Supplementary Figure 1. Biochemical characterization of the AKT degrader MS21

A. Co-crystal structure of AZD5363 (yellow) in complex with AKT1 (cyan, PDB:4GV1) indicates that hydroxyl group (red circled) is solvent-exposed position.

B. Chemical structure of MS21N1 and MS21N2.

C. Immunoblotting (IB) analysis of PC-3 cells treated with DMSO or indicated compounds at 0.1 µM, 0.3 µM, or 1 µM for 24 hr.

D. IB analysis of indicated protein levels in BT474 cells were treated with DMSO, VHL-1, AZD5363 or MS21 at 0.1 µM, 0.3 µM, or 1 µM for 24 hr. Cells treated with VHL-1 at 1 µM for 24 hr were used as control.

E. IB analysis of indicated protein levels in BT474 cells were 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.

F. IB analysis of indicated protein levels in BT474, PC-3, and MDA-MB-468 cells were treated with DMSO or MS21 at 1 µM for 24 hr.
Supplementary Figure S2

A

-Log10 p-Value (MS21N1/DMSO)

Log2Ratio (MS21N1/DMSO)

B

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C

D

-Log10 p-Value (MS21/MS21N1)

Log2Ratio (MS21/MS21N1)

E

-Log10 p-Value (MS21/DMSO)

Log2Ratio (MS21/DMSO)
Supplementary Figure 2. AKT degrader downregulates AURKB.

A. Mass Spectrometry analysis of PC-3 cells treated with DMSO, MS21 (1 µM), and MS21N1 (1 µM) for 8 hr. Proteins differentially expressed in MS21N1 treated cells versus DMSO treated cells. Volcano plots of the −log10 (p value) vs the log2 fold change, proteins with −log10 (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.

B. IB analysis of indicated protein levels in BT474, PC-3, and MDA-MB-468 cells were treated with DMSO or MS21 at 1 µM for 24 hr.

C. IB analysis of indicated protein levels in RT-4 (TSC1 null cells) cells were treated with DMSO, AZD5363 or MS21 at 1 µM or 5 µM for 24 hr.

D-E. Mass Spectrometry analysis of PC-3 cells treated with DMSO, MS21 (1 µM), and MS21N1 (1 µM) for 16 hr. (B) Proteins differentially expressed in MS21 treated cells versus MS21N1 treated cells. (C) Proteins differentially expressed in MS21 treated cells versus DMSO treated cells. Volcano plots of the −log10 (p value) vs the log2 fold change, proteins with −log10 (p value) > 1.3 (p < 0.05) 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.
Supplementary Figure S3

A. Bar graph showing cell viability % (Normalized to DMSO treatment) for PC-3 cells treated with different concentrations of DMSO for 5 days. The graph compares PC-3-Vec, PC-3-AURKB, PC-3-AURKB-T73E, and PC-3-AURKB-T73A.

B. Western blot images showing HA, AURKB, and β-actin expression in PC-3 cells treated with different concentrations of DMSO.

C. Western blot images showing HA, AURKB, and β-actin expression in BT474-Vec, BT474-AURKB, BT474-AURKB-T73E, BT549-Vec, BT549-AURKB, and BT549-AURKB-T73E cells treated with different concentrations of DMSO.

D. Western blot images showing AURKB and β-actin expression in BT474 and BT549 cells treated with different concentrations of DMSO.

E. Bar graph showing relative AURKB mRNA levels in PC-3 cells treated with DMSO or M321.

F. Diagram showing predicted sites for AURKB phosphorylation by AKT.

G. Western blot images showing p-AKT, T-AKT, p-AURKB(T232), T-AURKB, and β-actin expression in PC-3 and BT474 cells treated with insulin for different time points.
Supplementary Figure 3. AURKB downregulation contributes to AKT degrader inhibits cancer cell proliferation.

A. Quantification of cell viability percentage in indicated cells were treated with DMSO or MS21 at 0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM for 5 days and 15 days. Cells were trypsinized and counted at 5 days and 15 days, and normalized to DMSO treatment.

B. IB analysis of HA-AURKB, HA-AURKB-T73E, and HA-AURKB-T73A protein levels in PC-3 cells.

C. 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.

D. IB analysis of HA-AURKB and HA-AURKB-T73E protein levels in BT474 and BT549 cells.

E. qRT-PCR analysis of AURKB mRNA levels in PC3 cells were treated with DMSO or MS21 at 1 µM for 24 hr.

F. Scan AURKB-Human protein for phosphorylation sites on Scansite 4.0 website and illustrate a potential AKT phosphorylation site-Threonine 73(DILTRHFTIDDFEIG).

G. IB analysis of indicated protein levels in PC-3 cells and BT474 cells were treated with Insulin (200 µg/mL) for 0, 10, 30, 60 mins after starvation for overnight.
Supplementary Figure 4. MS21 selectively degrades AKT and inhibits cancer cells proliferation.

A. 1-3 × 10^3 PC-3 cells were seeded in 96-well plates in triplicates and treated at the indicated compound concentrations for 72 hr. Cell confluence was determined by MTS assay. The concentration for 50% of maximal inhibition of cell proliferation (GI_{50}) values were determined by fitting to a standard four-parameter logistic using GraphPad Prism® v5.

B. BT474 cells were treated with AZD5363, MS21, GDC0068, or INY-03-041 at 0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM for 2 weeks. Cells were stained with Crystal Violet.

C. PC-3, MDA-MB-468, and BT474 cells were treated with MS21N1 or VHL-1 at 0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM for 2 weeks. Cells were stained with Crystal Violet.

D. IB analysis of indicated protein levels in PC-3 cells were treated with MS21 or MS21N2 at 1 µM for 24 hr.

E. PC-3 cells were treated with MS21N2 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.

F. IB analysis of indicated protein levels in indicated cells transfected with a pool of siAKT1, siAKT2, siAKT3 for 72 hr and 96 hr. Scramble siRNAs were used as control.

G. Growth curve of indicated cells transfected with a pool of siAKT1, siAKT2, siAKT3, and cell numbers were collected at 72 hr, 96 hr and 120 hr time point. Scramble siRNAs were used as control. Multiple t-test was performed. (* < .05, ** < 0.01, *** < 0.001, n =3).

H. PC-3 cells and MDA-MB-468 cells were treated with DMSO or indicated compounds at 0.3 µM, 1 µM, 3 µM, or 10 µM for 24 h and lysates tested for T-AKT and downstream signaling to PRAS40 and S6.
**Supplementary Figure S5**

A. Western blot analysis showing changes in protein levels in MDA-MB-468 and PC-3 cells treated with DMSO, MS21, and AZD-M1.

B. Graph depicting the relative AKT level (%) in MDA-MB-468 cells treated with MS21 over 24 hours. The DC_{50} is 94 nM.

C. Heatmap displaying the changes in protein expression for various targets in PC-3 and MDA-MB-468 cells treated with DMSO, MS21, and AZD-M1.

D. Heatmap illustrating the expression levels of genes such as Axl, MCT4, EphA2, HIF1a, MMP14, Paxillin, Vimentin, HMHA1, IDO, Caveolin1, NDRG1-pT346, EphA2-pY588, SHP2-pY542, and Atg7 in MDA-MB-468 cells treated with MS21, AZD, DMSO.

E. Additional Western blot analysis showing changes in protein levels in MDA-MB-468 cells treated with DMSO, MS21, and AZD-M1.

F. Image showing cell culture plates with treatment groups for MDA-MB-468-VHL cells.
Supplementary Figure 5. VHL expression level affects the effectiveness of VHL-recruiting AKT degraders

A. IB analysis of the indicated cells treated with indicated compounds for 24 hr.

B. (Top) IB analysis of MDA-MB-468 cells were treated with MS21 at 1 nM, 3 nM, 10 nM, 30 nM, 0.1 µM, 0.3 µM, 1 µM, 3 µM or 10 µM for 24 hr. DC₅₀ were determined by fitting to a standard four-parameter logistic using GraphPad Prism® v5. (Bottom) Graph depicts the DC₅₀ of MS21.

C-D. Reverse phase protein array (RPPA) analysis of PC-3 cells and MDA-MB-468 cells treated with DMSO or indicated compounds at 1 µM for 24 hr. (C) RPPA heatmap shows PI3K/AKT pathway signaling alterations due to drug treatment. (D) RPPA heatmap shows other significantly altered proteins, with red arrow indicating VHL.

E. IB analysis of indicated proteins in MDA-MB-468-Vec cells and MDA-MB-468-VHL cells.

F. MDA-MB-468-VHL cells 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.
Supplementary Figure 6. AKT degrader inhibits xenografted tumor growth in vivo

A. Female Nude mice (Foxn1nu, athymic nude) bearing established MDA-MB-468 xenograft were treated with AZD5363 (25 mg/kg, daily, n=6), MS21 (75 mg/kg, daily, n=7), or vehicle control (daily, n=7). 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. AZD treatment and AZD vs. vehicle treatment.

B. Representative IHC staining of Cleaved-Caspase3 in PC-3 tumors from nude mice treated with vehicle, AZD5363 or MS21. Scale bars represent 50 µm.

C. Percentage of Cleaved-Caspase-3 IHC score in indicated tumors. Unpaired t-test with Welch’s correction was used to compare IHC scores.

D. Body weight changes of mice at the end of the in vivo experiment. Sidak’s multiple comparisons test was used following one-way ANOVA for statistics (n = 8~9). The P value was calculated between day 0 and day 21.

E. Nonfasted wild-type mice (n=5 per group) were treated with Vehicle, AZD5363 (25 mg/kg) and MS21 (75 mg/kg), and monitored their blood glucose levels over time after acute treatment.
Supplementary Figure S7

Panel A shows the effects of AZD5363 and MS21 on cell lines with different genetic alterations involving HER2, PIK3CA, PTEN, and BRAF when treated with DMSO at 0.3 µM and 10 µM. The cell lines include:

- HCC1143 cells (AKT1)
- BT549 cells (PTEN)
- BxPC3 cells (BRAF)
- HCC1395 cells (PTEN)

Panel B includes:

- BT474 cells (HER2, PIK3CA)
- HCC1937 cells (PTEN, PIK3CA)
- SKBR3 cells (HER2)
- MCF-7 cells (PIK3CA)
- ZR75-1 cells (PTEN)
- T47D cells (HER2, PIK3CA)
- SKOV3 cells (HER2, PIK3CA)

All cell lines were treated with AZD5363 and MS21 at concentrations ranging from 0.3 µM to 10 µM. The images illustrate the effect of these treatments on cell proliferation.
Supplementary Figure 7. Colony formation assay analysis of MS21 and AZD5363 treatment in different cell lines.

A. Cell lines have better response to MS21 than AZD5363. Indicated cells 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. Blue character indicated sensitive cells and red character indicated resistant cells.

B. Cell lines have similar response to MS21 than AZD5363. Indicated cells 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. Blue character indicated sensitive cells and red character indicated resistant cells.

C. Cell lines are resistant to MS21. Indicated cells 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. Blue character indicated sensitive cells and red character indicated resistant cells.

D. Cell lines have weak resistant to MS21. Indicated cells 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. Blue character indicated sensitive cells and red character indicated resistant cells.
Supplementary Figure S8

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\[ p=2.25 \times 10^{-2} \text{ OR}=3.74 \]

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\[ p=3.9 \times 10^{-3} \text{ OR}=15.06 \]

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Supplementary Figure 8. PI3K/AKT pathway mutant cell lines are sensitive to MS21 and AKT phosphorylation regulates AKT degradation

A. Summary of cell lines that are sensitive or resistant to AZD5363 treatment and their mutation status on the PI3K/PTEN and RAS pathways. Fisher’s exact test was performed to identify any enrichment of resistant cell lines between mutated cell lines and wide type cell lines. Cell numbers are shown with white color on the columns.

B. Summary of the cell lines with mutations in the PI3K/PTEN pathway and PI3K/PTEN pathway without RAS pathway mutations, and their sensitivity status to MS21 or AZD5363 treatment. Fisher’s exact test was performed to identify if there is any difference of specificity between MS21 and AZD5363 treatments in mutated cell lines. Cell numbers are shown with white color on the columns.

C. IB analysis of indicated protein levels in MS21-sensitive and MS21-resistant cells were treated with DMSO or MS21 at 1 µM for 24 hr.

D. IB analysis of indicated protein levels in MS21-sensitive and MS21-resistant cells were treated with DMSO or GDC0941 at 1 µM for 24 hr.
Supplementary Figure S9

A

Resistant
Sensitive

Akt_pS473
Akt_pT308
Akt
HER2
EGFR_pY1173
EGFR
HER3
JNK_pT183_Y185
MAPK_pT202_Y204
p38_alpha_MAPK
p38_MAPK
p38_pT180_Y182
PRAS40_pT246

Protein expression
of genes in CCLE

B

MiaPaca-2 cells
(PANC-1)

DMSO
MS21
Insulin
Insulin+MS21

p-Akt(S473)
p-Akt(T308)
T-Akt
p-PRAS40(T246)
T-PRAS40
p-S6(S240/244)
T-S6
p-Erk
T-Erk

β-actin

C

MiaPaca-2 cells (KRAS)

DMSO
MS21-1 µM
IGF-100 ng/mL
Combination

D

PC-3-DMSO
PC-3-MS21
U87MG-DMSO
U87MG-MS21
456-DMSO
456-MS21

T-Akt
p-Akt(S473)
p-PRAS40(T246)
T-PRAS40
p-S6(S240/244)
T-S6
PTEN
β-actin
Supplementary Figure 9. PI3K/AKT pathway mutant cell lines are sensitive to MS21 and AKT phosphorylation regulates AKT degradation

A. RPPA profiling of AKT phosphorylation and other signaling events in twenty cancer cell lines extracted from Broad Institute Cancer Cell Line Encyclopedia (CCLE) database that overlapped with the 38 cell line panel screened for sensitivity to MS21, associated with the response status to MS21 treatment, KRAS mutations, BRAF mutations, PIK3CA mutations, PTEN, HER2+, AKT1 and tissue origin of the cell lines. Red asterisk label indicates cell lines which are more sensitive to AKT degrader MS21 than AZD5363.

B. Cells were serum-starved overnight before treatment. Cells were pretreated with MS21 (1 µM) for 6 hr and treated with 10 µg/ml insulin for 1 hr in combination, and total cell lysates were analyzed by Immunoblotting.

C. MiaPaca-2 Cell were treated with DMSO, MS21(1 µM), IGF(100 ng/mL), or combination for 2 weeks. Cells were stained with Crystal Violet.

D. IB analysis of indicated protein levels from indicated cell lines treated with DMSO or MS21 (1 µM) for 24 hr.
Supplementary Figure S10

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A375 (BRAF)  MiaPaca-2 (KRAS)  SK-MEL-239 (BRAF)

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MiaPaca-2 (KRAS)  SK-MEL-239 (BRAF)

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HT29 (BRAF, PIK3CA)  DBTRG (BRAF, PTEN)
Supplementary Figure 10. Trametinib treatment increases AKT phosphorylation and improves AKT degradation in the presence of MS21 in a wide variety of cancer cell lines

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

B. IB analysis of indicated protein levels in indicated cells treated with Trametinib (10 nM), MS21 (1 µM), or combination for 24 hr.

C. IB analysis of indicated protein levels in indicated cells treated with Trametinib (10 nM), MS21 (1 µM), or combination for 24 hr.
Supplementary Figure 11. Colony formation assay and quantitative analysis of a combinational treatment with MS21 and Trametinib in different cell lines

A-B. (A) 1-3 × 10³ A375 cells, MiaPaca-2 cells, SK-MEL-239 cells, PANC-1 cells, SW1990 cells and PANC10.05 cells were seeded in 6-well plates in triplicates and treated with DMSO, Trametinib (20 nM), MS21 (3 µM) or combination for 10 days, and then 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. Analysis of the MYC status and its relationship to sensitivity to MS21 and ASD5363 shows no significant correlation or relationships. (MYC mutation is defined as MYC amplification and point mutation.)
Supplementary Figure S12

Percentages of gene and mutations and types thereof on the PI3K/PTEN pathway for cancers that have no co-occurring RAS pathway mutations.
Supplementary Figure 12.

A. PieDonut plot showing percentage of different PI3K/PTEN pathway gene alterations in the patient samples having mutations in PI3K/PTEN pathways without co-occurring RAS pathway mutations, and the types of alteration including mutation, CNA (amplification or deletion) and fusion.