SUPPLEMENTARY MATERIALS AND METHODS

Patient samples

Human adrenocortical tissue samples were collected under the clinical protocol entitled "Prospective comprehensive molecular analysis of endocrine neoplasms" (Clinical Trial Registration number NCT01005654). The ethical approval was granted by the Institutional Review Board, National Cancer Institute, NIH, and the NIH Office of Human Subject Research. All participants provided written informed consent.

Cell culture

Human ACC cell lines, SW13 and NCI-H295R, were purchased from the American Type Culture Collection™ (CCL-105, CRL-2128; Manassas, VA, USA) and cultured in 5% CO_2 atmosphere at 37 °C in Dulbecco's Modified Eagle Medium (11195-065, Thermo Fisher Scientific, MA, USA) supplemented with 2.5% Nu-Serum (355100, Corning, MA, USA) and 0.1% Insulin-Transferrin-Selenium (41400045, Thermo Fisher Scientific, MA, USA). Cell lines were authenticated by short tandem repeat profiling. NCI-H295R cells used to generate human ACC xenografts were transfected with a linearized pGL4.51[*luc2*/CMV/Neo] vector (9PIE132, Promega) encoding the luciferase reporter gene *luc2* (*Photinus pyralis*) and maintained in the above medium with up to 500 µg/mL of G-418 antibiotic (11811-023, Gibco, MA, USA) for selection.

Small molecule inhibitors and *in vitro* treatments

Small molecule inhibitors Ganetespib (STA-9090; S1159), Luminespib (NVP-AUY922; S1069), HSP990 (NVP-HSP990; S7097), PIK-75 (PIK-75 HCl; S1205) and BGT226 maleate (NVP-BGT226; S2749) were purchased from Selleck Chemicals LLC (Houston, TX, USA). These inhibitors were

dissolved in dimethyl sulphoxide (DMSO) for *in vitro* experiments. For all the *in vitro* experiments, we assessed the effects of the HSP90 inhibitors (STA9090, AUY922, and HSP990) and the inhibitors of the p110α subunit of PI3K (PIK75 and BGT226), as monotherapy, and in combination using multiple concentrations to validate the synergistic antiproliferative effect. The effective concentrations of the HSP90 (16nM-20nM) and PI3K (15nM-20nM) inhibitors were used in subsequent *in vitro* studies.

Quantitative high-throughput drug screening and combination matrix analysis

The National Center for Advancing Translational Sciences (NCATS) Pharmaceutical Collection (NPC) and the Mechanism Interrogation PlatE (MIPE) library, which in total consisted of 4,991 approved and investigational compounds, were primarily screened against SW13 and NCI-H295R cell lines using their cell viability measured by CellTiter-Glo® (Promega, Madison, WI), as described in our previous studies [1,2]. Next, a pair-wise combination matrix drug screening of the most efficacious 31 drugs from the primary quantitative high throughput screening (qHTS) was performed against NCI-H295R to identify the synergistic anti-proliferative effect. Plating of compounds was done in matrix format using acoustic droplet ejection and numerical characterization of synergy, additivity, and/or antagonism was conducted as described in the previous studies [1,2].

Gene expression profiling

Publicly available genome-wide mRNA expression data were downloaded and analyzed from three Gene Expression Omnibus (GEO) cohorts (GSE10927, GSE12368, and GSE75415) using embedded interactive statistical software (GEO2R). The relative mRNA expressions of genes of

interest in ACC samples were compared to those of adrenocortical adenoma (ACA) and/or normal adrenal tissue samples. The *p* values were adjusted for a false discovery rate using Benjamini-Hochberg method.

We obtained and analyzed transcriptomic and relevant clinical data of primary human ACC samples from the Cancer Genomics Atlas (TCGA) database for Adrenocortical Carcinoma (total samples, N=79) from C-Bioportal (http://www.cbioportal.org), hosted by the Computational Biology Center at Memorial-Sloan-Kettering Cancer Center for Cancer Genomics. Query was performed for somatic mutations, copy number alterations, mRNA expression, and protein expression of the key genes involved in the regulatory functions of HSP90 (HSP90AA1, HSP90AB1, HSP90B1, TRAP1), AKT2, PIK3CA, and CDK1 and percentage alterations of each gene in the database and mutual exclusivity or co-occurrence was evaluated.

Cell Proliferation Assay

ACC cells NCI-H295R (n=6000 cells/well) and SW-13 (n=2000 cells/well)) were seeded in 96-well plates and 48 hours later were treated in triplicates with a series of concentrations of either vehicle (DMSO), HSP90 inhibitor (STA-9090, HSP990 or NVP-AUY922), and PI3K inhibitor (PIK-75 or BGT226) individually or in combinations for 7 days. The antiproliferative activity was evaluated using the CyQuant® Cell Proliferation Assay (Invitrogen™ Corp., Carlsbad, CA) as per the manufacturer's protocol, quantified by SpectraMax i3x 96-well fluorescence plate reader (Molecular Devices, Sunnyvale, CA) at 485nm/538nm. To confirm the synergy, we used the Chou-Talalay method to assess the treatment efficacy in at least 5 different concentrations (based on IC50 values of the drugs) of each of the HSP90 and PI3K inhibitors in monotherapy and combination therapy. Each experiment was repeated at least three times. Efficacy indicated by

the combination index (CI) was compared to cells treated with a single drug. CI < 1 indicated synergy; CI = 1 indicated an additive effect; and CI > 1, indicated an antagonistic effect.

Clonogenic Assay

ACC cells were seeded in triplicate in 6-well plates (1000 cells/well) and allowed to grow for 1 week followed by treatment with drug(s) alone or in combination or with the vehicle in complete media. Growth media with vehicle or drug(s) were replaced twice every week. Treated or untreated cells were allowed to form colonies for another 3 weeks, and then fixed with 0.4% buffered paraformaldehyde and then stained with 0.5% crystal violet prepared in 25% methanol for 10 min. The colonies were counted and photographed using a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA) and quantified manually for the number of colonies.

Three-dimensional multicellular aggregates (MCA)

To create 3-dimensional multicellular aggregates (MCA), SW13 or NCI-H295R (1×10^5 cells/0.5 mL) cells were seeded in an Ultra-Low Cluster, 24-well plate (Costar catalog #, Corning, NY), incubated at 37°C with 5% CO2 for two weeks. Next, we treated these MCAs with monotherapy and in combination using the above inhibitors for 3 weeks. The MCAs or tumor spheroids were treated continuously with intermittent change of growth media with or without inhibitors. Spheroids were observed and images were captured under the light microscope (magnification 40x) and compared for their sizes and integrity.

Apoptosis Assay

The caspase 3/7 activity was estimated to quantify the apoptotic potential and was evaluated using the Caspase-Glo 3/7 Assay kit (G8091, Promega North America, Madison, WI) according to the manufacturer's protocol. Briefly, SW13 (6×10^3 cells/well in 100 µl) and NCI-H295R (6×10^3

cells/well in 100 µl) were seeded in a 96-well white-walled clear bottom plate (Lonza, Allendale, NJ) were incubated for 24 hours at 37°C in 5% CO2 and then treated with the standardized concentrations of inhibitors or respective vehicle. After 48 and 72 hours of treatment, 100 µl of the Caspase-Glo 3/7 reagent was added to each well including blank wells containing only culture medium, vehicle control, and treated cells in culture medium, contents were gently mixed using a plate shaker for 30 seconds. Plates were covered with aluminum foil and incubated at room temperature for 30 minutes. Luminescence was measured by the SpectraMax i3Max plate reader (Molecular Devices, Sunnyvale, CA) per the manufacturer's protocol.

Invasion/migration Assay

We assessed cellular migration and invasion by using the Corning Biocoat cell culture 24-multiwell insert system (354578, 354480, Corning, Glendale, AZ) according to the manufacturer's protocol. Briefly, cells were plated on 6-well plates in triplicates and treated with inhibitors. Cells were trypsinized and plated in transwell chambers at a density of 1×10^5 cells in 0.5 mL serum-free medium. For cell invasion transwell coated with Matrigel was used and was rehydrated by adding warm media and incubating the trans-well at 37^0C for 2 hours. The bottom chambers were filled with DMEM supplemented with 10% FBS as a chemoattractant. The SW13 and NCI-H295R cells were allowed to migrate for 24 and 48 hours, respectively. The membranes of the chambers were fixed and stained with Diff-Quik (cat. #65044-93, Dade Behring, Newark, NJ, USA). Cells were imaged at 20× magnification. The migrated and invaded SW13 and NCI-H295R cells were counted in three random fields per well. Quantitative analysis was done by capturing the images under a brightfield microscope at magnification 200X and counting the cells in each treatment

well using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Western Blotting

Total protein was extracted from cells (SW13 and NCI-H295R, treated or untreated with either STA9090 or PIK75/BGT226 or both for 48 hrs and 72hrs) using RIPA lysis buffer (ThermoFischer Scientific, MA) with Halt protease inhibitor (ThermoFischer Scientific, MA), and protein concentration was determined using Bradford assay. An equal amount of protein concentration (50µg) from each treatment condition was resolved by electrophoresis using SDS-PAGE in 4-20% tris-glycine gels. We blocked the nonspecific binding to the membrane with 5% BSA in TBS-Tween buffer, then incubated the membrane at 4 °C overnight with specific primary antibodies for AKT (1:2000, cat #13038, Cell signaling technology, CA), phospho-AKT(1:2000, cat# 4060, Cell signaling technology, CA), mTOR(1:2000, cat# 2983, Cell signaling technology, CA), phospho-mTOR(1:2000, cat #2971, Cell signaling technology, CA), 4eBP1(1:2000, cat#2855, Cell signaling technology, CA), GSK3a/b(1:2000, cat# 5676, Cell signaling technology, CA), cleaved-caspase3(1:2000, cat #9664, Cell signaling technology, CA), caspase3(1:2000, cat # 9665, Cell signaling technology, CA), cleaved PARP(1:2000, cat#5625, Cell signaling technology, CA), N-cadherin(1:2000, cat#13116, Cell signaling technology, CA), vimentin (1:2000, cat#5741, Cell signaling technology, CA) and Actin (1:2000, cat# 12262, Cell signaling technology, CA). The membranes were incubated with horseradish-peroxidase-conjugated IgG secondary antibodies. Protein bands were analyzed using an enhanced chemiluminescence (ECL) reagent (Pierce, ThermoFisher Scientific, MA), and images were captured in ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions.

Cell cycle analysis

Cells (SW13 and NCI-H295R) treated or untreated with either STA9090 or PIK75/BGT226 or both for 48 hrs) were trypsinized, counted (1×10^6 cells/500 µl for analysis), washed twice with phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol and stained with FxCycle™ Propidium Iodide/RNase Staining Solution (Thermofisher Scientific, MA) followed by quantification of stained cells by flow cytometry analysis using the BD Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data were generated for at least 20,000 events per sample. The cell cycle of the gated PI distribution was analyzed using FlowJo software (Becton Dickinson and Company, USA).

RNA sequencing analysis

Gene expression profiling was performed using bulk RNA sequencing in ACC cells (NCI-H295R) treated with 20nM of HSP90 or PI3K inhibitors alone or in combinations, for 48 hours, followed by mRNA extraction (RNeasy Mini Kit, 74104, Thermofisher Scientific) and quantification by Nanodrop 1000 (Thermofisher Scientific). The integrity of isolated RNA was evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA sequencing was performed at the Sequencing Facility, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research. Sequencing. Total RNA-Seq samples were pooled and sequenced on NovaSeq 6000 SP using Illumina Stranded Total RNA Ligation with Ribo-Zero Plus and paired-end sequencing. The samples had 34 to 79 million pass filter reads with more than 89% of bases above the quality score of Q30. Reads of the samples were trimmed for adapters and low-quality bases using Cutadapt before alignment with the reference genome (hg38) and the annotated transcripts using STAR. The average mapping rate of all samples was 89% and unique alignment was above 14%.

The mapping statistics were calculated using Picard software. The samples have 0.12% ribosomal bases. The percentage of coding bases was between 5-33% and percentage of UTR bases was 12-88%, and mRNA bases were between 18-94% for all the samples. Library complexity was measured in terms of unique fragments in the mapped reads using Picard's Mark Duplicate utility. The samples had 12-58% non-duplicate reads. In addition, the gene expression quantification analysis was performed for all samples using STAR/RSEM tools. Both the normalized count and the raw count were provided as part of the data delivery. We subsequently used the RNA-Seq workflow module in Partek Genomics Suite 6.6 (Partek Inc.; http://www.partek.com/) and the NIH Integrated Data Analysis Platform (NIDAP, Plantanir Technologies, version 5.341.0) for downstream analysis and visualization of differentially expressed genes, at FDR less than 5% and a fold change (≥ 1.5) cutoff. The analysis was based on RPKM units (reads per kilobase per million mapped reads) and the gene counts were fitted to a negative binomial generalized linear model with DESeq2.

Pathway analysis

The Ingenuity Pathway Analysis (IPA, Qiagen Inc.) was used to identify significantly upregulated or downregulated signaling pathways in each of the treatment groups. Gene lists obtained from our RNA sequencing data analyses were uploaded into the IPA tool to analyze their relationship with molecular pathways by calculating the pathway enrichment scores. IPA maintains a large-scale pathway network derived from the Ingenuity Knowledge Base, a large collection of observations in various experimental contexts with nearly 5 million findings curated from biomedical literature or integrated from third-party databases. The enrichment score and P values in IPA were calculated using Fisher's exact test.

Autophagy flux analysis

Autophagic activity in the live ACC cells was evaluated using an Autophagy detection kit (ab139484, Abcam) as per the manufacturer's protocol. Briefly, SW13 and NCI-H295R cells were seeded in 8-chambered cover-glass slides (80827, ibidi GmbH., Germany) and grown to 50% confluency, and then treated with monotherapy of HSP90 or PI3K inhibitors or the combination of both, for 48 to 72 hours respectively. Cells were washed twice with the assay buffer, incubated with 100µl of Microscopy Dual Detection Reagent containing GFP (1:500), DAPI (1:1000), and 5% FBS, for 30 minutes at 37C, protected from light. Cells were then washed twice with Assay Buffer and observed for the accumulation of the GFP (colocalizes with the autophagosome marker LC3) in the spherical vacuoles. Images were captured at magnification 400X by confocal microscope (Nikon CSU-W1 SoRa, Nikon Instruments Inc. NY). Rapamycin (500nM) was used as a positive control, whereas Chloroquine (120nM) was used as a negative control.

***In vivo* study with human ACC xenografts**

The protocol designed to study the *in vivo* efficacy of the combination of STA9090 and BGT226 in mice with human ACC xenografts was approved by the Animal Care and Use Committee, National Cancer Institute, National Institutes of Health (NIH). Mice were maintained according to NIH Animal Research Advisory Committee guidelines. NCI-H295R cells with luciferase reporter (5×10^6) prepared in 100µl Matrigel (Corning® Matrigel® Matrix (Cat # 354234, Corning, NY, USA) and serum-free growth media (50:50) were subcutaneously injected into the unilateral flank of 8-weeks old non-obese diabetic/severe combined immunodeficiency mice (NOD/SCID gamma, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, Jackson Laboratory, Bar Harbor, ME) to form ACC xenografts. Five mice were housed per cage and given regular food and water ad libitum. The tumor burden of

ACC xenografts was monitored weekly using the Xenogen IVIS Spectrum *in vivo* imaging system (PerkinElmer, Shelton, CT, USA). After 3 weeks, mice with ACC xenografts were randomized into four treatment groups (n=10 per group) with intraperitoneal injections three times per week for 7 weeks of either vehicle, STA9090 (8mg/kg body weight, prepared in 1:1 PEG300:Distilled H2O), BGT226 (4mg/kg body weight, prepared in 1:1 PEG300:Distilled H2O) and combination of STA9090 (8mg/kg body weight) and BGT226 (4mg/kg body weight). We performed a live bioluminescence imaging study under general anesthesia using the Xenogen IVIS Spectrum in vivo imaging system (PerkinElmer, Shelton, CT, USA). We injected 200µl of 15mg/ml luciferin/ kg body weight of mouse, intraperitoneally 10 minutes prior to the live-imaging study. Next, the animals were anesthetized in a plastic chamber filled with a 2.5% isoflurane/oxygen/air mixture. This was maintained using a nose-cone delivery system during imaging. Tumor volume was also measured [Volume (cm^3) = 1/2 (Length x $Width^2$] using vernier calipers once a week. After 7 weeks, mice were euthanized, once the tumors reached a total volume of 2 cm^3. The tumors obtained were either flash-frozen in liquid N_2 or fixed in 10% formalin, for future analysis.

Patient-derived Organoids

Following surgical resection, tumor tissues were washed twice with 1% Penicillin/Streptomycin in PBS anti-biotic solution (15070063, Gibco). Tissues were dissected to remove necrotic or non-cancerous regions then minced into 1-2 mm pieces, transferred into tubes containing tumor dissociation kit (130-095-929, Miltenyi Biotec, MD, USA) and homogenized using a gentle MACS Octo Dissociator with Heaters (130-096-427, Miltenyi Biotec) according to manufacturer's instructions. The digested solution was filtered using a 70 µM cell strainer (130-098-462, Miltenyi) and centrifuged. A red blood cell lysis was performed using ACK lysing buffer (118-156-721,

Quality Biological, Gaithersburg, MD, USA). The resulting suspension was centrifuged, resuspended, counted, and seeded (10 million cells/ml) in non-tissue culture treated 96-well plates (351172, Corning, Bedford MA, USA) with a mixture of 50% growth medium (Advanced DMEM/F12, 12634-010, Gibco, MA, USA) and 50% Growth Factor Reduced (GFR) Matrigel (356321, Corning, Bedford MA, USA) in 1 µL droplets and incubated at 37^0C for 30 minutes for formation of patient-derived organoid (PDO) domes. Following gel curation, 200 µL growth media was added to each well. Treatments were performed after ACC PDO formation, approximately after 1-2 weeks. The ACC PDOs were treated with STA9090 or BGT226 monotherapy and in combination for 7 days. Treatment efficacy was evaluated by assessing cell viability using CellTiter Glo (G7570; Promega, Madison, WI, USA) according to the manufacturer's protocol.

Immunohistochemistry

Human ACC or adrenocortical adenoma (ACA) tissue samples and PDOs were formalin-fixed, embedded in paraffin, and 5-µm-thick sections were used for hematoxylin and eosin (H&E) staining. Immunohistochemistry (IHC) analysis was performed for the proteins using their respective primary antibodies, HSP90 (1:1000, Cell Signaling Technology), phospho-AKT (1:1000, Cell Signaling Technology), CDK1 (1:1000, Cell signaling technology, location) and vimentin (1:1000, Cell Signaling Technology, location), and IHC staining kit (Dako Envision Dual link System-HRP, Agilent Technologies Inc., Santa Clara, CA). Antigen retrieval was performed with citrate buffer in pressurized steam at 120 °C for 10 minutes. The endogenous peroxidase activity was blocked with 6% hydrogen peroxide (H325-500, Fisher Scientific, MA, USA). The primary antibodies were diluted in Dako background reducing diluent and incubated overnight at 4°C in a humidified chamber followed by incubation with anti-rabbit peroxidase-labeled polymer for 30

minutes. Expression of the target proteins was developed using the chromogen 3,3'-diaminobenzidine (DAB; EnVision + Kit system HRP) and then counterstained with hematoxylin. Images were captured using a light microscope (Zeiss Axio, Carl Zeiss Microscopy, LLC, white plains, NY) at a magnification of 200x for analysis.

Statistical analysis

Gene expression profiling data from GEO databases were analyzed using embedded interactive statistical software (GEO2R). The *p* values were adjusted for the false discovery rate using the Benjamini-Hochberg method. For the *in vitro* experiments, and the associations between mRNA expression and clinical features, the student's t-test was used to compare the mean between groups that were normally distributed, or the Mann–Whitney *U* test was used to compare continuous variables with non-parametric distribution. A two-tailed *p*-value less than 0.05 was considered statistically significant. Analysis of variance (ANOVA) with post-hoc tests to compare the mRNA expression and *in vivo* luciferase activity between treatment groups. We used Pearson's and Spearman's correlation to assess the correlations between continuous variables such as mRNA expression data in TCGA cohort with parametric and non-parametric distribution, respectively. We compared the estimated overall survival (OS) and disease-free survival (DFS) of patients with primary ACC in TCGA cohort using the Kaplan-Meier method with a log-rank test in a dichotomized cohort using the median as a cutoff for mRNA expression of the selected genes. All the statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, La Jolla, CA, USA) and the IBM SPSS Statistics 29.0 (IBM, Inc, Amork, NY).

Table S1. Patient characteristics for the respective patient-derived organoids (PDOs).

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	47	45	30	67	53
Gender	F	F	F	F	F
Year of diagnosis	2016	2014	2023	2022	2023
Tumor type and origin	Recurrent, metastatic ACC to right adrenal gland.	Metastatic ACC to liver	Primary left ACC	Recurrent/metastatic adrenal bed ACC (testosterone-secreting)	Primary left adrenal ACC
Local Treatment	Left adrenalectomy and nephrectomy, left abdominal mass resection, tumor thrombectomy, left lower lung lobectomy	Right adrenalectomy and nephrectomy, 2017 liver and right diaphragm resection, 2021 liver resection	ND	Adrenalectomy, 2022 lung nodule	None
Radiotherapy	No	No	No	No	No
First line treatment	EDP	Mitotane	EDP and mitotane	EDP and mitotane	ND
Second line treatment	EDP and temozolamide	Pembrolizumab	ND	ND	ND
Third line treatment	N/A	EDP followed by sunitinib	ND	ND	ND
Histology	6.2 cm. right ACC, lymphovascular invasion with retroperitoneal soft tissue metastasis	ND	11.5 cm. ACC, 10% necrosis, extension to left renal vein, periadrenal and renal hilar tissue, negative lymph node involvement	ACC involving soft tissue, kidney with tumor deposits in renal pelvis, perirenal+pelvic adipose tissue, pancreas, spleen, and diaphragm with tumor deposits, liver, and pertoneal metastasis	10.8 cm. ACC with unremarkable kidney, pancreas, spleen, omentum, 4 benign lymph nodes
Pathogenic Variants	*CTNNB1, FANCL*	ND	*CTNNB1, RB1*	*TP53*	*ARID1A, CTNNB1, TP53, MEN1,*

					RB1, ATRX, CREBBP, MSH2, JAK1, SETD2
Copy Number Variation	*CDKN2A loss, CDKN2B loss*	ND	ND	ND	ND

ND, Not Defined; N/A Not available; EDP, Etoposide, Doxorubicin and Cisplatin; ACC, Adrenocortical Carcinoma.

SI REFERENCES

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