring KRAS-BRAF mutation are highly addicted to AKT maintenance of Aurora kinase B and that a large percentage of human cancers have a genotype that could benefit from AKT degradation therapy.

**Results:**

**Creation and biochemical characterization of the AKT degrader MS21.**

To create effective hetero bi-functional small-molecule degraders of AKT, we selected several AKT inhibitors including AZD5363, a highly potent and selective ATP-competitive pan-AKT inhibitor (24), as a binder to AKT (Fig. 1A). The co-crystal structure of AKT1 in complex with AZD5363 (34) indicates that the hydroxyl group of AZD5363 is solvent-exposed (Supplementary Fig. S1A). We
therefore replaced the hydroxyl group with a piperazine moiety with the outer amino group of the piperazine moiety as the tethering site to attach an E3 ligase ligand via a linker. We designed and synthesized a series of bivalent compounds by exploring various linkers and E3 ligase ligands including VHL-1, which binds the E3 ligase VHL (35), and thalidomide/pomalidomide, which bind to the E3 ligase CRBN (36). We then assessed the effects of these compounds on reducing AKT protein levels using an antibody that detects all three isoforms of AKT (T-AKT) and on inhibiting downstream signaling. From these studies, we identified MS21, which recruits the VHL E3 ligase, as the lead compound (Fig. 1A). We also designed and synthesized MS21N1 (Supplementary Fig. S1B), which contains a diastereoisomer of VHL-1 that is incapable of binding the VHL E3 ligase (37), as a negative control for MS21. Because MS21N1 and MS21 share the same AKT binding moiety and linker, MS21N1 was expected to display similar inhibition effect towards AKT kinase activity as MS21, but be incapable of inducing AKT degradation. In addition, we also designed MS21N2, with three methyl groups attached to the parent AKT-inhibitor portion of MS21 to ablate the binding ability to AKT isoforms without impairing its binding to VHL E3 ligase based on the inspection of key interactions displayed in co-crystal structure of AKT1 in complex with AZD5363 (34). (Supplementary Fig. S1B, bottom).

We next assessed binding affinities of MS21, MS21N1 and MS21N2 to AKT1, AKT2 and AKT3 using a competitive binding assay (Fig. 1B), which quantitatively measures the ability of the test compounds to compete with an immobilized, active-site directed ligand (38). Compared to the AKT inhibitor AZD5363 ($K_d = 1.8 \pm 2.8$ nM), the AKT degrader MS21 and its negative control MS21N1 maintained high binding affinities to AKT1 albeit they displayed 2 to 5-fold weaker affinities (MS21: $K_d = 10 \pm 4.3$ nM, MS21N1: $K_d = 3.8 \pm 3.5$ nM, Fig. 1B). As for AKT2, MS21 ($K_d = 360 \pm 396$ nM) and MS21N1 ($K_d = 260 \pm 374$ nM) exhibited 16 to 22-fold weaker binding affinities than AZD5363 ($K_d = 16 \pm 18$ nM). We also found that MS21 ($K_d = 46 \pm 43$ nM) and MS21N1 ($K_d = 24 \pm 26$ nM) displayed 5 to 10-fold weaker binding affinities to AKT3 comparing with AZD5363 ($K_d = 4.8 \pm 4.6$ nM). As expected, MS21 and MS21N1 maintained high binding affinities ($K_d < 50$ nM) to AKT1 and AKT3, and moderate...
binding affinities \( (K_d < 500 \text{ nM}) \) to AKT2, while MS21N2 exhibited no significant binding to three AKT isoforms \( (K_d > 10 \text{ µM}) \) (Fig. 1B). Overall, these data have demonstrated that MS21 is an AKT targeting degrader with high binding affinities, and MS21N1 and MS21N2 are suitable control compounds for MS21 to use for cellular efficacy assessments.

We then evaluated the effects of MS21 on reducing AKT protein levels and inhibiting downstream signaling by immunoblotting in two cancer cell lines: PC-3 (a PTEN mutant prostate cancer cell line) and BT474 (a PIK3CA\(^{K111N}\) mutant and HER2 positive breast cancer cell line). We first treated PC-3 cells with MS21 at a wide range of concentrations for 24 h, and determined that the DC\(_{50}\) value (the drug concentration that results in 50% protein degradation) for MS21 in PC-3 cells was 8.8 nM (Fig. 1C). At 0.1 µM at this time point, MS21 not only diminished total T-AKT protein levels but also inhibited signaling to the AKT substrate PRAS40 (T246) and the downstream target of p70 S6 kinase S6 (S240/244) comparably to AZD5363 (Supplementary Fig. S1C). MS21 degradation of AKT protein occurred gradually over 24 h and led to a gradual reduction in downstream signaling, while the AKT inhibitor AZD5363 rapidly inhibited signaling downstream that led to feedback activation of PRAS40 and S6 phosphorylation by 24 h (Fig. 1D). We observed that MS21 reduced T-AKT protein level significantly at as early as 4 h and near completely at 8 h, and that MS21’s degradation effect lasted for at least 24 h in PC-3 cells (Fig. 1D). In addition, MS21 effectively reduced AKT protein levels and inhibited downstream signaling in BT474 cells (Supplementary Fig. S1D and S1E). MS21 concentration- and time-dependently induced AKT degradation in BT474 cells, with the maximum degradation effect apparent at 1 µM (Supplementary Fig. S1D) and 24 h (Supplementary Fig. S1E). Substantial T-AKT degradation was evident at 0.3 µM for 24 h or at 1 µM for 12 h. Similar to PC-3 cells, MS21 inhibited p-PRAS40, p-S6 and p-FOXO as effectively as AZD5363 did in BT747 cells and MDA-MB-468 cells when treated at 1 µM for 24 h, while the total protein level of PRAS40, S6, and FOXO were not altered by MS21 treatment (Fig. 1D and Supplementary Fig. S1C-S1F). Collectively, these results indicate that MS21 is an effective AKT degrader.
To confirm that the mechanism of action of the AKT degradation induced by MS21 is mediated through the VHL E3 ligase and proteasome, we performed a set of competition assays by pretreating BT474 cells with Ac-VHL-Me (a VHL ligand) (39), MLN4924 (a NEDD8-activating enzyme inhibitor that blocks cullin neddylation and inactivates cullin RING ligases (CRLs) (40), MG132 (a proteasome inhibitor) (41) and AZD5363 for 2 h, respectively (Fig. 1E). Pretreatment with Ac-VHL-Me (1 µM), which competes with MS21 binding the VHL E3 ligase, significantly blocked the AKT degradation induced by MS21, indicating that the endogenous VHL E3 ligase engagement by MS21 is required for AKT degradation. Pretreatment with MLN4924 (1 µM) also substantially rescued the AKT protein levels, indicating that the CRL complex is involved in MS21-induced AKT protein degradation. Similarly, pretreatment with MG132 (20 µM) blocked the AKT degradation induced by MS21, demonstrating that MS21’s AKT degradation effect is mediated by the proteasome. Moreover, pretreatment of AZD5363 (1 µM) also led to AKT protein recovery but retained the inhibition of the downstream signaling pathway, suggesting that AZD5363 and MS21 compete for the same binding site on AKT. Overall, these results demonstrated that MS21 induced AKT protein degradation in a VHL E3 ligase- and proteasome-dependent manner.

MS21 selectively degrades three AKT isoforms AKT1, AKT2, AKT3 and alters levels of PDCD4 and AURKB through inhibition of AKT downstream signaling

To assess selectivity of MS21, we took an unbiased and proteome-wide approach and conducted quantitative tandem mass tag mass spectrometry (TMT-MS)-based proteomics analysis. We compared the effects of MS21 treatment to control treatments (MS21N1 and DMSO) in PC-3 cells. Based on our time course study results (Fig. 1D), we chose to treat PC-3 cells with MS21, MS21N1 or DMSO at 1 µM for 8 h to achieve near complete degradation of AKT by MS21 and capture the primary and immediate consequences of MS21 versus MS21N1 and DMSO controls. We first analyzed the treatment samples using western blotting and confirmed that the MS21 treatment effectively degraded AKT and inhibited downstream signaling while the MS21N1 or DMSO treatment did not (Fig. 1F).
Analysis of the protein changes measured by mass spectrometry (MS) found that the MS21 treatment led to a significant change in protein levels of AKT1, AKT2, AKT3 while the MS21N1 or DMSO treatment did not result in a change for any proteins (Fig. 1G and Supplementary Fig. S2A). Among the approximately 5,000 proteins detected in this study, AKT1, AKT2 and AKT3 were the top hits with their protein levels reduced most by MS21 (Fig. 1G). The parent compound inhibitor AZD5363 is known to also inhibit S6K and PKA (24,34). To assess MS21 for off target inhibition of these kinases we tested the signaling effects of MS21 and AZD5363 in RT-4 cells (TSC1 null cells) that have undetectable phospho-AKT. In these cells, MS21 did not degrade S6K or PKA as shown by proteomics analysis and western blot (Supplementary Table 1 and Supplementary Fig. S2B-S2C), nor did it alter S6K or PKA downstream signalling while the parent compound kinase inhibitor AZD5363 inhibited S6 phosphorylation at a concentration of 5 µM (Supplementary Fig. S2B and S2C)(34), suggesting the AKT degrader has low off-target effects on these kinases. However, independent MS experiments of MS21 treated PC-3 cells found that the MS21 treatment led to an increase in PDCD4 and a decrease in Aurora kinase B (AURKB) protein levels (Supplementary Fig. S2D and S2E). Previous reports have shown that PDCD4 is a substrate of AKT and p70 S6 kinase, which phosphorylate PDCD4 on serine 67 to promote its degradation by βTRCP-mediated proteasome degradation, which is consistent with the increased level of PDCD4 we detected after reducing AKT levels with MS21 (42-45). Immunoblot blot analysis of PC-3 cells treated with DMSO or MS21 at 1µM for 24 hr confirmed that PDCD4 was upregulated in PC-3 cells treated with MS21 (Fig. 2A). In addition, treatment with AKT inhibitor AZD5363, PI3K inhibitor GDC-0941(46), FDA approved PI3K inhibitor BYL719 (Alpelisib) (15,47) or a previously reported AKT degrader INY-03-041 (33) also significantly upregulated PDCD4 in PC-3 cells (Fig. 2A), further suggesting that PDCD4 is a downstream target of the PI3K/AKT pathway. On the other hand, AURKB is not known to be a substrate of AKT or another kinase on the PI3K pathway. Immunoblot analysis confirmed that MS21 downregulated AURKB in PC-3 cells after AKT degradation, consistent with our proteomics analysis. Moreover, treatment with AZD5363, GDC0941, BYL719 or INY-03-041 also downregulated AURKB in PC-3 cells to various extents (Fig. 2A), with the pan class I PI3K inhibitor...
GDC0941 downregulating AURKB to nearly the same extent that MS21 did. Thus, it is likely that the changes in AURKB and PDCD4 induced by MS21 are not off-target effects, but rather downstream “on-target” effects of PI3K/AKT pathway inhibition. Taken together, our proteomic study results suggest that MS21 is a highly selective AKT degrader.

**AURKB, an AKT substrate that is depleted after AKT degradation, is responsible for the growth inhibition seen with MS21**

AURKB, a member of the Aurora Kinase family of serine-threonine kinases, is a cell cycle gene whose mRNA, protein and kinase activity peak during mitosis to function as a key regulator of mitosis (48-50) that is needed for accurate chromosomal segregation. AURKB is often overexpressed in cancer and is required for cell division in mouse embryonic fibroblasts in which Cre-mediated deletion results in massive chromosomal abnormalities, apoptosis, and failure of cytokinesis (51). Genome-wide CRISPR screens performed on hundreds of cancer cell lines indicates that deletion of AURKB is an essential gene in virtually all cells tested (52). Inhibitors of AURKB are highly toxic to proliferating cells (53). Thus, the growth inhibitory effects of MS21 we have observed in PC-3 cells could be the result of depletion of AURKB.

Measurement of cell cycle changes in PC-3 cells after MS21 treatment for 48 hr showed that MS21 lowered G1 and S phase compared to DMSO or AZD5363 but increased G2/M phase (41.23%) compared to DMSO (19.3%) or AZD5363 treatment (20.75%) (Fig. 2B and 2C), and cells accumulated >G2/M hyperploid DNA content cells (34.98%) at 48hr compared to DMSO (17.55%), which is consistent with the phenotype of AURKB ablation or inhibition (51), suggesting that the AKT degrader inhibited cell proliferation through AURKB. Next, we tested the ability of AURKB to restore colony formation for cell lines (PC-3, BT474 or BT549) treated with MS21 through overexpression of exogenous HA-tagged AURKB protein and found that cells with AURKB overexpression were resistant
to the MS21 treatment compared to control cells expressing empty vector (Fig. 2D and Supplementary Fig. S3A-S3D), demonstrating that AURKB downregulation contributes to the inhibition effect of MS21.

Next, we decided to investigate how AKT pathway could regulate AURKB. After MS21 treatment, we assessed AURKB mRNA and found that in contrast to the effect on AURKB protein, the mRNA was increased by two fold indicating that the depletion of AURKB protein that we observed was not likely through alteration of the mRNA or the cell cycle (Supplementary Fig. S3E), but rather raised the possibility that PI3K/AKT could regulate AURKB at the protein level. Using the Scansite search tool, we found that AURKB protein has a potential threonine (T73) phosphorylation site of AKT (Supplementary Fig. S3F), suggesting that AURKB could be phosphorylated directly by AKT. To this end, we first performed an in vitro kinase assay with recombinant AKT protein and AURKB protein to test if AKT could directly phosphorylate AURKB in vitro. AURKB has a known autophosphorylation site (AURKB-T232) (54) which is also an RRxT site that was detected by the phospho-AKT substrate motif (RXXS*/T*) antibody when incubated with ATP (Fig. 2E). To eliminate this AURKB-T232 autophosphorylation, we used a specific AURKB kinase inhibitor barasertib (55,56) to pretreat AURKB for 10 min in vitro before ATP incubation, which inhibited autophosphorylation of AURKB. After inhibition of AURKB autophosphorylation, we added recombinant AKT into the kinase assay, and found that AKT could induce AURKB phosphorylation and that this phosphorylation could be inhibited by AKT kinase inhibitor AZD5363 pretreatment (Fig. 2E). These data imply that AKT directly phosphorylates AURKB at Threonine 73 site.

To test if PI3K/AKT signaling affects AURKB autophosphorylation, we treated PC-3 cells and BT474 cells with insulin over a time course. However, we did not see AURKB autophosphorylation (p-T232) altered by insulin (Supplementary Fig. S3G). To exclude the possibility that T232 autophosphorylation may affect the p-AKT substrate antibody detection, we have mutated Threonine 232 site into Alanine (T232A) on HA-tagged AURKB and HA-tagged AURKB-T73A (non-phosphorylatable mutant) vector system, and performed an IP pulldown experiment in 293T cells with HA antibody after
insulin stimulation for 0, 10, 30, and 60 mins. As shown in Figure 2F, insulin stimulation upregulates HA-AURKB-T232A phosphorylation over the time course while it does not induce phosphorylation of HA-AURKB-T232A/T73A, showing that AURKB was indeed phosphorylated at T73A by insulin stimulation in vivo. We also overexpressed HA-tagged AURKB in 293T cells and PC-3 cells and found that insulin stimulated phosphorylation of AURKB was blunted by pretreatment with PI3K inhibitor GDC0941 or AKT inhibitor AZD5363 (Fig. 2G and 2H). Additionally, endogenous AURKB was also phosphorylated by insulin treatment and blocked by AZD5363 inhibitor in PC-3 cells (Fig. 2I). Moreover, the AURKB T73A mutation led to less cell viability compared to PC-3 cell overexpressing wide type AURKB or AURKB (T73E), a mutant that mimics constitutive phosphorylation (Fig. 2D, Supplementary Figure S3A and S3B), while overexpression of AURKB (T73E) mutant in PC-3 cells, BT474 cells or BT549 cells leads to increased stability of AURKB and increased cell viability upon MS21 treatment compared to control cells expressing empty vector (Fig. 2D, 2J, Supplementary Figure S3A-S3D). Furthermore, proteasome inhibitor MG132 treatment together with GDC0941 rescued the protein levels of AURKB downregulated by GDC0941 treatment, suggesting that PI3K/AKT pathway inhibition reduced AURKB protein stability by increasing its proteasome-mediated degradation (Fig. 2K). We therefore conclude that AKT phosphorylation of AURKB at threonine 73 leads to AURKB stability and MS21 treatment inhibits AKT function to destabilize AURKB to inhibit cancer cell growth.

**MS21 is superior to AZD5363 for inhibiting the proliferation of PI3K/PTEN pathway mutant tumor cell lines in vitro.**

To assess the phenotypic effect of MS21, we compared MS21 to AZD5363 for their effect on tumor cell growth in vitro. MS21 displayed much better cell proliferation inhibition activity than AZD5363 in PTEN mutant cell lines PC-3 and MDA-MB-468 as shown by the colony formation assay for 12 days (Fig. 3A and 3B). Consistently, treatment of PC-3 or MDA-MB-468 cells with AZD5363 (1 µM) or MS21 (1 µM) for 48, 72, 96 hr clearly showed that MS21 promoted sustained destabilization of AKT for 96 hr and durable inhibition of p-AKT and downstream signaling in contrast to the weaker effect
on downstream signals seen with AZD5363. MS21 also downregulated AURKB over the time course while AZD5363 was less effect at lowering AURKB (Fig. 3C). Even in a short term proliferation assay (72 h treatment), MS21 showed a potent and superior inhibitory effect compared to AZD5363 with a GI\textsubscript{50} of 0.18 µM versus >8 µM in PC-3 cells and 0.49 µM vs >8 µM in MDA-MB-468 cells, respectively (Fig. 3D), and similar results were observed in the MTS assay (CellTiter 96\textsuperscript{®} AQueous One Solution Cell Proliferation Assay, Promega, Supplementary Fig. S4A). Moreover, MS21 also showed a superior inhibitory effect in BT474 cells than a previously reported GDC0068-based and Cereblon-recruiting AKT degrader (33), INY-03-041 (Supplementary Fig. S4B). To rule out the potential VHL-independent or AKT-independent off-target effects that may be caused by MS21, we also compared MS21 to MS21N1 or VHL-1 control in PC-3 cells, MDA-MB-468 cells and BT474 cells by colony formation assay in vitro. As shown in Supplementary Fig. S4C, MS21N1 and VHL-1 treatment did not inhibit cell proliferation of any of the three cell lines. In addition, we treated PC-3 cells with MS21 and MS21N2 at 1 µM, and found that MS21N2 did not degrade AKT compared to MS21 since it lacks the binding ability to AKT (Supplementary Fig. S4D). In addition, we have conducted colony formation assay experiments with MS21 and MS21N2 in PC-3 cells, and our data shown that MS21N2 does show any effect on PC-3 cells (Supplementary Fig. S4E), suggesting tumor cells growth inhibiting effect of MS21 is VHL recruiting-dependent and AKT binding-dependent.

To investigate whether cancer cell proliferation inhibition is due to downregulation of AKT, we knocked down AKT (all three isoforms) in PC-3 cells using siRNAs and reduced the expression level of AKT by almost 9-10 times (Supplementary Fig. S4F). Indeed, AKT knockdown (KD) inhibited the proliferation of PC-3 cells (Supplementary Fig. S4G), which is consistent with previous reports (57). The percentage of cell death determined by DRAQ7 assay was also increased by MS21 treatment compared to AZD5363 or MS21N1 (Fig. 3E).

MS21 treatment reduced AKT protein and downstream signaling in PC-3 and MDA-MB-468 cells but the dose needed to inhibit AKT downstream signaling was higher in MDA-MB-468 cells than in PC-3
cells, which was consistent with its reduced potency in the cell proliferation assays (Supplementary Fig. S4H). Because we observed that MS21 diminished AKT downstream signaling (p-PRAS40 and p-S6) in PC3 cells but was less effective in MDA-MB-468 cells (Supplementary Fig. S4H, and Supplementary Fig. S5A), we decided to investigate the different potencies of MS21 in PC-3 and MDA-MB-468 cells. In MDA-MB-468 cells, MS21 reduced AKT protein levels in a concentration-dependent manner at 24 h albeit at a higher dose range than seen with PC-3 with the DC50 value of MS21 in MDA-MB-468 cells at 94 nM (Supplementary Fig. S5B). Therefore, we performed a Reverse Phase Protein Array (RPPA) analysis of PC-3 and MDA-MB-468 cells treated with DMSO, AZD5363 and MS21 at 1 µM, respectively, and alterations of AKT levels and downstream signaling were consistent with our western blot results (Supplementary Fig. S5A and S5C). Importantly, we found that MDA-MB-468 cells have less endogenous VHL protein compared to PC-3 cells (Supplementary Fig. S5D), which may explain why MS21 did not degrade AKT in MDA-MB-468 cells as effectively as in PC-3 cells. To test whether the lower level of VHL is responsible for the lower AKT degradation potency of MS21 in MDA-MB-468 cells, we overexpressed VHL in MDA-MB-468 cells. We found that increased expression of VHL increased AKT degradation (Supplementary Fig. S5E) and enhanced cell growth inhibition (Supplementary Fig. S5F) by MS21 in MDA-MB-468 cells, compared to the control cells lacking the VHL expression cassette, confirming that the VHL expression level is an important factor affecting the effectiveness of VHL-recruiting AKT degraders.

**MS21 inhibits xenograft tumor growth in vivo.**

We next assessed in vivo pharmacokinetic (PK) properties of MS21 and AZD5363 in mice. Following a single intraperitoneal (IP) injection of MS21 at a dose of 75 mg/kg, the Cmax (the maximum concentration) of 10 µM was achieved at 1 h in plasma, and high plasma concentrations (> 1 µM) were maintained for over 12 hr (Fig. 4A). Furthermore, mice treated with MS21 at this dose was well tolerated since no clinical signs were observed, demonstrating that MS21 is bioavailable in mice and suggesting that it is suitable for in vivo efficacy studies. Mouse PK properties of AZD5363 were also evaluated under
the same conditions following a single IP injection at 25 mg/kg. The $C_{\text{max}}$ of approximately 8 $\mu$M in plasma was achieved, which is comparable to that of MS21 (Fig. 4A). To assess the effect of MS21 on tumor growth in vivo, we generated a PC-3 xenograft model by injecting PC-3 cells to nude mice and administered once daily via IP injection of vehicle (n = 8), AZD5363 (25 mg/kg) (n = 9), AZD5363 (75 mg/kg) (n = 6) or MS21 (75 mg/kg) (n = 8) to test mice. Once daily 75 mg/kg IP administration of MS21 for 21 days resulted in more than 90% tumor growth inhibition relative to vehicle treatment ($P = 1.44 \times 10^{-10}$), which was significantly more effective than the approximately 50% inhibition effect by AZD5363 (25 mg/kg) ($P = 1.05 \times 10^{-8}$) treatment or the 75% inhibition effect by AZD5363 (75 mg/kg) ($P = 0.0082$) (Fig. 4B). Consistently, MS21 (75 mg/kg) treatment inhibited more than 80% tumor growth of MDA-MB-468 xenograft model compared to vehicle treatment, and was significantly ($P = 3.53 \times 10^{-8}$) more effective than 50% inhibition effect by AZD5363 (25 mg/kg) (Supplementary Fig. S6A). Western blot analysis of the tumor samples isolated from different treatment groups at the end of the 21-day study showed that the T-AKT protein levels in the tumors from MS21 treated mice was reduced compared to that from vehicle or AZD5363 treated mice (Fig. 4C). This result is consistent with IHC staining of T-AKT expression (MS21 versus vehicle: $P < 0.0001$, MS21 versus AZD5363: $P = 0.002$) (Fig. 4D and 4E). In addition, the MS21 treatment strongly reduced p-AKT compared with the vehicle or AZD5363 treatment (Fig. 4C), and inhibited the downstream signaling (such as p-S6) as effectively as the AZD5363 treatment (Fig. 4C-4E). Furthermore, Ki67 staining was substantially decreased ($P < 0.0001$) in MS21 treated tumors compared to the vehicle or AZD5363 treatment, suggesting that MS21 inhibits PC-3 cell proliferation in vivo (Fig. 4D and 4E). Moreover, the apoptosis marker cleaved caspase-3 was somewhat upregulated in the MS21 treatment group compared with the vehicle or AZD5363 treatment group (Supplementary Fig. S6B and S6C). We also determined the drug levels in the plasma and tumor samples isolated at 2 hr post the last dose from the mice treated with MS21 at the 75 mg/kg (once daily IP injection) for 5 days. The concentration of approximately 5 $\mu$M in plasma was achieved in this study (Fig. 4F), which is consistent with the results from our mouse PK study (Fig. 4A). Although the concentration of MS21 in tumor samples was lower than that in plasma samples, the concentration of approximately
500 nM was maintained in tumors, which is significantly higher than the DC_{50} and GI_{50} values of MS21 in PC-3 cells and sufficient to induce AKT degradation and inhibit tumor cell proliferation in vivo. Taken together, these results have established a consistent in vivo PK/PD relationship for MS21. Furthermore, MS21 was well tolerated at the efficacious dose (75 mg/kg once daily IP) by the test mice, which did not exhibit any appreciable weight loss ($P = 0.126$) or other overt toxicities (Supplementary Fig. S6D). Mice treated with MS21 did not gain body weight, which could be due to inhibition of tumor growth through AKT degradation, while the vehicle treated mice did (Supplementary Fig. S6D). Collectively, our results indicate that MS21 is efficacious in vivo with minimal toxicity at the efficacious dose and an excellent chemical tool for further in vivo studies.

We also treated nonfasted wild-type mice with both AZD5363 (25 mg/kg) and MS21 (75 mg/kg), and monitored their blood glucose levels over time after acute treatment. As expected, both AZD5363 and MS21 caused substantial increases in blood glucose levels within 2 hr and this hyperglycemia resolved gradually after 3-8 hr without additional intervention, suggesting that PI3K/AKT signaling had been reactivated in muscle and liver despite the presence of either drug (Supplementary Fig. S6E). Notably, AZD5363 treatment increased glucose level more than MS21 treatment did and also with more rapid kinetics, perhaps reflecting that small molecule inhibitor AZD5363 has a faster distribution than MS21 in vivo. We have no expectation of any new “off-target” toxicities with MS21 as we have not seen any evidence for them in our cell and mouse studies.

Cancer cell lines with PI3K/PTEN pathway mutations tend to be highly sensitive to MS21 unless they also contain RAS pathway mutations.

To screen cancer cell lines for their sensitivity to MS21 and any potential relationship to driver mutations on the PI3K/PTEN pathway, we compared the cellular proliferation inhibition effects of MS21 and AZD5363 at various concentrations ranging from 0.1 to 10 µM using colony formation assays in a panel of 38 cancer cell lines without known mutations in VHL (Fig. 5A and Supplementary Table 2),
derived from human breast cancer, pancreatic cancer, prostate cancer, melanoma, glioblastoma and ovarian cancer. The assays were performed over 10-14 days depending on the cell line (Fig. 3A and 3B, Supplementary Fig. S7A-D). These cell lines have a wide range of driver mutations including alterations of the PI3K/PTEN and RAS pathways. Based on their sensitivity to MS21, these 38 cancer cell lines can be categorized into two groups (Fig. 5A). Cell lines in group 1 are sensitive to MS21 (Fig. 3A, 3B, 5A and 5B, Supplementary Fig. S7A-B). Within this group, there are two subgroups. The first subgroup consists of eight cell lines that were measurably more sensitive to MS21 than AZ5363, such as PC-3, MDA-MB-468, HCC1143, BT549, BxPC3, HCC1395, BT474 and HCC1937 cells (Fig. 3A, 3B and 5B, Supplementary Fig. S7A). As illustrated in Fig. 5A (see sensitive lines labeled with an asterisk) and shown in Fig. 5B, MS21 inhibited growth of the 8 cell lines in the first subgroup by 90-100% at 1 µM while AZD5363 inhibited this subgroup’s growth only by 40-50%. The second subgroup contains eleven cell lines that are highly sensitive to both MS21 and AZD5363. They include SKBR3, MCF-7, ZR75-1, SKOV3, IGROV-1, SUM149, MDA-MB-453, HCC70, LNCAP, T47D and HCC1954 cells (Fig. 5B, Supplementary Fig. S7B). MS21 and AZD5363 inhibited growth of these cell lines by 90-100% at 1 µM. In some cases from this subgroup, MS21 was slightly more effective than AZD5363. Cell lines in group 2 are resistant to MS21 and also have two subgroups of resistant growth phenotypes. The first resistant subgroup contains sixteen cell lines that are resistant to both MS21 and AZD5363 (Fig 5A and 5B). They include DBTRG, Colo205, SW620, ASPC1, Panc10.05, SW1990, MiaPaca2, OVCAR-3, HCT116, SW480, Panc-1, MDA-MB-231, SKMEL239, A375, U87MG and MDA-MB-436 cells (Fig. 5B, Supplementary Fig. S7C). MS21 and AZD5363 inhibited growth of these cells by only 20-40% from 1 to 10 µM (Fig. 5B). Cell lines in the second resistant subgroup are less resistant to MS21 and AZD5363 than the first resistant subgroup. They include HT29, HPAF11 and CFPAC-1 cells (Fig. 5B, Supplementary Fig. S7D). MS21 inhibited growth of these cells by around 50% at 1 to 10 µM (Fig. 5B) and was slightly more effective than AZD5363 in HT29 and CFPAC-1 cells (Supplementary Fig. S7D).
We next analyzed the gene mutation status of these cell lines for the PI3K/PTEN pathway (defined as genetic alterations of HER2, PIK3CA, PTEN or AKT1) and RAS pathway (defined as genetic alterations of KRAS and BRAF) summarized in Fig. 5A. Remarkably, almost all (17 out of 19) of the cell lines that were resistant to MS21 in Group 2 (including melanoma, glioblastoma, colon cancer, breast cancer cells) were KRAS mutant or BRAF mutant even if they contained a PI3K/PTEN pathway alteration, while only one RAS pathway mutant line (BRAF mutant Bx-PC-3) was among the 19 sensitive cell lines. By comparing resistance detected in seventeen of eighteen KRAS or BRAF mutant lines versus only two of twenty RAS or BRAF wild type lines, resistance to MS21 showed a strong association with RAS pathway mutation that was highly significant (p=9.73×10^{-8}, OR=113.45) (Fig. 5A-5C). A previous study with the AKT kinase inhibitor AZD5363 has also seen a significant correlation between the presence of a RAS pathway mutation and resistance to AZD5363, which we detected as well (p=3.91×10^{-4}) (Supplementary Fig. S8A) (24).

Cell lines that were sensitive to MS21 showed a very strong tendency to be mutated for PI3K/PTEN pathway genes. Of the nineteen sensitive cell lines, eighteen had a mutation of the PI3K/PTEN pathway, while among the nineteen resistant lines seven had a PI3K/PTEN pathway genetic alteration with six of these having a co-occurring RAS pathway mutation. By comparing sensitivity detected in eighteen of twenty-five PI3K/PTEN pathway mutant lines versus only one of thirteen PI3K/PTEN pathway wild type lines, sensitivity to MS21 showed a strong association with PI3K/PTEN pathway mutation that was highly significant (p = 1.81×10^{-4}, OR = 27.76, Fisher's Exact test) (Fig. 5A-5C). Response to AZD5363 was observed in ten of twenty-five cell lines mutated in the PI3K/PTEN pathway and in none of the wild type lines, which also showed a significant association of PI3K/PTEN pathway alteration to AZD5363 sensitivity as expected (p = 6.91×10^{-3}, Fisher's Exact test) (Supplementary Fig. S8A) (24). Thus, with the eight additional PI3K mutant treatment sensitive cell lines that occurred with MS21 versus AZD5363, we observed a significant association of MS21 to improved sensitivity relative to AZD5363 (p = 2.25×10^{-2}, OR=3.74) (Supplementary Fig. S8B).
Remarkably, of the nineteen cell lines in the panel mutated within the PI3K/PTEN pathway without a co-occurring RAS pathway mutation, eighteen were sensitive to MS21, while of the nineteen cell lines that were either wild type for the PI3K/PTEN pathway or had a mutation with a co-occurring RAS pathway mutation, only one was sensitive to the small molecule. These data demonstrate a striking correlation of sensitivity to MS21 treatment with cell lines that are mutated on the PI3K/PTEN pathway without co-occurring mutation of KRAS or BRAF ($p = 1.03 \times 10^{-8}$, OR = 199.65, Fisher's Exact test) (Fig. 5A-5C). A similar trend was also observed for AZD5363 but not to the same extent ($p = 1.95 \times 10^{-4}$, Fisher's Exact test). Because MS21 was able to inhibit eight more of the cell lines in this subset than AZD5363 was able to inhibit, MS21 was significantly better than AZD 5363 at inhibiting the growth of tumor cell lines in the subset with PI3K/PTEN pathway but not RAS pathway mutations ($p = 3.9 \times 10^{-3}$, OR = 15.06) (Supplementary Fig. S8B).

**MS21 is unable to degrade AKT effectively in most KRAS or BRAF mutant cell lines**

One potential explanation for the resistance of KRAS or BRAF mutant cell lines to MS21 is that MS21 does not degrade AKT effectively in these cell lines. In contrast to the near complete degradation of AKT induced by MS21 in RAS pathway wild type PC-3 and SKOV-3 cells, MS21 induced incomplete degradation of AKT isoforms in KRAS mutant cell lines (MDA-MB-231, HCT116, SW620, MiaPaca2) or BRAF V600E mutant cell lines (HT29, DBTRG, A375, Colo-205) (Fig. 5D). We hypothesized that MS21 might not bind AKT in these resistant cell lines as effectively as in sensitive cell lines. To test this hypothesis, we determined cellular target engagement effects of the AKT binding moiety of MS21, AZD5363, in PC-3 cells (PTEN null, sensitive to MS21) and MiaPaca2 cells (KRAS G12C mutant, resistant to MS21), which was the cell line that was most resistant to degradation of AKT, using a Cellular Thermal Shift Assay (CESTA) assay, an assay for evaluating drug and target interactions in cells (58). We found that the AKT ligand AZD5363 of MS21 significantly enhanced AKT stability in PC-3 cells but not in MiaPaca-2 cells (Fig. 5E), suggesting that AZD5363 can engage AKT more effectively in PC-3 cells than MiaPaca-2 cells.
Furthermore, AURKB was also significantly downregulated in those MS21 sensitive cells (BT474, MDA-MB-468, PC-3, HCC1937, BT549) but not in MS21 resistant cells (SW1990, HT29, Panc-1, A375, Colo205) (Supplementary Fig. S8C). PDCD4 was also significantly upregulated in MS21 sensitive cells but not in MS21 resistant cells, showing that MS21 is unable to degrade AKT effectively in most KRAS or BRAF mutant cell lines and that degrading AKT regulates AURKB and PDCD4 levels in a wide variety of sensitive cell lines. Similarly, GDC0941 treatment also downregulated AURKB and upregulated PDCD4 in MS21 sensitive cells but did so modestly or not at all in MS21 resistant cells (Supplementary Fig. S8D), suggesting that sensitive cell lines strongly regulate AURKB and PDCD4 levels through the PI3K pathway whereas resistant lines do not.

**AKT phosphorylation regulates AKT degradation**

We next explored the possibility that AKT phosphorylation could account for the resistance to MS21. AKT kinase function is activated following phosphorylation of two residues: the threonine residue (T308) in the activation loop of the AKT kinase domain by phosphoinositide-dependent kinase 1 (PDK1) and the serine residue (S473) within the hydrophobic motif of the AKT regulatory domain that can be phosphorylated by a number of kinases, most prominently mTOR complex 2 (mTORC2) (4). Phosphorylation at both sites is responsible for a major conformational change of the protein that allows for ATP to bind the ATP pocket and for the substrate-binding region to engage a substrate (59,60). Since ATP-competitive inhibitors selectively and preferentially bind to the phosphorylated form of AKT (61) and the MS21 ligand for AKT AZD5363 can preferentially engage AKT in PTEN mutant PC-3 cells relative to KRAS mutant MiaPaca-2 cells, we decided to examine the AKT phosphorylation level in a small panel of MS21 resistant and sensitive cells. Compared to PC3 and MDA-MB-468 cells, all KRAS mutant cells including MiaPaca-2 and BRAF mutant cells had a lower level of AKT phosphorylation at both pS473 and pT308 sites (Fig. 6A). A phospho-protein profiling of 31 of the cell lines from CCLE also indicated that the AKT phosphorylation level (AKT_pS473 and AKT_pT308) in the resistant group was generally lower than the sensitive group with some outliers ($P = 9.7e-03$ and $P = 6.6e-03$, respectively)
(Fig. 6B and Supplemental Fig. S9A). No significant difference was detected for receptor tyrosine kinase (RTK) expression levels between the sensitive and resistant groups, including HER2, EGFR, Insulin receptor and IGF-1R (Fig. 6A and supplementary Fig. S9A). We therefore hypothesized that lower AKT phosphorylation signaling might be responsible for the reduced target engagement of MS21 and decreased AKT degradation activity. To this end, we treated resistant cell lines MiaPaca2 (KRAS G12C) and A375 (BRAF V600E) with IGF-1 to upregulate AKT phosphorylation, and found that IGF-1 indeed enhanced the degradation effect of MS21 (Fig. 6C). In addition, insulin treatment in MiaPaca2 (KRAS G12C) and another resistant line SW620 (KRAS G12V) also led to activated AKT phosphorylation and enhanced AKT degradation by MS21 (Supplementary Fig. S9B). The findings that IGF-1 or insulin are able to enhance MS21 degradation of AKT in the resistant KRAS or BRAF mutant cell lines, suggest that the low level of AKT degradation is due to the relatively low level of AKT phosphorylation. However, induction of phosphorylation of AKT and subsequent degradation by IGF-1 treatment was not sufficient to cause sensitivity in RAS pathway mutant MiaPaca2 cells (Supplementary Fig. S9C).

For the MS21-resistant cell lines with high levels of AKT phosphorylation like U87MG (PTEN mutant glioblastoma (1)) and MDA-MB-436 cells (PTEN mutant (62) and BRAF mutant triple negative breast cancer(63)), MS21 degraded AKT as effectively as in PC-3 cells (Fig. 5B, Supplementary S7C and S9D). The ability of MS21 to degrade AKT in resistant PTEN and RAS/BRAF mutant cell lines that have high baseline phospho-AKT is consistent with the ability of IGF-1 or insulin to stimulate AKT degradation in cell lines that have a low baseline of phospho-AKT (Fig. 6C, Supplementary Fig. S9A and S9B). These findings indicate that AKT phosphorylation is required for its degradation but that AKT degradation is not alone sufficient to cause growth inhibition in resistant lines.

**MEK inhibition increases AKT phosphorylation and improves AKT degradation by MS21.**

It has been reported that MEK inhibition induces expression and activation of several RTKs, which further trigger the downstream activation of AKT in cancer cell lines (64-66). We therefore hypothesized
that MEK inhibition, which signals downstream of KRAS and BRAF, might enhance MS-21 dependent degradation of AKT by activating AKT phosphorylation. To this end, we tested the ability of MEK inhibitor trametinib to increase AKT phosphorylation in MS21 resistant KRAS and BRAF mutant cell lines A375 (BRAF V600E mutant), MiaPaca-2 (KRAS G12C mutant), and SK-MEL-239 cells (BRAF V600E mutant). Indeed, p-AKT was increased over the time course of trametinib treatment in all of these cell lines (Supplementary Fig. S10A). Additionally, trametinib treatment more robustly increased p-AKT levels in resistant cancer cells with mutations of both the RAS and PI3K/PTEN pathways that demonstrated resistance to MS21, including HT29 (PIK3CA P449T, BRAF V600E, T119S), HPAF11 cells (KRAS G12D, PIK3CA amplified, AKT1 amplified pancreatic cancer cells), MDA-MB-436 cells (PTEN loss and BRAF mutant triple negative breast cancer cells), and HCT116 cells (PIK3CA H1047R and KRAS G13D colon cancer cells) (Fig. 6D). Interestingly, the increase of AKT phosphorylation started after 24 hr of treatment and reached the maximum level at 48 hr or 72 hr in almost all of the cell lines tested, suggesting trametinib treatment leads to a slow reprogramming and feedback activation of AKT phosphorylation which has been previously described with MEK inhibitors (64,65,67).

We then combined MS21 and trametinib together in MS21-resistant cell lines and found that MEK inhibition by trametinib promoted MS21 degradation of AKT in all of the cell lines that were tested regardless of the RAS or PI3K/PTEN pathway genotypes (HT-29, MDA-MB436, A375, MiaPaca-2 and SK-MEL-239 cells) (Fig. 6E, Supplementary Fig. S10B). In addition, we found that MS21 and trametinib combination treatment also led to modestly enhanced AKT degradation and inhibition of downstream signaling as measured by p-PRAS40 and total AURKB protein (Fig. 6E and Supplementary Fig. S10C) in two cell lines with both RAS and PI3K/PTEN pathway mutations-DBTRG (BRAF and PTEN mutant glioblastoma cells) and HT29 (PIK3CA P449T, BRAF V600E, T119S) cell lines. These findings suggest that increasing AKT phosphorylation with trametinib leads to improved AKT degradation in the presence of MS21 in a wide variety of cancer cell lines; however, the level of trametinib induced AKT phosphorylation varied as did the amount of AKT degradation and inhibition of downstream signaling.
including AURKB, suggesting that the combination only modestly improved the reduction of PI3K signaling.

**Inhibition of MEK can overcome resistance to MS21**

Because most cell lines that were resistant to MS21 had a mutation in KRAS or BRAF and it is well established that tumor cells with these mutations maintain dependence on signaling through MEK and ERK, we tested the possibility that the enhancing AKT degradation and inhibiting ERK signaling with trametinib could have a growth inhibiting effect on cells and overcome resistance to MS21 as a single agent. Therefore, we examined the ability of trametinib, MS21, or the combination to inhibit the growth of six tumor cell lines that demonstrated resistance to MS21 and had no co-occurring mutation on the PI3K/PTEN pathway (Fig. 5A). For these growth experiments, cells were treated for 10 days and then allowed to recover for a subsequent 10 days without any inhibitor in order to discriminate between the ability of the drugs to block colony formation altogether versus to slow colony growth. Interestingly, two of the pancreatic cancer cell lines with single mutations (KRAS G12D), SW1990 and PANC10.05, were very sensitive to the combination treatment of MS21 and trametinib (Supplementary Fig. S11A and S11B), suggesting combination treatment could improve the growth inhibiting effect of single agent trametinib in some type of cancer cells that carry KRAS mutations. On the other hand, the combination treatment did not have an enhanced inhibition effect in cell lines with **BRAF** V600E mutations (A375, SK-MEL-239) or with **KRAS** G12C MiaPaca-2 or **KRAS** G12D PANC-1 cell lines (Supplementary Fig. S11A and S11B), suggesting these cell lines do not depend on PI3K/AKT pathway for growth or that the extent of AKT degradation was insufficient, which could clearly be the case for MiaPaca-2 (Supplementary Fig. S10B). Overall these findings show that half of **KRAS** mutant cell lines tested were sensitive to the combination treatment but that none of the **BRAF** mutant cells were.

We next speculated that the combination of an AKT degrader and MEK inhibitor could lead to more effective cancer cell line growth inhibition in MS21-resistant cancer cells with mutations on both
the PI3K and RAS pathways (Fig. 5A). We reasoned that this could be the case because both pathways acted as drivers of oncogenesis and/or tumor progression during tumor evolution. To test this hypothesis, we combined the MEK inhibitor trametinib with MS21 in tissue culture wells side by side with either drug alone or vehicle in cell lines, including HT29 cells (PIK3CA P449T, BRAF V600E, T119S colon cancer cell line), HPAF11 cells (PIK3CA amplified, AKT1 amplified, KRAS G12D pancreatic cancer cells), HCT116 cells (PIK3CA H1047R and KRAS G13D colon cancer cells), MDA-MB-436 cells (PTEN null, BRAF G30_A33del triple negative breast cancer cells) and DBTRG (PTEN loss, BRAF V600E mutant glioblastoma). Compared to the incomplete cell growth inhibition of single drug treatment, growth of HT29 and HPAF11 cells was completely inhibited by the combination treatment (5 nM trametinib and 1µM MS21) (Figure 7A and 7B). Growth in MDA-MB-436 cells and HCT116 cells was also completely inhibited by the MS21 and trametinib combination treatment albeit at a slightly higher concentration of trametinib (20 nM) and MS21 (3 µM) (Figure 7A and 7B), suggesting that different cell lines may require different thresholds of the ERK pathway and AKT pathway for growth. Therefore, combining MS21 and trametinib was effective in four out of five cell lines carrying mutations in both the PI3K and KRAS pathways. To rule out other possible mechanisms of resistance, we examined the relationship between MYC mutation, which is known to cause resistance to PI3K inhibition (14), and resistance to MS21, but did not see any evidence of a correlation in our panel of cell lines of which 16 have documented MYC amplification or point mutation (Supplementary Fig. S11C and Supplementary Table 2). Collectively, these data suggest that this group of cell lines largely remain addicted to both AKT and MEK signaling and that the combination of an AKT degrader and MEK inhibitor could provide a novel therapeutic approach for cancers with dual mutations activating the RAS and PI3K/PTEN pathways if the toxicity of the combination were tolerable, although investigation of the combination of MEK and AKT inhibitors in the clinic has seen substantial toxicity with limited anti-tumor efficacy (68,69).

**Determination of the mutation frequency of PI3K/PTEN pathway without concomitant mutation of the RAS pathway in human cancer.**
We next wanted to determine the frequency of PI3K/PTEN pathway mutations in human cancer that occur without mutations in the RAS pathway to estimate the patient population that could benefit from therapy with an AKT degrader. To determine the frequency of cancers that have mutation of the RAS and PI3K/PTEN pathways, we analyzed a publicly available cancer patient cohort (n = 44,347 patients / 46,697 samples in 176 studies) across different histologies from the cBioPortal for Cancer Genomics (70,71) and identified a total 15,279 (34%) of queried patients that have somatic genetic variant in at least one gene from PI3K/PTEN pathway (PTEN, PIK3CA, AKT1, ERBB2, PIK3R1) or RAS pathway (HRAS, KRAS, NRAS, BRAF, NF1, and SOS1). We defined tumors as double mutant tumors when tumors have somatic genetic variants in at least one gene from PI3K/PTEN pathway as well as in at least one gene from RAS pathway. In this cohort, 8,233 (18.6%) patients have only PI3K/PTEN pathway mutated in their tumors, 5,259 (11.9%) with only RAS pathway mutated, and 1,787 (4.0%) with both pathways mutated (Fig. 7C). Among the patients with mutations only in PI3K/PTEN pathway, PIK3CA (41.3%), PTEN (27.5%) and ERBB2 (13.7%) are the three most frequently mutated genes in the PI3K/PTEN pathway (Supplementary Fig. S12A). Based on these results, there are many patients with tumors carrying mutations on just the PI3K/PTEN pathway that could benefit from treatment using an AKT degrader such as MS21.

Discussion:

Pursuit of a small molecule agent that effectively treats cancers driven by activation of the PI3K-PTEN-AKT-mTOR signalling axis has been ongoing for over 20 years. The ATP-competitive AKT kinase inhibitors are a promising class of agents that inhibit the activated form of the kinase, which may explain their improved selectivity for tumors with lesions on that pathway and their tolerability in patients relative to prior agents. Here, we show that the AKT degrader MS21 degrades AKT when it is
phosphorylated by the PI3K signalling pathway kinases PDK1 and TORC2 and that this form of degradation preferentially inhibits the growth of tumor cell lines that have alterations on the PI3K/PTEN pathway in large part due to the ability of MS21 to lower the level of the AKT substrate AURKB in cells.

Study of three initial PI3K/PTEN pathway mutant cell lines (PC3, MDA-MB-468, and BT474) showed that MS21 degraded all three isoforms of AKT and inhibited downstream signalling and growth in tissue culture (Fig. 1-3). In all three cases, the cell lines were more responsive to MS21 than they were to the AKT inhibitor AZD5363 in terms of growth inhibition and the induction of cell death, and the level of expression of VHL affected the potency of MS21 in terms of AKT degradation and cell growth inhibition. The ability of MS21 to inhibit cell growth over the course of two weeks in our colony formation assays was consistent with the signaling kinetics of MS21 treatment from 48 to 96 hr, which showed that the AKT degrader had more potent and durable pharmacological effects on downstream signaling than the AKT inhibitor. Similar results were also seen with another AKT degrader INY-03-041 based on GDC0068 (33). Xenografts of PC-3 and MDA-MB-468 in nude mice showed that MS21 effectively inhibited tumor growth relative to vehicle and AZD5363 controls by inhibiting proliferation without increasing blood glucose as much as AZD5363 did (Fig. 4). Using an unbiased mass spectrometry approach to detect protein changes in PC-3 cells treated with MS21, we determined that in addition to degrading AKT, the known AKT substrate PDCD4 was elevated and the protein Aurora kinase B was reduced. Other PI3K and AKT targeting agents also altered the expression of these two proteins in a similar manner. We also found that AURKB was a substrate for AKT and was phosphorylated in cells in a PI3K and AKT dependent manner. Wild type and a phospho-mimicking mutation of AURKB were able to rescue PC-3 cells from cell growth inhibition by MS21, suggesting that much of the therapeutic benefit of MS21 was through lowering AURKB, which is an essential gene for cell proliferation (Fig. 2). Cell cycle analysis confirmed that MS21 treatment induces G2/M arrest and hyperploidy.
Examination of MS21’s ability to inhibit cell growth in thirty-eight different cell lines determined that it inhibited the growth of seventeen of eighteen PI3K/PTEN pathway mutant cell lines that did not have RAS pathway mutations. Importantly, MS21 showed substantial improvement over AZD5363 for inhibiting tumor cell lines with PI3K/PTEN pathway mutations, and this improved potency is likely the case for other AKT competitive inhibitors based on comparison of our results to those of others in overlapping cell lines and could reflect the different kinetics of pathway inhibition and enhanced reduction of AURKB achieved with the degrader relative to the inhibitor (24-26). On the other hand, cell lines in the group with RAS pathway mutations were nearly all resistant to MS21 (seventeen of eighteen) even in the setting of a co-occurring PI3K/PTEN pathway mutation. MS21 degraded AKT well in sensitive cell lines but did not degrade AKT or degraded AKT poorly in many of the cell lines that were resistant (Fig. 5). Examination of the mechanisms of resistance revealed that many of the resistant cell lines had low levels of AKT phosphorylation and that this was associated with RAS mutation without co-occurring PI3K/PTEN pathway mutation (Fig. 6; Supplementary Fig. S8-9). Stimulation of AKT phosphorylation in these cell lines enhanced AKT degradation but was not sufficient to inhibit cell growth or dampen signalling downstream of AKT, which showed that lack of degradation was not the only cause of resistance. Moreover, we identified cell lines with mutations on the RAS and PI3K/PTEN pathways where AKT was degraded effectively that still displayed resistance. We therefore tested the MEK inhibitor trametinib to see if it could overcome resistance to MS21 not only because of its selective ability to inhibit MEK signalling to ERK in these lines but also because of its ability to induce AKT phosphorylation and thereby AKT degradation. We saw that trametinib did in fact stimulate AKT phosphorylation and degradation in multiple cell lines tested with RAS pathway mutations; however, the impact on downstream AKT signalling was modest. Moreover, we saw that resistance appeared to be a result of the MAPK pathway since we saw that a total of four of the five cell lines with RAS and PI3K/PTEN pathway mutations were sensitive to the combination of the AKT degrader and MEK inhibitor (Fig. 7). Interestingly, MS21 could not enhance the ability of trametinib to inhibit the growth of two cell lines with only BRAF mutations. On the other hand, the combination enhanced inhibition of
growth over either drug alone in two of four KRAS mutant cell lines, which is consistent with cell
dependency on KRAS activation of RAF and PI3K effector signals, which is well established (72).
Moreover, resistance in the remaining two KRAS mutant cell lines could be due to a failure to sufficiently
degrade AKT, which was the case in one of them, MiaPaca-2 (Fig. S10B). The two glioblastoma cell
lines (U87MG (PTEN mutant); DBTRG (PTEN and BRAF mutant)) in this study were the only ones with
PI3K/PTEN pathway mutations with resistance to MS21 or the combination of MS21 and trametinib,
suggesting that something about their lineage and/or signals in addition to AKT and MAPK are driving
tumor cell survival and growth. While targeting the MAPK and AKT pathways simultaneously is a
theoretically attractive therapeutic combination based on the ability of trametinib to inhibit MAPK
signalling and induce measurable AKT degradation, attempts to inhibit both pathways in vivo with kinase
inhibitors have been toxic and were therefore not explored in this study. Future examination of a larger
panel of cancer cell lines is likely to reveal other mechanisms of resistance to the AKT degrader.

Our findings suggest that tumors with PI3K/PTEN pathway mutations without RAS pathway
mutations, which are quite common, may be predictably sensitive to treatment with MS21 (Fig. 7). Our
analysis of human tumors that have been genotyped identified that approximately 19% of human cancers
have a mutation on the PI3K/PTEN pathway without an overlapping mutation of the RAS pathway.
Therefore, we suspect that MS21 or another AKT degrader could be useful for the treatment of nearly one
fifth of all cancer patients. Of course MS21 requires the presence of intact VHL to be effective and hence
would not be effective in VHL mutant cancers, which is very often the case in renal cell carcinoma.

Our data has shown that the AKT degrader that we have developed has reduced off target effects
compared to the parent compound. We are seeing very specific efficacy in cell culture experiments in
tumor lines that have PI3K pathway mutations in the absence of RAS pathway mutations. This is
associated with the finding that the AKT degrader does not degrade S6K or PKA while the parent
compound kinase inhibitor inhibits S6K and PKA (24). In addition, we unexpectedly found a novel AKT
kinase substrate, AURKB, in the course of profiling of proteins altered by MS21. Our data show that
AKT phosphorylation leads to AURKB stabilization and that PI3K or AKT inhibition destabilizes AURKB to inhibit cancer cell growth. The mechanism of AURKB stability regulated by AKT and the E3 ligase which is responsible for degradation need to be further investigated.

In summary, we provide compelling data supporting MS21 as a potent, selective, and highly efficient AKT degrader that utilizes VHL to recruit ubiquitin ligases to ubiquinate AKT for digestion by the proteasome. Degradation of AKT led to effective inhibition of downstream signalling and the identification of a new AKT substrate phosphoprotein AURKB that is required for cell viability. Mutation of the threonine 73 phosphorylation site to glutamate to mimic constitutive phosphorylation completely inhibited the growth inhibition effects of MS21. These data suggest that AURKB depletion in cells and the consequential effect on the cell cycle is the likely reason for the selective manner in which PI3K/PTEN pathway cancers respond to the AKT degrader MS21. Our study lays a solid foundation for the clinical development of an AKT degrader for the treatment of human cancers with PIK3CA, PTEN, HER2 and AKT1 alterations based on the idea that MS21 can selectively degrade phosphorylated but not unphosphorylated AKT.

Materials and Methods:

Compounds and antibodies

GDC-0068 (Catalog #S2808) and Barasertib (AZD1152-HQPA) (Ca# S1147) were purchased from Selleckchem Company, and AZD-5363(Catalog #AK316078) was purchased from Ark Pharm, Inc. The rest of the inhibitors were provided by the Stand Up To Cancer (SU2C) PI3K Dream Team. Antibodies used include phospho-AKT(Thr308) (Cat# 4056), phospho-AKT(Ser473) (Cat# 9271), AKT (Cat#9272, recognizes all three isoforms of Akt), AKT1 (2H10) (Cat# 2967), AKT2 (Cat# 3063), AKT3 (Cat# 8018), phospho-PRAS40(Thr246) (Cat# 2997), PRAS40 (Cat# 2691), phospho-S6(Ser240/244) (Cat# 5364), S6 (Cat# 2217), phospho-GSK-3α/β (Ser21/9) (Cat# 8566), p-Erk (T202/Y204) (Cat# 4370), EGFR (Cat#
4267), HER2 (Cat# 2165), IGF-1Rβ (Cat# 9750), VHL (Cat# 68547), AURKB (Cat# 14475), Aurora A (Cat# 91590), PKA C-α (Cat# 4782), phosphor-VASP(Ser157) (Cat# 3111), VASP (Cat# 3112), p70S6K (Cat# 9202), phospho-GSK-3α/β (Ser21/9) (Cat# 8566), phospho-Akt Substrate (RXXS*/T*) (Cat# 9614), HA-Tag (C29F4) (Cat# 3724) and PTEN (XP) (Cat# 9188) from Cell Signalling Technology, HER3 (#05-390) from Millipore company, IRβ (611276) from BD Transduction Laboratories, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat# 2-RGM2) from Advanced ImmunoChemical and β-actin (Cat# A5316) from Sigma. For IHC antibodies, Cleaved-Caspase 3 (Cat# 9664) was from Cell signalling technology and Ki-67 (Cat# VP-RM04) was from Vector Laboratories. For IP antibodies, Anti-HA rat monoclonal antibody (11867423001) was from Roche/Sigma. Secondary Antibodies: Mouse (Thermo 31432), Rabbit (Thermo 31460).

Cell Culture and Treatment

All cell lines were purchased from ATCC and authenticated. Cell lines were regularly tested in the lab for mycoplasma. All cells were cultured at 37°C and 5% CO₂. BT549, HCT116, PANC1, MiaPaca2 and SK-MEL-239 were cultured in 1X RPMI 1640 medium (Corning, 10-040-CV) with 10% fetal bovine serum (Atlanta Biologicals S11150) and 1X Penicillin/Streptomycin. RT4 cells were cultured in McCoy's 5a Medium Modified with 10% fetal bovine serum and 1X Penicillin/Streptomycin. All the other cell lines were cultured in 1X DMEM (Corning, 10-013-CV) with 10% fetal bovine serum (Atlanta Biologicals S11150) and 1X Penicillin/Streptomycin. Cells were split using 0.05% or 0.25% trypsin (Corning 25-051-Cl or 25-053-Cl, respectively) before they reached full confluence and media was changed every 3-4 days.

Immunoblotting

Cells were lysed in 2X sample buffer (125 mM Tris-HCl at pH 6.8, 10% βME, 2% SDS, 20% glycerol, 0.05% Bromophenol Blue, 8 M urea). Protein lysates were loaded into 4-12% Bis-Tris gels and resolved by electrophoresis. Samples were then blotted on PVDF membrane (Millipore IPVH00010) using the wet transfer technique (Invitrogen). Membranes were blocked in 5% milk-TBST for 1 hour, washed in TBST
for 10 min, and incubated in primary antibody in 5% milk-TBST or 5% BSA-TBST at 4 °C for 16 h. Membranes were rinsed (3 x 6 min) in TBST and incubated in horseradish peroxidase-conjugated secondary antibodies in 5% milk-TBST for 1 hour and rinsed again in TBST (3 x 6 min). Membranes were visualized using the chemiluminescence system (Thermo 34080, 37075) on autoradiography film (Denville E3018).

**AKT binding affinity assays**

AKT binding affinities were determined with KINOMEscan assay by DiscoverX Company. KINOMEscan™ is based on a competition-binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active-site directed ligand. The assay is performed by combining three components: DNA-tagged AKT; immobilized ligand; and a test compound. The ability of the test compound to compete with the immobilized ligand is measured via quantitative PCR of the DNA tag. For most assays, kinase-tagged T7 phage strains were prepared in an E. coli host derived from the BL21 strain. E. coli were grown to log-phase and infected with T7 phage and incubated with shaking at 32°C until lysis. The lysates were centrifuged and filtered to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1% BSA, 0.05% Tween 20, 1 mM DTT) to remove unbound ligand and to reduce non-specific binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20% SeaBlock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT). Test compounds were prepared as 111X stocks in 100% DMSO. Kds were determined using an 11-point 3-fold compound dilution series (top concentration=30000 nM in this case) with three DMSO control points in duplicates. All compounds for Kd measurements are distributed by acoustic transfer (non-contact dispensing) in 100% DMSO. The compounds were then diluted directly into the assays such that the final concentration of DMSO was 0.9%. All reactions performed in polypropylene 384-well plate. Each was a final volume of 0.02 ml. The
assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05% Tween 20, 0.5 μM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

**TMT based proteomics global profiling sample preparation**

The cell pellets were suspended in 8M Urea, 50mM Tris-HCl pH8.0, reduced with dithiothreitol (5mM final) for 30min at room temperature, and alkylated with iodoacetamide (15mM final) for 45min in the dark at room temperature. Samples were diluted 4-fold with 25mM Tris-HCl pH8.0, 1mM CaCl2 and digested with trypsin at 1:100 (w/w, trypsin : protein) ratio overnight at room temperature. Peptides were desalted on homemade C18 stagetips. 100 µg of each peptide sample was labeled with TMT reagent following manufacture’s instruction. The mixture of labeled peptides were desalted and fractionated into 24 fractions in 10 mM trimethylammonium bicarbonate (TMAB) buffer containing 5-40% acetonitrile. Trypsin was purchased from Promega. All chemicals were HPLC-grade unless specifically indicated. TMT11plex Isobaric Label Reagent was purchase from Thermo Fisher (Cat. A34808).

**TMT based Mass spectrometry analysis**

Dried peptides were dissolved in 0.1% formic acid, 2% acetonitrile. For global profiling samples, peptide concentration was measured with Pierce™ Quantitative Colorimetric Peptide Assay (Thermofisher). 0.5 µg of each fraction was analyzed on a Q-Exactive HF-X coupled with an Easy nanoLC 1200 (Thermo Fisher Scientific, San Jose, CA). Peptides were loaded on to a nanoEase MZ HSS T3 Column (100Å, 1.8 μm, 75 μm x 150 mm, Waters). Analytical separation of all peptides was achieved with 100-min gradient. A linear gradient of 5 to 10% buffer B over 5 min, 10% to 31% buffer B over 70 min and 31% to 75% buffer B over 15 minutes was executed at a 300 nl/min flow rate followed a ramp to 100%B in 1 min and 9-min wash with 100%B, where buffer A was aqueous 0.1% formic acid, and buffer B was 80% acetonitrile and 0.1% formic acid. LC-MS experiments were also carried out in a data-dependent mode with full MS (externally calibrated to a mass accuracy of <5 ppm and a resolution of 120,000 for TMT-
labeled samples at m/z 200) followed by high energy collision-activated dissociation-MS/MS with a resolution of 30,000 for TMT-labeled global samples at m/z 200. High energy collision-activated dissociation-MS/MS was used to dissociate peptides at normalized collision energy of 32 eV (for TMT-labeled sample) in the presence of nitrogen bath gas atoms. Dynamic exclusion was 45 or 20 seconds.

Mass spectrometry analysis

Peptides were cleaned up by C18 stage tips and the concentration was determined (Peptide assay, Thermo 23275). The clean peptides were dissolved in 0.1% formic acid and analyzed on a Q-Exactive HF-X coupled with an Easy nanoLC 1200 (Thermo Fisher Scientific, San Jose, CA). 0.5 µg of peptides were loaded on to an Acclain PepMap RSQC C18 Column (150 mm × 75 μm ID, C18, 2 μm, Thermo-Fisher). Analytical separation of all peptides was achieved with 130 min gradient. A linear gradient of 5 to 30% buffer B over 110 min was executed at a 300 mL/min flow rate followed a ramp to 100% B in 5 min, and 15-min wash with 100% B, where buffer A was aqueous 0.1% formic acid, and buffer B was 80% acetonitrile and 0.1% formic acid.

LC-MS experiments were performed in a data-dependent mode with full MS (externally calibrated to a mass accuracy of < 5 ppm and a resolution of 60,000 at m/z 200) followed by high energy collision-activated dissociation-MS/MS of the top 20 most intense ions with a resolution of 15,000 at m/z 200. High energy collision-activated dissociation-MS/MS was used to dissociate peptides at a normalized collision energy of 27 eV in the presence of nitrogen bath gas atoms. Dynamic exclusion was 30.0 seconds. There were two biological replicates for one treatment and each sample was subjected to three technical LC-MS replicates.

Raw proteomics data processing and analysis:

Mass spectra were processed, and peptide identification was performed using the MaxQuant software version 1.6.10.43 (Max Planck Institute, Germany). All protein database searches were performed against the UniProt human protein sequence database (UP000005640). A false discovery rate (FDR) for both
peptide-spectrum match (PSM) and protein assignment was set at 1%. Search parameters included up to two missed cleavages at Lys/Arg on the sequence, oxidation of methionine, and protein N-terminal acetylation as a dynamic modification. Carbamidomethylation of cysteine residues was considered as a static modification. Peptide identifications are reported by filtering of reverse and contaminant entries and assigning to their leading razor protein. The TMT reporter intensity found in MaxQuant was for quantitation. Data processing and statistical analysis were performed on Perseus (Version 1.6.10.50). Protein quantitation was performed using TMT reporter intensity found in MaxQuant and a two-sample t-test statistics on two biological replicates was used with a p-value of 1% to report statistically significant protein abundance fold-changes.

**Colony formation assay**

Cells were cultured for 12-17 days in the presence of different compounds. Media with compound was replenished every two days. At the end of the experiment, media was aspirated and viable cells were stained with 0.5% crystal violet dye. Quantification analysis was performed as previously described (73). All the cell lines (total 38 cell lines) tested in colony formation assay were included in the final report, and no cell line was excluded from the study.

**Proliferation and apoptosis assays**

Experiments were carried out in 96-well plates in triplicates (Corning 720089). A total of $1-3 \times 10^3$ cells per well were grown in the presence of 1 µM of AZD5363 (Selleckchem), 1 µM of MS21 or 1 µM of MS21N1. Cells were then monitored for 3–4 days using the IncuCyte live cell imaging system (Essen BioScience, Ann Arbor, MI, USA), which was placed in a cell culture incubator operated at 37 °C and 5% CO₂. Cell confluence was determined using calculations derived from phase-contrast images readings on an IncuCyte ZOOM (Essen Biosciences) on live cells over time. For measurement of cell death DRAQ7 (Cell Signaling # 7406) at 1.5 µM was included in the medium and apoptotic red counts were measured in IncuCyte™ FLR automated incubator microscope.

**MTS assays**
The 96-well plates were seeded with 6,000 cells per well in a volume of 200 µL per well followed by incubation at 37 °C under 5% CO₂ overnight. Compounds were diluted in dimethyl sulfoxide (DMSO) to generate the desired stock concentrations then added into the well. All treatments were tested in quadruplicates. After 4 days incubation, relative numbers of viable cells were analyzed using CellTiter 96 AQueous One solution cell proliferation Assay (Promega) in accordance with the manufacturer’s protocol. Absorbance was measured on a Wallac Multilabel Reader (PerkinElmer). The concentration of drug resulting in IC₅₀ was calculated from a 4-parameter curve analysis (XLfit, IDBS software) and was determined from a minimum of 3 experiments. For cell lines that failed to achieve an IC₅₀, the highest concentration tested (10 µM) is listed.

**Reverse phase protein array (RPPA):**

RPPA was performed at MDACC Functional Proteomics core facility as previously described. Briefly, cellular proteins were denatured by 1% SDS, serially diluted, and spotted on nitrocellulose-coated slides. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. The signal obtained was amplified using a Dako Cytomation–catalyzed system and visualized by DAB colorimetric reaction. The slides were analyzed using customized Microvigene software (VigeneTech Inc., Carlisle, MA). Each dilution curve was fitted with a logistic model (“SuperCurve Fitting” developed at MDACC) and normalized by median polish. The data clustering was performed using Cluster 3.0 (centered by gene; followed by hierarchically clustering by gene and array using complete linkage) and TreeView. RPPA data have been deposited in the GEO database (GEO accession number: GSE141079).

**Statistical Analysis of RPPA data on MDA-MB-468 cell lines and PC-3 cell lines**

We performed unpaired two-sample Wilcoxon test between RPPA data from two 2 independent experiments of MDA-MB-468 cell lines treated by DMSO and from two 2 independent experiments of MDA-MB-468 cell lines treated by AZD5363. We visualized only protein differentially expressed with FDR at 5% using the function Heatmap Annotation of R Complex Heatmap library (Version 2.0.0). We performed the same type of analysis between RPPA data from two independent experiments of MDA-
MB-468 cell lines treated by DMSO and from two independent experiments of MDA-MB-468 cell lines treated by MS21. We then performed these three types of analysis on RPPA data from PC-3 cell lines. We finally compared using anova test between RPPA data from 6 independent experiments performed on MDA-MB-468 cell lines (two treated by DMSO, two treated by AZD5363, two treated by MS21) and from 6 independent experiments performed on PC-3 cell lines (two treated by DMSO, two treated by AZD5363, two treated by MS21). We visualized only protein differentially expressed with FDR at 5% and log fold change of more than 0.5 using the function Heatmap Annotation of R Complex Heatmap library (Version 2.0.0).

**RPPA data analysis from CCLE cell-lines**

We extracted the protein expression of nineteen proteins (AKT, AKT_pS473, AKT_pT308, EGFR, HER2, HER3, JNK_pT183_Y185, MAPK_pT202_Y204, p38_alpha_MAPK, p38_MAPK, p38_pT180_Y182, PRAS40_pT246) from the CCLE Reverse Phase Protein Array (RPPA) data (version 10/03/2018) found on the portal Cancer Cell Line Encyclopedia (CCLE) (https://portals.broadinstitute.org/ccle). We merged our list of 38 cell-lines with information about the known pathogenic mutations with RPPA data of CCLE 899 cell-lines using R DescTools (version 0.99.2), dplyr (version 0.8.3), tidyverse (version 1.2.1), and stringr (version 1.4.0) libraries. Only 20 of the 38 cell lines have the CCLE RPPA data and annotation for pathogenic mutations.

**Cell cycle analysis**

After 48 hours treatment with DMSO, AZD5363, or MS21, PC-3 cells were incubated with 10 µM EdU for 1 hour. The cells were then trypsinized, washed with 1% BSA in 1X PBS, and fixed with 4% paraformaldehyde for 15 min at room temperature. Following a second wash with 1% BSA in 1X PBS, the cells were permeabilized with 0.5% Triton, and a Click reaction with 2.5 µM Alexa Fluor™ 488 picolyl azide was performed according to manufacturer’s instructions for 30 min at room temperature in the dark. The cells were then washed with 0.5% Triton and stained with 2 µM SYTOX™ AADvanced™ Dead Cell Stain supplemented with 100 µg/mL RNase A for 30 min at room temperature in the dark.
Flow cytometry and analysis were then performed using a Guava® easyCyte™ 8HT Flow Cytometer, InCyte™, and GraphPad Prism.

**Generation of AKT knockdown cells:**

siRNA-mediated AKT knockdown was achieved with the Validated MISSION® Predesigned siRNA (MISSION RNAi, Sigma-Aldrich) as listed: AKT1 siRNA validated (NM_005163: SASI_Hs01_00105953); AKT2 siRNA validated (NM_001626: SASI_Hs01_00035055); AKT3 siRNA Rosetta Predictions (NM_005465: SASI_Hs01_00122808); AKT3 siRNA Rosetta Predictions (NM_005465: SASI_Hs01_00122809); MISSION® siRNA Universal Negative Control #1 (SIC001). A mixed pool of 100 picomolar of each siRNAs was transfected into indicated cells cultured in 6-well plates using Lipofectamine RNAiMAX Transfection Reagent (5 µL). After 48 hrs transfection, cell lysates were collected for western blot analysis and cells were counted for proliferation curve.

**Generation of AURKB overexpressing PC-3 cells:**

For stable overexpression of AURKB, AURKB(T73E), AURKB(T73A) in PC-3 cells, a Lentiviral plasmid p7052 pHAGE-P-CMVt-N-HA-GAW-AURKB (Cat# 100142) was purchased from Addgene. AURKB (T73E), AURKB (T73A), AURKB (T232A), and AURKB (T232A/T73A) mutations were made using QuickchangeII XL site directed mutagenesis kit (Agilent, Cat# 200522). Lentiviral plasmids were transfected into the packaging cells 293T cells together with two other plasmids: packaging DNA (psPAX2) and envelope DNA (pMD2G) through Lipofectamine transfection. After 48 hrs transfection, viruses were collected, filtered, and used to infect PC-3 cells in the presence of 8-10 µg/mL polybrene for 24 hrs. The infected PC-3 cells were selected with 4 µg/mL puromycin.

**Immunoprecipitation (IP) experiments:**

Cell lysates (1000 µg) were diluted with IP buffer, and then pre-cleared by incubation with 2 µg of isotype-matched IgG and 40 µL protein G-linked agarose beads (Roche Diagnostics) for 2 hr at 4°C. After pre-clearing, lysates were incubated with 1 µg specific antibody overnight at 4°C, followed by incubation with 50 µL protein-G linked agarose beads for 1 hr. The beads were then pelleted at 1000 rpm and washed with cold IP buffer (3 x 10 min). The washed immunoprecipitated complex was mixed with 2X
loading buffer (100 mM Tris-HCL [pH 6.8], 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) at a 1:1 ratio and denatured by boiling for 5 min, followed by western blot analysis with specific antibodies as described above.

**AKT kinase assay:**

Recombinant active full-length human Akt1/PKB protein (400 ng) (Cat#14-276, Millipore Sigma) was incubated with 200 µM ATP (Cat#20-306, Sigma) and 500 ng recombinant active full-length human AURKB protein (Cat#14-835, Millipore Sigma) in 50 µl 1 × kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2, Cat#9802, Cell Signaling Technology). Reactions were incubated at 30°C for 30 min and terminated by addition of Laemmli SDS sample dilution buffer. Proteins were separated by 4-12% NuPAGE Bis-Tris gels, and phosphorylation of AURKB was visualized by immunoblotting with anti-Phospho-AKT Substrate (RXXS*/T*) (110B7E) Rabbit mAb (Cat#9614, Cell Signaling Technology).

**Cellular thermal shift assay (CESTA):**

PC-3 cells and MiaPaca-2 cells were treated with DMSO or AZD5363 at 1 µM for 6 hr, and cell lysates were collected and processed following the protocol which has described before (58).

**VHL Expression Plasmid and generation of VHL stable expressing cells:**

The expression plasmid for VHL (Catalog# BC058831) was purchased from Applied Biological Materials Inc. (Richmond, BC, CANADA). VHL gene was inserted into a Lentivirus vector-pLenti-GIII-CMV-RFP-2A-Puro, and it was transfected into the packaging cell line 293T together with two other plasmids: packaging DNA (psPAX2) and envelope DNA (pMD2G) through Lipofectamine transfection. After 48 hr viruses were collected, filtered, and used to infect target cells in the presence of 8-10 μg/mL polybrene for 24 hr. The infected MDA-MB-468 cells were selected with 6 μg/mL puromycin.

**Mouse pharmacokinetic study**

Compounds AZD5363 and MS21 (in their HCl salts) were dissolved in 5% NMP, 5% Solutol HS-15, and 90% normal saline as formulation. Six male Swiss Albino mice (6 weeks old) were administered
intraperitoneally with solution formulation of compound AZD5363 at dose of 25 mg/kg or MS21 at the dose of 75 mg/kg. Plasma samples (approximately 60 µL) were collected from three mice at each of six time points (0.5, 1, 2, 4, 8 and 12 h). Plasma was harvested by centrifugation of blood and stored at -70 ± 10 °C until analysis. Compound concentrations in plasma at each time point are average values from 3 test mice. Error bars represent ± SEM. Experiments involving mice were performed according to the Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Xenograft studies

40 male immunocompromised NU/J mice (6 weeks old) (The Jackson Laboratory) were engrafted with PC-3 human prostate cancer cells (10 animals per group) or 24 female immunocompromised NU/J mice (6 weeks old) (The Jackson Laboratory) were engrafted with MDA-MB-468 human breast cancer cells (8 animals per group). After tumor volumes reach ~100 mm³, mice were randomized into different treatment arms and administrated with vehicle control, 25 mg/kg of AZD5363, 75 mg/kg of AZD5363 or 75 mg/kg of MS21 daily for 3-4 weeks via intraperitoneal (IP) injections. Cohort size was based on a power calculation to have at least 80% power to detect the difference observed with AZD5363 treatment vs. vehicle with a P value of less than 0.05. Attrition of the mice due to death of unknown causes occurred with no more than 2 deaths per arm. The experimenters were not blinded. Tumor volume was calculated as follows: tumor size (mm³) = (longer measurement × shorter measurement²) × 0.5. Tumor sizes will be recorded every other day over the course of the studies. Engraftment and monitoring of tumor growth and toxicity in mice will be performed using procedures that are already described in our protocol. Treatments will be discontinued if toxicity or distress is encountered. All procedures involving mice and experimental protocols (LA13-00024) were approved by the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai (ISMMS).

Bioanalysis of MS21 in mouse plasma and tumor samples

Mice were treated with 75mg/kg of MS21 via i.p. injection once daily for five days. On day 5, mice were euthanized 2 hr after the last dose. For plasma preparation, ~200 µL of blood was collected in an
Eppendorf tube pre-treated with EDTA. Samples were centrifuged at 3,000 rpm for 10 min at 4 °C, and
the resulting supernatant was collected in a clean Eppendorf tube and stored at −80 °C. Tumors were
harvested immediately after animals were euthanized and then cut into smaller specimens, snap-frozen in
liquid nitrogen and stored at −20 °C. All tumor samples were homogenized in 80:20 (vol/vol)
water:acetonitrile at a 1:9 (wt/vol) ratio. Total homogenization dilution was 10×. All tumor samples were
diluted 5× in plasma and analyzed against plasma calibration curves. MS21 concentrations in plasma and
tumor samples were analyzed using LC-MS. A Mac Mod Ace C18 column (2.1 × 50 mm, 3 μm) was used
for LC. Mobile phase A: 95:5:0.1 (vol/vol/vol) water:acetonitrile:formic acid. Mobile phase B: 50:50:0.1
(vol/vol/vol) methanol:acetonitrile:formic acid. API 5500 was used for MS/MS analysis. Electrospray
was used for the ionization method (positive ion).

Immunohistochemistry

Paraffin sections were dewaxed as previously described. If necessary, antigen retrieval was performed,
typically, the sections were boiled in 0.01 M sodium citrate, pH 6.0, 0.05% Tween-20 twice for 10 min,
and then cooled at room temperature for 20 min. After that, the sections were washed with ddH2O for 3
times of 5 min each and then washed with PBS for 5 min. HRP conjugated secondary antibody was used
to detect the signal. Endogenous HRP was blocked with 3% H2O2 in PBS or methanol for 10min. Then
the slide was washed in PBS for 3 times of 5 mins each. At this time, the section on slide was circled with
a PAP pen.

Mutational data

All cases reported with mutation in genes belonging to PI3K/PTEN pathway (PTEN, PIK3CA, PIK3R1,
AKT1, and ERBB2) and in genes belonging to RAS pathway (HRAS, KRAS, NRAS, BRAF, NF1, and
SOS1) were downloaded from the curated set of non-redundant studies of cBioPortal (www.cbioportal.org) on February 14th, 2020 (http://bit.ly/39uAr2a). This dataset consisted of 46,697 samples from 44,347 patients from 176 studies. 18,595 (42%) of queried patients (19,292 (41%)
of queried samples) have at least one mutation in the genes listed in PI3K/PTEN and RAS pathways. We
considered that 1) any amplification of PTEN, PIK3R1, HRAS, KRAS, NRAS, BRAF, NF1, and SOS1
are not driver mutations; 2) any deletion of PIK3CA, ERBB2, AKT1, HRAS, KRAS, NRAS, BRAF, and SOS1 are not driver mutations. Consequently, we removed these mutations as potential pathogenic. We considered NF1 gene as driver gene when there are two driver mutations in samples (n=38 patients have double mutations). The tumors were then categorized as 1) containing only mutations in the genes of the PI3K/PTEN pathway, 2) only mutations in genes of the RAS pathway, or 3) double mutants when they have variations in at least one of genes of the PI3K/PTEN pathway as well as in at least one of genes of the RAS pathway.

**Statistical analysis**

Aside from traditional Mann-Whitney (non-parametric), Spearman correlation test (non-parametric), student t-tests (parametric) to compare two data sets, and Chi-squared test (non-parametric), parametric statistical methods were used in order to make appropriate multiple comparisons of repeated measures of data (following 1-way or 2-way ANOVA). Graphpad Prism was used to make these simple predetermined statistical comparisons. Tukey’s Multiple Comparisons Correction: Used for making all possible pairwise comparisons in a data set. Dunnett’s Multiple Comparisons Correction: Used for comparing all samples to a control sample, but not for comparing the non-control samples to one another. Sidak’s Multiple Comparisons Correction: Used when specific multiple comparisons are pre-selected. Fischer’s Exact Test: Used to analyze items in a contingency table.

**Visualization**

We produced plots using several libraries on R (version 3.6.0 (2019-04-26)) and the framework RStudio (version 1.1.419). We used the function Heatmap Annotation of R Complex Heatmap library (Version 2.0.0) with R circlize (version 0.4.8) and data.table (version 1.12.2) libraries to visualize the known pathogenic mutations found in 38 cell-lines and their CCLE RPPA data. We ordered the cell lines according to their categories established by the colony formation assays, KRAS mutations, BRAF mutations, NF1 mutation, PIK3CA mutations, PTEN mutations, the status HER2+ of breast cancer cell-lines, and then tissue type. We put protein names as provided by CCLE for the heatmap of RPPA dataset.

**Code available**
The scripts used to analyze the RPPA dataset and visualize data are accessible to this URL https://github.com/parsonslabmssm/Akt_degrader.

Author contributions
J.X., X.Y., J.L., R.P. and J.J. conceived and designed the experiments. X.Y. and Y.S. synthesized the compounds. J.X., K.C. and R. Z. performed mechanism of action studies, WB experiments, proliferation and apoptosis assays, qRT-PCR, IHC and in vivo experiments. J.X. and A.B. performed colony formation and A.L. performed quantification analysis. Y.S. and R.Q. performed IHC staining. L.W., L.X., and X.C. performed Mass Spectrometry assay and data analysis. E.S., K.M.C., and A.L. performed Incucyte assay and FACS assay and analyzed the data. T.M. analyzed the RPPA, CCLE and human mutational data. X.W. and P.P. provided Ras/Braf mutant cancer cell lines and helped with CESTA assay. J.X., X.Y., T.M., J.J. and R.P. analyzed the data and co-wrote the paper. All authors discussed the results and commented on the manuscript. We thank members of the R.P. laboratory for technical assistance and critical reading of the manuscript.

Reference:


Figure Legends:

Figure 1. AKT degrader reduces AKT protein levels through E3 ubiquitin ligase.

A. Chemical structures of AZD5363 (428.92 Da) and MS21 (1107.6 Da).

B. AKT binding affinities of AZD5363, MS21, MS21N1 and MS21N2. AKT binding affinities were determined using a competitive binding assay (KINOMEscan™) in triplicate for the three different AKT isoforms. The lowest concentration points represent the DMSO control points. The remaining amount of DNA tagged kinase on the beads after competition assay was washed, eluted and measured by qPCR. The values were then normalized to that of DMSO control (%) (Signal %; y-axis) and plotted against the corresponding compound concentration in M in log10 scale (x-axis). Error bars represent SEM in triplicated independent experiments.

C. (Top) Immunoblot (IB) analysis of PC-3 cells treated with MS21 at 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM or 1 µM for 24 hr. DC<sub>50</sub> was determined by fitting to a standard four-parameter logistic using GraphPad Prism® v5. Antibody that detects all three isoforms of AKT (T-AKT) was used. (Bottom) Graph depicts the DC<sub>50</sub> of MS21 for PC-3 cells based on scanning of immunoblot for T-AKT.

D. IB analysis of PC-3 cells treated with DMSO or the indicated compounds at a fixed concentration of 1 µM for 1, 2, 4, 8, 12, or 24 hr in a time-dependent manner. Cells treated with VHL-1 at 1 µM for 24 hr were used as control.

E. MS21 degrades AKT via VHL, cullin and proteasome. BT474 cells were pre-treated with DMSO, Ac-VHL-Me (1 µM), MLN4924 (1 µM), MG-132 (20 µM) or AZD5363 (1 µM) for 2 h, before being treated with the 1 µM of MS21 compounds for 24 h. Indicated proteins from cell lysates were detected by IB.

F-G. Mass Spectronomy analysis of PC-3 cells treated with DMSO, MS21 (1 µM), and MS21N1 (1 µM) for 8 hr. (F) IB analysis of indicated proteins in indicated treatment. (G) Proteins differentially expressed
in MS21 treated cells versus MS21N1 treated cells. Volcano plots of the $-\log_{10}$ (p value) vs the log2 fold change, proteins with $-\log_{10}$ (p value) $>2$ ($p<0.01$) and log2 fold change $>1$ or $<-1$ were considered significantly changed upon the treatment indicated. $P$ values were calculated from the data of two biological and three technical replicates.
Figure 2. AKT degrader inhibits cancer cell proliferation by destabilizing Aurora kinase B.

A. IB analysis of indicated protein levels in PC-3 cells were treated with DMSO or indicated compounds at 1 µM for 24 hr.

B-C. (B) Cell cycle analysis by flow cytometry of PC-3 cells after 48 hr treatment with DMSO, AZD5363, MS21, or GDC-0941. Histograms are representative of triplicates. (C) Quantification of cell cycle phases of PC-3 cells after 48 hr treatment as indicated. Percentages reflect the mean of triplicates, and error bars indicate SEM.

D. Indicated cells were treated with DMSO or MS21 at 0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM for 2 weeks. Cells were stained with Crystal Violet.

E. *In vitro* AKT/PKB phosphorylation of AURKB. Recombinant active full-length human AKT1/PKB protein (400 ng) and recombinant active full-length human AURKB protein (500 ng) were incubated with or without 200 µM ATP in 50 µL 1 × kinase buffer at 30°C for 30 min. 600 µM of Barasertib or AZD5363 were pre-incubated with recombinant protein for 20 min before ATP was added into the reactions. IB analysis of phosphorylation of AURKB using anti-Phospho-AKT Substrate (RXXS*/T*) antibody after kinase assay.

F. IP of HA-AURKB protein and IB analysis of phospho-AKT substrate in 293T-AURKB-T232A or 293T-AURKB-T73A/T232A cells treated with Insulin (200 µg/mL) for 0, 10, 30, 60 mins after starvation for overnight.

G. IP of HA-AURKB and IB analysis of phosphor-AKT substrate in 293T-HA-AURKB cells pretreated with or without GDC0941 (1 µM) for 10 mins and followed by insulin (10 µg/mL) stimulation for 10 mins.

H. IP of HA-AURKB and IB analysis of phospho-AKT substrate in PC-3-AURKB cells treated with DMSO or AZD5363 (1 µM) for 2 hr.
I. IP of endogenous AURKB and IB analysis of phospho-AKT substrate in PC-3 cells pretreated with or without AZD5363 (1 µM) for 20 mins and followed by insulin (200 µg/mL) stimulation for 10 mins.

J. Indicated cells were treated with DMSO or MS21 at 3 µM for 24 hr and lysates tested for AURKB, T-AKT.

K. IB analysis of AURKB protein in PC-3 cells treated with GDC0941 (1 µM) and MG132 (10 µM) for indicated times.
Figure 3. AKT degrader inhibits cancer cell proliferation.

A-B. PC-3 cells (A) and MDA-MB-468 cells (B) were treated with DMSO, AZD5363 or MS21 at 0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM for 2 weeks. Cells were stained with Crystal Violet.

C. IB analysis of indicated protein levels in PC-3 and MDA-MB-468 cells were treated with DMSO, AZD5363, or MS21 at 1 µM for 48, 72, or 96 hr.

D. 1-3 × 10^3 PC-3 or MDA-MB-468 cells were seeded in 96-well plates in triplicates and treated at the indicated compound concentrations. Cells were monitored using the IncuCyte® live cell imaging system (Essen Bioscience™, Ann Arbor, MI) which was placed in a cell culture incubator operated at 37 °C and 5% CO². Cell confluence was determined using calculations derived from phase-contrast images. The concentration for 50% of maximal inhibition of cell proliferation (GI₅₀) values were determined by fitting to a standard four-parameter logistic using GraphPad Prism® v5. Graphs depicting the GI₅₀ of AKT degraders MS21 for these cancer cell lines were shown.

E. MDA-MB-468 and PC-3 cells treated with DRAQ7 and 2.5 µM of MS21N1, AZD5363, or MS21 to monitor fold change in cell death using the IncuCyte® Live-Cell Analysis System at 24, 48, 72, 96 and 120 hr (Student’s t-test, * < .05, ** < 0.01, *** < 0.001, n =3).
Figure 4. AKT degrader inhibits xenografted tumor growth in vivo

A. PK analysis of AZD5363 and MS21. Plasma concentrations of MS21 was collected following a single IP injection at a dose of 75 mg/kg over 12 hr in male Swiss Albino mice, and plasma concentrations of AZD5363 was collected following a single IP injection at a dose of 25 mg/kg over 12 hr.

B. Nude mice (Foxn1mu, athymic nude, Stock No: 002019) bearing established PC-3 xenograft were treated with AZD5363 (25 mg/kg, daily), AZD5363 (75 mg/kg, daily), MS21 (75 mg/kg, daily), or vehicle control. Data points represent mean tumor volume +/- S.E.. An ANOVA test (univariate test) was performed to identify if the profiles of tumor growth across different treatments have equal levels. P-Value indicated significant tumor suppression MS21 vs. Vehicle or AZD treatment.

C. IB analysis of indicated protein levels in the xenograft PC-3 tumors from nude mice treated with vehicle, AZD5363 or MS21.

D. Representative IHC staining of AKT, p-S6, and Ki67 in PC-3 tumors from nude mice treated with vehicle, AZD5363 or MS21. Scale bars represent 50 µm.

E. Percentage of AKT, p-S6, and Ki67 IHC score in indicated tumors. Unpaired t test with Welch’s correction was used to compare IHC score.

F. MS21 concentrations in the plasma and tumor samples isolated from mice treated once daily with 75 mg/kg via i.p. injection for 5 days at 2 hr after the last dose (n = 6).
Figure 5. PI3K/PTEN pathway mutant cell lines are sensitive to MS21 unless they also have a KRAS or BRAF mutation.

A. Summary of the sensitivity to MS21 treatment in the cell lines tested by colony formation assay, and status of KRAS mutations, BRAF mutations, PIK3CA mutations, PTEN mutations, HER2+ status, AKT1 mutations and tissue origin of the cell lines. Cells are categorized into two groups: resistant and sensitive. Red asterisk label indicates cell lines which are more sensitive to AKT degrader MS21 than AZD5363.

B. Cellular activity of AZD5363 and MS21 across a panel of indicated cell lines measured as the effects on cell viability tested and quantified in colony formation assay. Grey line indicated AZD5363 treatment and black line indicated MS21 treatment.

C. Analysis of cell lines that are sensitive or resistant to MS21 treatment based on their mutation status on the PI3K/PTEN pathway and Ras pathways. Fisher’s exact test was performed to identify any enrichment of sensitive or resistant cell lines between mutated and wide type cell lines. Cell numbers are shown with white color on the columns.

D. IB analysis of indicated protein levels in the indicated cells treated with DMSO or MS21 for 24 hr. Blue characters indicate sensitive cell lines and red characters indicate resistant ones.

E. Lysates from PC-3 and MiaPaca2 cells were treated with DMSO or MS21 at 1 µM for 4 hr at the indicated temperatures and then analyzed by immunoblot with T-AKT and β-actin antibodies.
Figure 6. AKT phosphorylation stimulates MS21 degradation of AKT

A. IB analysis of indicated protein expression in indicated cell lines.

B. The P-value of two-tailed Wilcoxon rank sum tests was performed on the RPPA data to compare the expression level of p-AKT (T308) and p-AKT (S473) between resistant group of 12 cell lines and sensitive group of 16 cell lines. Results in black dot represent the mean and error bars indicate ± SD.

C. Cells were serum-starved for overnight before treatment. Cells were pretreated with MS21 (1 µM) for 6 hr and treated with 100 ng/mL IGF-1 for 1 hr in combination, and total cell lysates were analyzed by Immunoblotting.

D. IB analysis of indicated protein levels in indicated cells treated with Trametinib at 10 nM for 24, 48, 72 hr.

E. IB analysis of indicated protein levels in the HT29 cells treated with DMSO, Trametinib at 5 nM, MS21 at 1 µM or combination treatment for 48 hr. MDA-MB-436 cells and A375 cells treated with DMSO, Trametinib at 20 nM, MS21 at 3 µM or combination treatment for 48 hr.
Figure 7. Resistance is converted to sensitivity by combining MS21 with trametinib in cancer cell lines with mutations of both the PI3K/PTEN and RAS pathways

A-B. (A) 1-3 × 10³ HT29, HPAF11, MDA-MB-436, HCT116 and DBTRG cells were seeded in 6-well plates in triplicates. HT29 and HPAF11 cells were treated with DMSO, Trametinib (5 nM), MS21 (1 µM) or combination for 10 days. MDA-MB-436 and HCT116 cells were treated with DMSO, Trametinib (20 nM), MS21 (3 µM) or combination for 10 days. After that, the treatments were removed and cells were cultured for 10 more days. (B) Quantitative analysis of the colonies by ImageJ. Tukey’s multiple comparisons test was used following two-way ANOVA for statistics.

C. Pie chart showing frequency of 44,347 patients in Cbio Portal depending on their pattern of mutations.
Figure 1

A

[Chemical structures of AZD5363 and MS21]

T-Akt
β-actin

p-PRAS40(T246)

MS21 in PC-3 cells

DC50 = 8.8 nM

B

qPCR Signal (%)

Log (compound concentration/nM)

AKT1

AZD5363 Kd = 1.8 ± 2.8 nM
MS21 Kd = 10 ± 4.3 nM
MS21N1 Kd = 3.8 ± 3.5 nM
MS21N2 Kd = 13 ± 9.1 nM

AKT2

AZD5363 Kd = 16 ± 10 nM
MS21 Kd = 960 ± 396 nM
MS21N1 Kd = 260 ± 374 nM
MS21N2 Kd > 30 μM

AKT3

AZD5363 Kd = 4.8 ± 4.6 nM
MS21 Kd = 46 ± 43 nM
MS21N1 Kd = 24 ± 26 nM
MS21N2 Kd = 11 ± 6.5 μM

D

PC-3 cells

24hr

T-Akt
p-PRAS40(T246)
T-PRAS40
p-S6(S240/244)
T-S6
β-actin

2hr 4hr 8hr 12hr 24hr

E

MS21-1μM

24hr

T-Akt
p-PRAS40(T246)
T-PRAS40
β-actin

BT474 cells

F

PC-3 cells

G

Log10 p-Value (MS21/MS21N1)

Log2Ratio (MS21/MS21N1)
Figure 2

A

DMSO 1 μM 3 μM 10 μM
MS21

PC-3 cells

AURKB
PDCD4

T-AKT
p-AKT

T-S6
p-S6

T-FOXO
p-FOXO
β-actin

B

DMSO
AZD5363
MS21

Percent cells (%)

C

D

PC-3-Vec cells
PC-3-AURKB cells

MS21

1 μM 3 μM 10 μM

E

AZD5363
Barasertib
ATP
Activity
Recombinant AURKB
Recombinant AKT
IB:phospho-AKT substrate

F

Insulin
200 μg/mL

IB: p-AKT substrate

293T-AURKB-T232A cells

G

Insulin-10 μg/mL
GDC0941-1 μM

IB: p-AKT substrate

IB: HA

293T-HA-AURKB cells

H

AZD5363 1 μM for 2 hr

IB: p-AKT substrate

IB: HA

PC-3-AURKB cells

I

Insulin-200 μg/mL
AZD5363-1 μM

IB: p-AKT substrate

IB: AURKB

PC-3 cells

J

PC-3
PC-3-AURKB
PC-3-AURKB-T73E
PC-3-AURKB-T73A

MS21

AURKB
T-AKT

β-actin

K

GDC0941
MG132

AURKB
p-AKT
T-AKT

β-actin

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Figure 3

A

PC-3 cells

DMSO 0.1µM 0.3µM

AZD5363 1µM 3µM 10µM

B

MDA-MB-468 cells

DMSO 0.1µM 0.3µM

AZD5363 1µM 3µM 10µM

C

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<td>MS21</td>
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T-AKT
p-AKT(S473)
AURKB
p-PRAS40(T246)
T-PRAS40
β-actin

PC-3 cells
MDA-MB-468 cells

D

Normalized Confluency%

Log Concentration (µM)

E

Percent of cell death (%)

PC-3 cells

DMSO
AZD5363
MS21
MS21N1

MDA-MB-468 cells

DMSO
AZD5363
MS21
MS21N1

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Figure 4

A. Plasma Concentration (nM) over Time (hr)

B. Tumor volume (mm^3) over Days of Treatment

C. Western Blots of PC-3 Tumors

D. IHC Staining of PC-3 Tumors

E. E. T-Akt IHC score (%)

F. Plasma Concentration (nM) vs. Tumor Concentration (nM)
Figure 5

A Resistant Sensitive

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<tr>
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<th>PTEN</th>
<th>HER2+</th>
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Figure 5B

Sensitive cells

Cell Viability (% of control)

Concentration/µM

Resistant cells

Cell Viability (% of control)

Concentration/µM

Figure 5C

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p=1.8x10-4, OR = 27.76

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<td>Resistance to MS21</td>
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p = 9.73x10-8, OR = 113.45

Figure 5D

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Figure 7

A) HT29 cells (BRAF, PIK3CA) and HPAF11 cells (KRAS, PIK3CA) treated with DMSO, Trametinib, MS21, and Trametinib+MS21.

B) Intensity Percent graphs for HT29 cells and HPAF11 cells treated with DMSO, Trametinib, MS21, and Trametinib+MS21.

C) Pie chart showing the distribution of cells with different mutational states: Double mutations in RAS & PI3K/PTEN pathways, Mutations in PI3K/PTEN pathway, Mutations in RAS pathway, and No mutation in RAS & PI3K/PTEN pathways.
AKT degradation selectively inhibits the growth of PI3K/PTEN pathway mutant cancers with wild-type KRAS and BRAF by destabilizing Aurora kinase B

Jia Xu, Xufen Yu, Tiphaine C Martin, et al.

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