Metabolic reprogramming ensures cancer cell survival despite oncogenic signaling blockade

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Supplemental Figure legends

**Supplemental Fig. S1. High content imaging to identify small molecule inhibitors that induce autophagy, Related to Figure 1**

**(A)** Schematic of high content imaging screen that measures p62 and p-S6, with magnification of lead small molecule inhibitors that decrease the expression of both proteins (list of drugs and targets is in Table S1).

**(B)** CYT387 inhibits JAK signaling in a dose-dependent manner in multiple human RCC cell lines. Immunoblots for p-JAK, total JAK, p-STAT3 and total STAT3. β-actin was used for loading control.

**(C)** CYT387 induces autophagy in multiple human MPN and RCC cells. LC3 processing and p-S6 evaluated by western blot following 24 hr of drug treatment.

**(D)** CYT387 induces reversible autophagy. ACHN cells were grown in the absence (control) or presence of 2 μM CYT387 for 24hr, followed by withdrawal of CYT387 for 24hr, lysed, and probed with antibodies to LC3, p-STAT3, total STAT3, p-S6, total S6, P-Akt Ser 473, and total AKT. β-actin as loading control.

**(E)** CYT 387 reduces proliferation in most human RCC and MPN cells lines. Pharmacological action of CYT387 in multiple human RCC cell lines, with induction of autophagy, as documented by LC3 immunoblots.

**(F)** Single agent CYT 387 treatment in most RCC cells lines demonstrates minimal effect on apoptosis. Bar graphs shows caspase 3/7 activation (apoptosis).
Supplemental Fig. S2. Validation of CYT387 as an inducer of autophagic flux, Related to Figure 1.

(A) ACHN cells stably expressing mCherry-GFP were treated with 2μM of CYT387 to document autophagic flux: yellow to red. Insert: high power. GFP-mCherry ratio measured by image analysis.

(B) Autophagic vacuoles stained with green with monodansylcadaverine (MDC). ACHN cells were treated with CYT387, in the presence or absence or absence of E64D and pepstatin. Cells were fixed, washed with PBS and observed directly under microscope and representative images are shown. The increase in MDC staining is quantified (bar graph; * p<0.001)

(C) Expression of LC3 (LC3-I and LC3-II) determined by immunoblotting in cell extracts from ACHN cells exposed to 2μM CYT387 for 24 hr in the presence or absence of E64d and pepstatin.

(D) Transmission Electron Microscopy (TEM) micrographs of CYT387-treated ACHN cells show increase in autophagosome, autolysosomes, lipid droplets and mitochondria (data represents >3 experiments).

**Figure panels:**
Left: Control: Low mag (1900x) + High mag (6800x) insert
Middle: CYT387 treated: Low mag (1900x) + High mag (6800x) insert
Green arrows: Autolysosome
Red Arrows: Autophagosome
Blue Arrows: Lipid Droplets
M: Mitochondria
Right: Pseudocolor of CYT387 treated (High mag insert from middle panel): Deep Blue: Mitochondria
Red: Autolysosome
Orange: Autophagosome
Bright Blue: Lipid Droplet
Supplemental Fig. S3. CYT387-induced inhibition of mTORC1 relieves the inhibitory feedback signal transmitted from mTORC1 to PI3K with consequent hyperactivation of PI3K and AKT, Related to Figure 2

(A-B): (A) Heatmap of kinase arrays shows time dependent decrease in p-S6 and subsequent increase in p-AKT Ser473 and Thr308 in ACHN cells at 24 hours and 72 hours after treatment.  
(B) ACHN cells were treated with 2µM CYT387 for 5, 24 and 72 hr and lysed and probed for LC3, p-AKT Thr 308, p-AKT Ser 473, total AKT, p-S6, total s6, p-STAT3, total STAT3 and β-actin.

(C) CYT387 treatment does not activate ERK signaling. ACHN cells treated with increasing dose of CYT387 (0-3µM) and probed for LC3B, p-ERK, total ERK, p-S6 and total s6. Tubulin as loading control.

(D) Schematic of chemical dissection of PI3K-AKT-mTOR pathway with GDC-0941 (pan-PI3K inhibitor), BX-795 (PDK1 inhibitor) and MK2206 (allosteric AKT inhibitor).

(E) Combination of GDC-0941, pan-PI3K inhibitor with CYT387 in ACHN cells for 24hr. Immunoblotted with indicated antibodies.

(F) Combination of BX-795 (3µM), PDK1 inhibitor with CYT387 (2µM) in ACHN cells for 24hr. Immunoblotted with antibodies shown.

(G, H) Stable mouse weights with treatment: Vehicle, CYT387 (50mg/kg), MK2206 (60mg/kg) and CYT387-MK2206 (50mg/kg+60mg/kg) combination. Body weights of mice bearing (G) ACHN, and, (H) SN12C tumors as indicated. Data are presented as mean ± SEM; ns: not significant.

(I) Tumor tissue from ACHN xenografts treated with the indicated drug regimens were evaluated by immunofluorescence for p-S6 and p-AKT
**Supplemental Fig. S4. Metabolic changes in vehicle treated compared to CYT387, MK2206 and CYT387+MK2206 treatment, Related to Figure 4**

(A-F) Metabolic pathway alterations in treated cells (CYT387, MK2206, CYT387+MK2206) compared to ACHN cells treated with vehicle (DMSO). Log fold change abundance of metabolites in glycolysis, pentose phosphate pathway, TCA cycle, amino acid, nucleotide biogenesis and neutral lipids are shown. Stars denote a significant difference of treated cells compared to vehicle (t-test p-value ≤ 0.05).

**Supplemental Fig. S5. Generation of bioactive eicosanoids from arachidonic acid, Related to Figure 4**

(A) Schematic of biosynthesis of eicosanoids from arachidonic acid.

(B) Levels of P450-derived and Arachidonic Acid-derived metabolites in ACHN human RCC cells treated with the MK2206-CYT387 combination

**Supplemental Fig. S6. Phospholipid derived fatty acid incorporation into lipid droplets is induced by CYT387-MK2206 co-treatment in ACHN, Related to Figure 5**

ACHN incubated with BODIPY-C12-HPC, a phospholipid containing a green fluorescent long chain fatty acid, for 16hrs with and without CYT387-MK2206 co-treatment. CYT387-MK2206 co-treatment led to a greater degree of incorporation of BODIPY-fatty acids into lipid droplets relative to vehicle ACHN, indicating CYT387-MK2206 co-treatment leads to an induction in phospholipid derived fatty acid to lipid droplet incorporation.

**Left panel:** Control (vehicle); **Right panel:** CYT387+MK2206.

Green: Bodipy-C12-HPC; Purple: wheat germ agglutinin to mark cell membrane

**Supplemental Fig. S7. Comparison of fatty acid driven OCR induction by CYT387+MK2206 treatment in ATG5 +/- and ATG5 -/- MEFs, Related to Figure 5.**

Fatty acid driven OCR was calculated as previously described (see Figure 5N) using the CPT-1a inhibitor, etomoxir, and normalized to the total mitochondrial OCR using Antimycin and Rotenone. The percent increase in fatty acid driven OCR in ATG5 +/- and ATG5 -/- by CYT387+MK2206 treatment is shown (n=2).
Supplemental Fig. S8. Glutamine fuel dependency as measured using the Seahorse XF Mito Fuel Flex assay, Related to Figure 5.
ACHN cells were treated with DMSO (control), CYT387+MK2206, for 24 h and OCR was measured before and after injection of glutaminase inhibitor BPTES (3 μM). The change in basal OCR after BPTES injection was normalized to the total change in OCR after inhibition of fatty acid oxidation, glutamine and pyruvate oxidation using Etomoxir, BPTES and UK5099, respectively (representative graph, n=2). The ability of ACHN cells to upregulate glutamine OCR, flexibility, is calculated by measuring the change in sensitivity to BPTES inhibition of OCR after blocking fatty acid and pyruvate oxidation.

Supplemental Fig. S9. Effect of inhibiting different PLA2 isoforms on lipid droplet number, Related to Figure 6
ACHN cells were treated with control, inhibitors; for calcium-sensitive PLA2 (cPLA2; cPLA2i), calcium-insensitive PLA2 (iPLA2; bromoenol lactone: BEL), CYT387+MK2206, CYT387+MK2206+cPLA2i, CYT387+MK2206+BEL for 24hrs, Bodipy 493/503 was added to visualize lipid droplets (n=2 experiments). Data are expressed as means ±SEM. *p=0.0065 CYT387+MK v CYT387+MK2206 +BEL; p=0.01 CYT387+MK2206 v CYT387+MK2206+cPLA2i

Supplemental Fig. S10. PLA2 inhibition by OOPEC blocks the generation of lipid droplets, Related to Figure 6
ACHN cells were treated with control, OOPEC, CYT387+MK2206, CYT387+MK2206+OOPEC added after 2hrs, CYT387+MK2206+OOPEC added after 2hrs+Etomoxir added after 8hrs, and monitored for 24hrs, Bodipy 493/503 (green) was added to visualize lipid droplets. Data are expressed as means ±SEM (n=2). p<.0001 OOPEC v CYT387+MK2206, OOPEC added at 2hrs v CYT387+MK2206, OOPEC added at 2hrs v CYT387+MK2206+OOPEC added at 2hrs+Etomoxir added at 8hrs.

Right panel: Bar graph with all conditions, for ease of viewing, all controls are in grey; Left panel: Line graph of CYT+MK+OOPEC added after 2hrs compared to CYT+MK+OOPEC added after 2hrs+Etomoxir added after 8hrs.
Supplementary Tables:

S1: Small Molecule Inhibitor library

S2-9: Phosphoproteomics Screen

S10-11: DAVID Analysis

S12: GSEA analysis across multiple datasets show enrichment for metabolic processes

S13: RNA Seq of CYT-treated ACHN cells, related to Table S12

S14: Metabolomics Profiling
A Schematic of high content imaging screen for autophagy flux inducers

116 clinically focused SMIs

B Human RCC and MPN cell lines

C Human RCC and MPN cell lines: Viability

D

E

F

Casae 3/7 Activity

Fold Change

0
1
2
3

Control

CYT387
A  
**GFP-mCherry-LC3 reporter**

Control  
CYT387

![Images of GFP-mCherry-LC3 reporter](image)

Ratio of GFP-mCherry

Control  
CYT

B  
**MDC autolysome tracking**

Control  
E64D+P  
CYT387  
CYT387+E64D+P

![Images of MDC autolysome tracking](image)

C  
**E64D/Pepstatin**

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D  
**Transmission Electron Microscopy**

Control  
CYT387  
CYT387
Supplementary Figure 4

Lue_Supplemental Fig. 3
A: Glycolysis  
B: Pentose Phosphate Pathway  
C: TCA Cycle  
D: Amino Acids  
E: Nucleotide Metabolism  
F: Neutral Lipids
% Beta-oxidation induction by CYT+MK

ATG5 +/+  ATG5 -/-
Glutamine Oxidation (% OCR)

- Control
- CYT+MK2206
LD Number

Mean FL intensity
## Gene Set Enrichment Analysis: Upregulated by CYT387 treatment

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## Gene Set Enrichment Analysis: Downregulated by CYT387 treatment

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