Supplemental information

Polyphenolic Proanthocyanidin-B2 Suppresses Proliferation of Liver Cancer Cells and Hepatocellular Carcinogenesis through Directly Binding and Inhibiting AKT Activity

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Supplemental figures legends

Figure S1. Characterization of OPC-B2 by high resolution LC-MS and inhibition of cell proliferation in different liver cancer cell lines, related to Figure 1.

A. Full scan (MS^1) of purified OPC-B2 from peanut skin using a high resolution Q-TOF-MS instrument. The difference of detected molecular ion and the theoretical value is 1.78 ppm in the negative ion mode. **B.** Cell viability of SMMC-7721 and LM3 cells treated with various concentrations of OPC-B2 at 24 and 48 hours. **C.** Dose-dependency on cell viability of different HCC cell lines treated with indicated concentrations for 48 hours. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure S2. OPC-B2 directly binds to AKT and inhibits its activity, related to Figure 2.

A. Immunoblots of cell lysates of Huh7 cells treatment with OPC-B2 (0, 50, and 75μg/ml) for 24 hours. **B.** Immunoblots of cell lysates of Huh7 cells treatment with 1X PBS or OPC-B2 (75μg/ml), followed by treatment with MK-2206 (0.5μM) for 24 hours. **C.** Cell numbers of Huh7 cells treated with control solvent or OPC-B2 (50 and 75μg/ml), followed by treatment with MK-2206 (2μM), measured by CCK8 kit. **D.** Immunoblot for upstream kinase expression of AKT in Huh7 cells treat with different concentrations of OPC-B2. **E.** Interactions between OPC-B2 and AKT1 amino acid residues. **F.** Immunoblot analysis of Huh7 cell lysates transfected with Myc-AKT1,

Myc-AKT1 (R86A), Myc-AKT1 (R273A) and Myc-AKT1 (K297A). **G.** Cell viability of Huh7 cells transfected with Myc-AKT1, Myc-AKT1 (K297A) and Myc-AKT1 (R86A) at different time points. **H.** Immunoblots of cell lysates of Huh7 cells transfected with myc-AKT1, myc-AKT1 (K297A) and myc-AKT1 (R86A), followed by treatment with DMSO or MK-2206 (0.5 μM) for 24 hours. The upper band is the exogenously overexpressed AKT1 protein and the lower band is the endogenous AKT1 protein. **I.** Cell numbers of Huh7 cells transfected with myc-AKT1, myc-AKT1 (K297A) and myc-AKT1 (R86A), followed by treatment with DMSO or MK-2206 (2 μM) for 72 hours. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure S3. OPC-B2 affects transcription and induces cell cycle arrest at G1 without affecting apoptosis, related to Figure 3.

A. Heat map of the clustering of differentially expressed genes (DEGs) transcripts in control and OPC-B2 (75 μg/ml) treated Huh7 cells. **B.** Volcano-plot of differentially expressed genes (DEGs) after log2 transformation. **C.** Cell cycle related genes expression profiles from clustering analysis of Huh7 cells treatment with OPC-B2 (75μg/ml). **D.** Enriched KEGG pathways of DEGs analysis. **E, F.** Cell cycle distribution of SMMC-7721 cells measured by flow cytometry after treatment with OPC. **G.** The protein expression levels of cyclin D1, phosphor-CDK4, and CDK4 were detected by western blotting assays after treatment with OPC-B2 (0, 25 and 75 μg/ml). **H**. Immunoblot analysis of proteins in apoptotic pathways in Huh7 cells treated with OPC-B2 (0, 50 and 75μg/ml) for 24 hours. **I**. Effects of OPC-B2 on apoptosis by Annexin-Ⅴ and propidium iodide (PI) double staining.

Figure S4. OPC-B2 promotes cyclin D1 degradation and ubiquitination, related to Figure 3.

A. Immunoblot analysis of Huh7 cells treated with OPC-B2 (0, 50, and 75μg/ml) for 24 hours. **B, C.** Cyclin D1 expression in Huh7 cells exposed to vehicle or OPC-B2 (50 μg/ml) for 24h, followed by treatment with cycloheximide (CHX, 10μg/ml). **D.** Polyubiquitination levels of affinity purified cyclin D1-Flag proteins were detected by western blotting in transfected SMMC-7721 cells upon treatment with 1X PBS or OPC-B2 (50 μg/ml). **E.** Immunoblot analysis of protein synthesis in Huh7 cells treated with OPC-B2 (0, 50, and 75μg/ml) for 24 hours. **F**. Quantification of total proteins in Huh7 cells after treatment with OPC-B2 (0 and 75μg/ml).

Figure S5. Metabolic flux from [U-^{13}C]-glucose in glycolysis and TCA cycle, related to Figure 4.

A, B Metabolic flux of glycolysis and TCA cycle. The ratio enrichment labelled carbons of different metabolites from [U-^{13}C]-glucose in glycolysis and TCA cycle upon treatment with OPC-B2 at dose of 0, 50 and 75 μg/ml for 12 (**A**) and 24 (**B**) hours. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure S6. Body weights, liver/body weight ratios, and enzymes of liver function of C57BL/6 mice, related to Figure 5.

A. Body weights of C57BL/6 mice during the experiment period. **B.** Liver /body weight ratios of mice. **C.** ALT, AST levels of C57BL/6 mice treated with the CK (control solvent), OPC-B2 or MK-2206. *$p < 0.05$.

Figure S1:

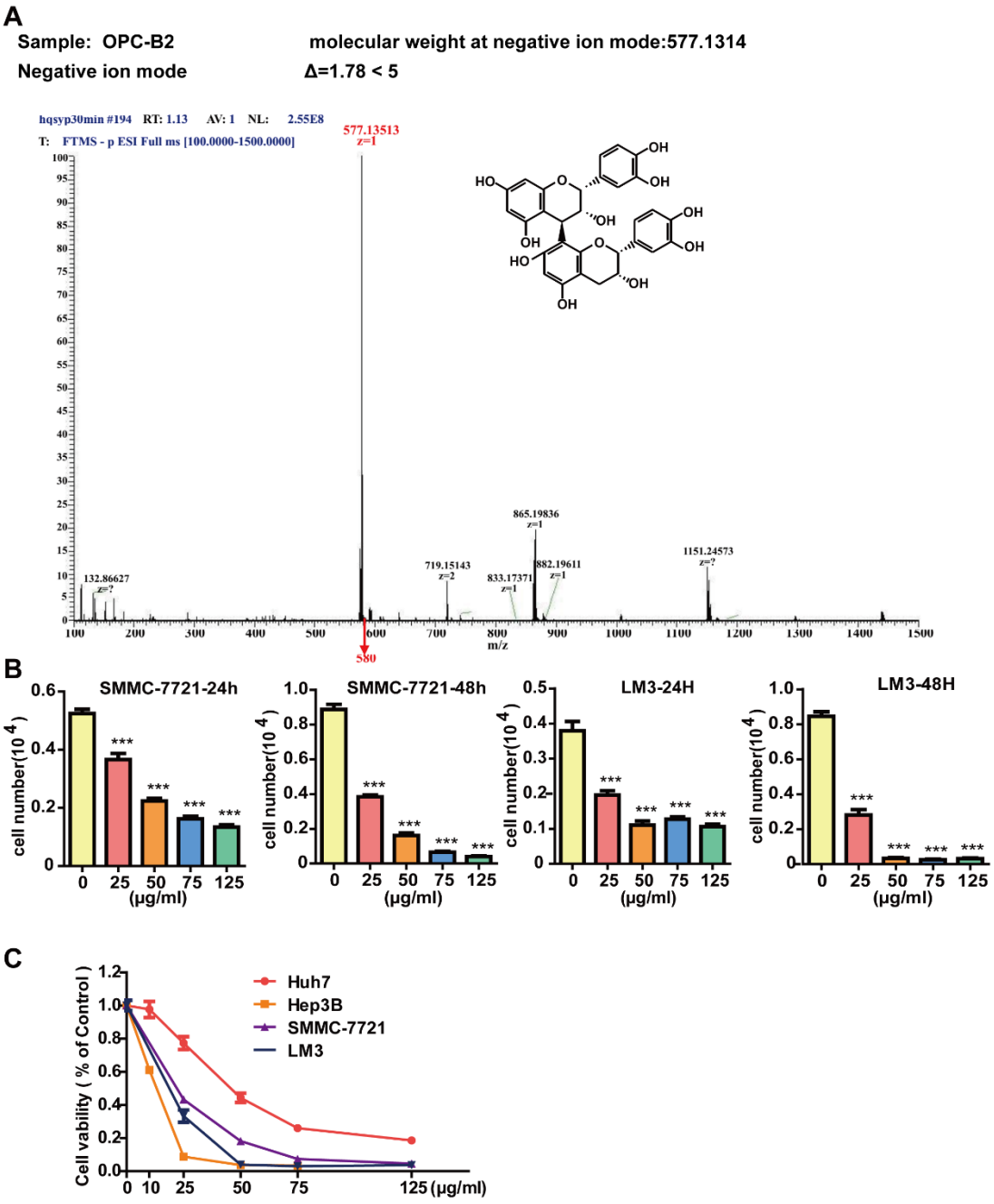
A
Sample: OPC-B2
molecular weight at negative ion mode:577.1314
Negative ion mode
Δ=1.78 < 5
hqsyp30min #194 RT: 1.13 AV: 1 NL: 2.55E8
T: FTMS - p ESI Full ms [100.0000-1500.0000]
577.13513
z=1
865.19836
z=1
719.15143
z=2
833.17371
z=1
882.19611
z=1
1151.24573
z=?
132.86627
z=?
580
m/z
B
SMMC-7721-24h
SMMC-7721-48h
LM3-24H
LM3-48H
cell number(10 4)
(μg/ml)
C
Cell vability (% of Control)
Huh7
Hep3B
SMMC-7721
LM3
(μg/ml)

Figure S2:

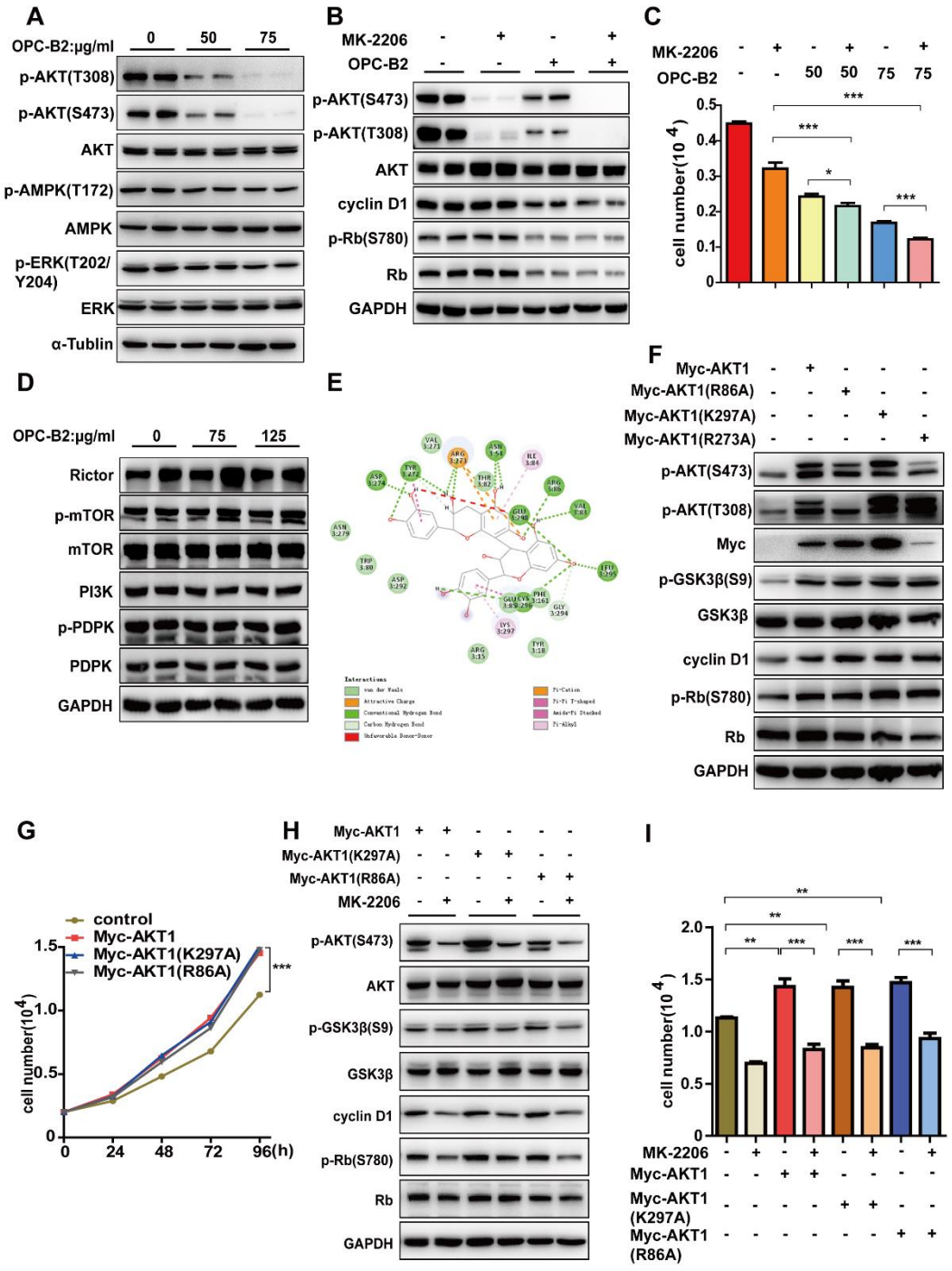
A
OPC-B2:μg/ml 0 50 75
p-AKT(T308)
p-AKT(S473)
AKT
p-AMPK(T172)
AMPK
p-ERK(T202/Y204)
ERK
α-Tublin
B
MK-2206 - + - +
OPC-B2 - - + +
p-AKT(S473)
p-AKT(T308)
AKT
cyclin D1
p-Rb(S780)
Rb
GAPDH
C
MK-2206 - + - + - +
OPC-B2 - - 50 50 75 75
cell number(10 4)
D
OPC-B2:μg/ml 0 75 125
Rictor
p-mTOR
mTOR
PI3K
p-PDPK
PDPK
GAPDH
E
F
Myc-AKT1 - + - - -
Myc-AKT1(R86A) - - + - -
Myc-AKT1(K297A) - - - + -
Myc-AKT1(R273A) - - - - +
p-AKT(S473)
p-AKT(T308)
Myc
p-GSK3β(S9)
GSK3β
cyclin D1
p-Rb(S780)
Rb
GAPDH
G
control
Myc-AKT1
Myc-AKT1(K297A)
Myc-AKT1(R86A)
cell number(10 4)
0 24 48 72 96(h)
H
Myc-AKT1 + + - - - -
Myc-AKT1(K297A) - - + + - -
Myc-AKT1(R86A) - - - - + +
MK-2206 - + - + - +
p-AKT(S473)
AKT
p-GSK3β(S9)
GSK3β
cyclin D1
p-Rb(S780)
Rb
GAPDH
I
cell number(10 4)
MK-2206 - + - + - + - +
Myc-AKT1 - - + + - - - -
Myc-AKT1 (K297A) - - - - + + - -
Myc-AKT1 (R86A) - - - - - - + +

Figure S3:

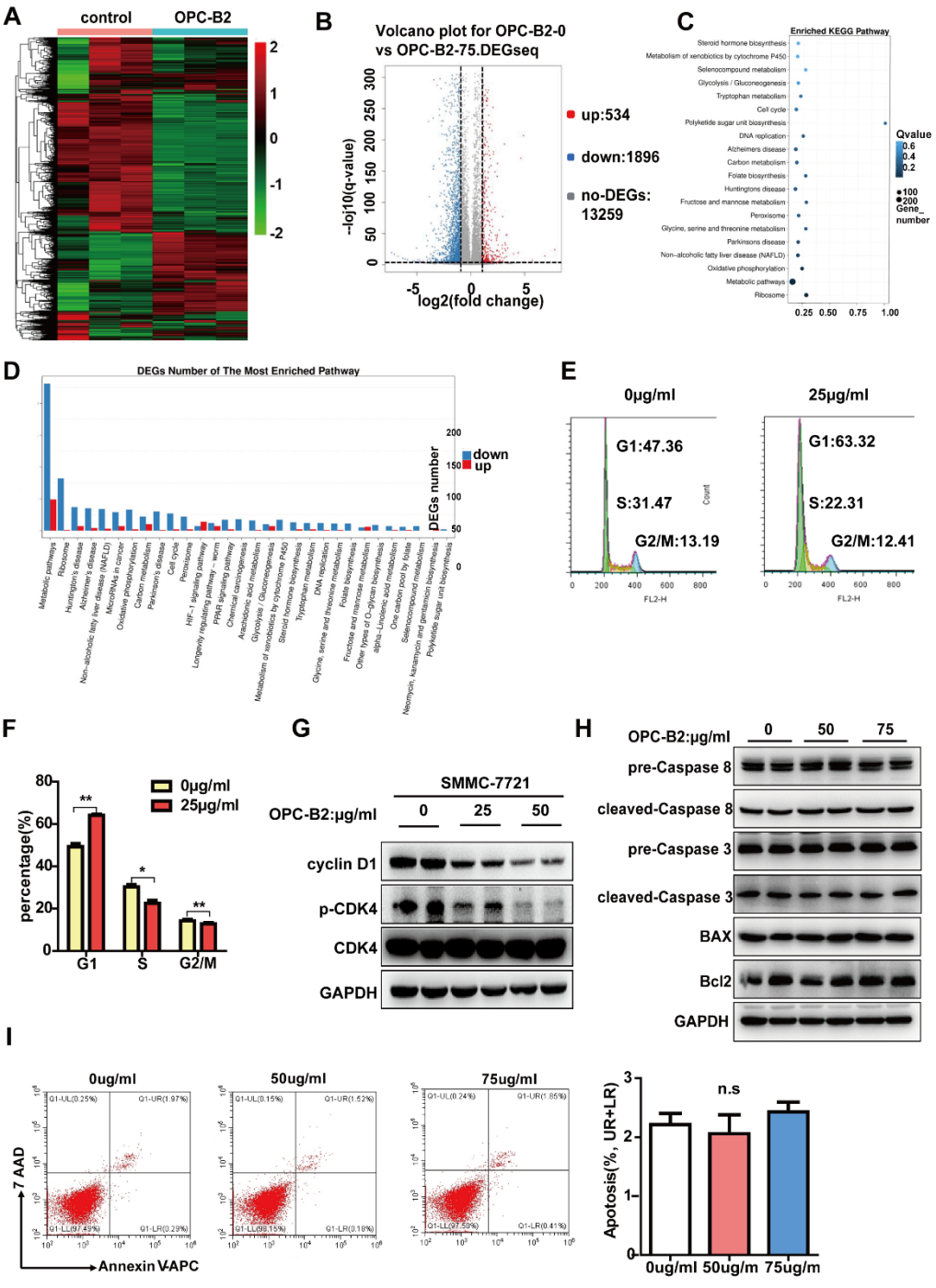
A
control
OPC-B2
2
1
0
-1
-2
B
Volcano plot for OPC-B2-0
vs OPC-B2-75.DEGseq
up:534
down:1896
no-DEGs:
13259
--log10(q-value)
log2(fold change)
C
Enriched KEGG Pathway
Steroid hormone biosynthesis
Metabolism of xenobiotics by cytochrome P450
Selenocompound metabolism
Glycolysis / Gluconeogenesis
Tryptophan metabolism
Cell cycle
Polyketide sugar unit biosynthesis
DNA replication
Alzheimers disease
Carbon metabolism
Folate biosynthesis
Huntingtons disease
Fructose and mannose metabolism
Peroxisome
Glycine, serine and threonine metabolism
Parkinsons disease
Non-alcoholic fatty liver disease (NAFLD)
Oxidative phosphorylation
Metabolic pathways
Ribosome
Qvalue
Gene_
number
D
DEGs Number of The Most Enriched Pathway
down
up
DEGs number
E
0μg/ml
25μg/ml
G1:47.36
S:31.47
G2/M:13.19
G1:63.32
S:22.31
G2/M:12.41
Count
FL2-H
F
0μg/ml
25μg/ml
percentage(%)
G1
S
G2/M
G
SMMC-7721
OPC-B2:μg/ml
0
25
50
cyclin D1
p-CDK4
CDK4
GAPDH
H
OPC-B2:μg/ml
0
50
75
pre-Caspase 8
cleaved-Caspase 8
pre-Caspase 3
cleaved-Caspase 3
BAX
Bcl2
GAPDH
I
0ug/ml
50ug/ml
75ug/ml
7 AAD
Annexin V-APC
n.s
Apotosis(%, UR+LR)
0ug/ml
50ug/m
75ug/m

Figure S4:

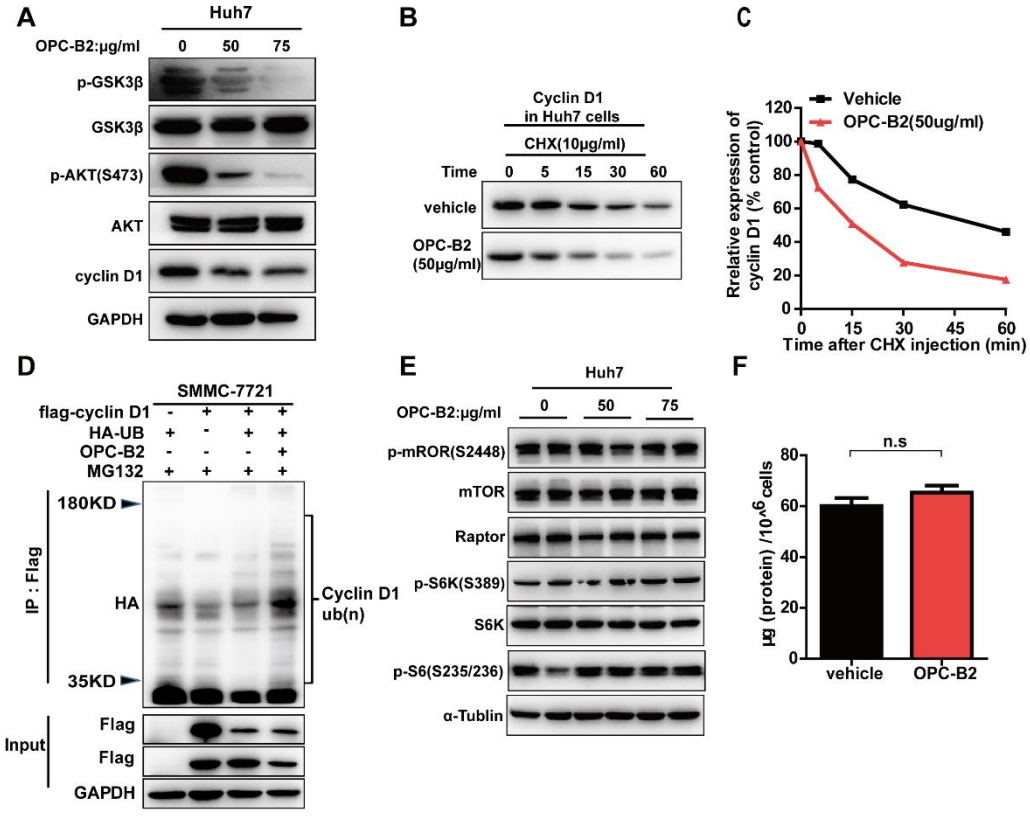
A
Huh7
OPC-B2:μg/ml 0 50 75
p-GSK3β
GSK3β
p-AKT(S473)
AKT
cyclin D1
GAPDH
B
Cyclin D1
in Huh7 cells
CHX(10μg/ml)
Time 0 5 15 30 60
vehicle
OPC-B2
(50μg/ml)
C
Vehicle
OPC-B2(50ug/ml)
Rrelative expression of
cyclin D1 (% control)
120
100
80
60
40
20
0
0 15 30 45 60
Time after CHX injection (min)
D
SMMC-7721
flag-cyclin D1 - + + +
HA-UB + - + +
OPC-B2 - - - +
MG132 + + + +
180KD
IP : Flag
HA
35KD
Cyclin D1
ub(n)
Input
Flag
Flag
GAPDH
E
Huh7
OPC-B2:μg/ml 0 50 75
p-mROR(S2448)
mTOR
Raptor
p-S6K(S389)
S6K
p-S6(S235/236)
α-Tublin
F
n.s
μg (protein) /10^6 cells
80
60
40
20
0
vehicle
OPC-B2

Figure S5:

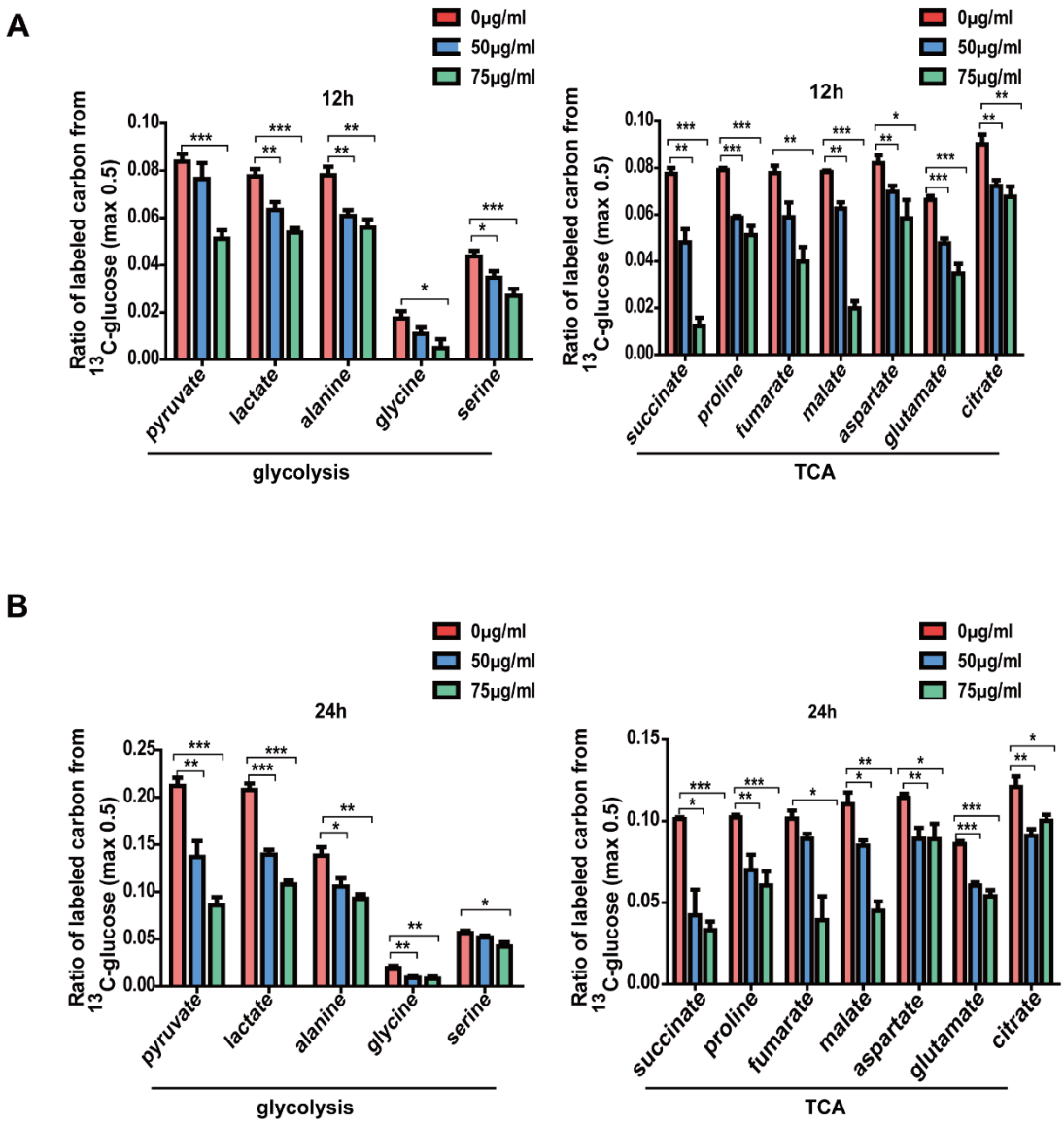
A
0μg/ml
50μg/ml
75μg/ml
12h
Ratio of labeled carbon from 13C-glucose (max 0.5)
pyruvate
lactate
alanine
glycine
serine
glycolysis
succinate
proline
fumarate
malate
aspartate
glutamate
citrate
TCA
B
24h

Figure S6:

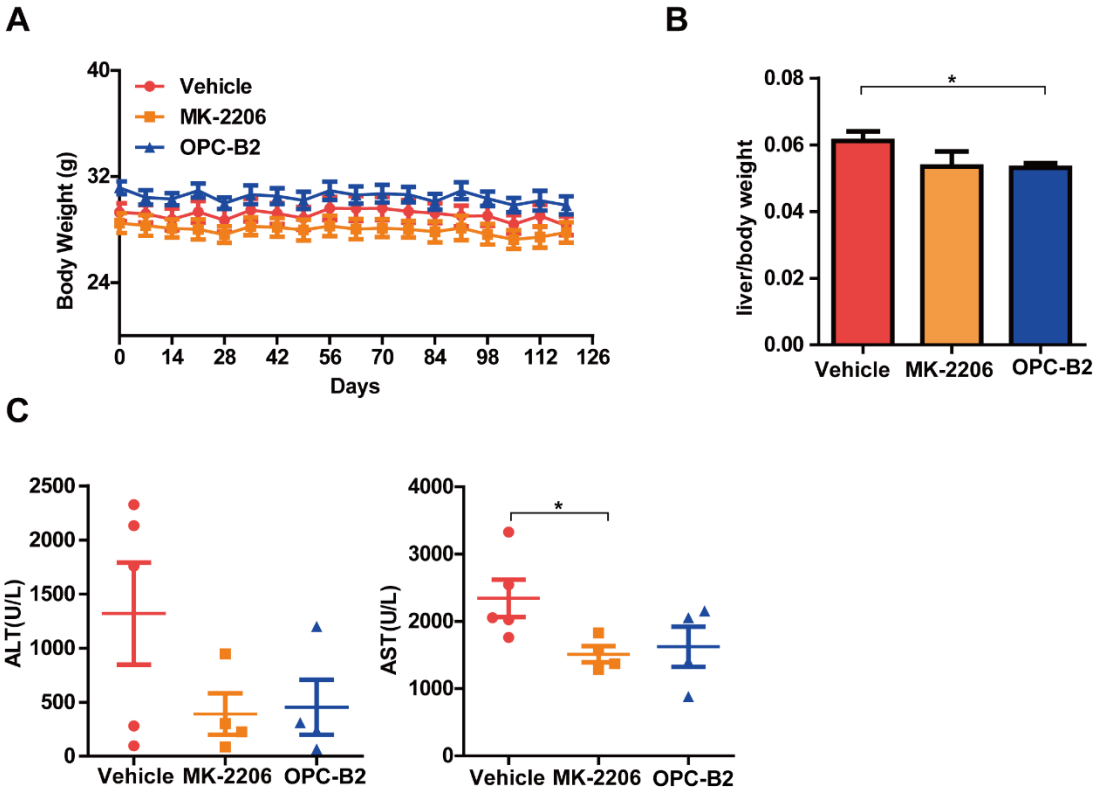
A
Vehicle
MK-2206
OPC-B2
Body Weight (g)
40
32
24
0
14
28
42
56
70
84
98
112
126
Days
B
liver/body weight
0.08
0.06
0.04
0.02
0.00
*
Vehicle
MK-2206
OPC-B2
C
ALT(U/L)
2500
2000
1500
1000
500
0
Vehicle
MK-2206
OPC-B2
AST(U/L)
4000
3000
2000
1000
0
*
Vehicle
MK-2206
OPC-B2

Table S1: The total interaction energy between ligands and receptor using molecular dynamic simulation

Ligand	Receptor	Total Interaction Energy (kcal/mol)	Total VDW Interaction Energy (kcal/mol)	Total Electrostatic Interaction Energy (kcal/mol)
OPC-B2	AKT1 (3O96)	-464.02753	-28.23306	-435.79447

Table S2: The specific information of the interaction sites and energies between the ligands and receptor in molecular dynamic simulation of OPC-B2 and AKT1

Ligands	Residue of 3O96	Interaction Energy (kcal/mol)	VDW Interaction Energy (kcal/mol)	Electrostatic Interaction Energy (kcal/mol)
OPC-B2	3_ASN54	-20.908554	-1.301405	-19.607149
	3_VAL83	2.324731	-0.402495	2.727226
	3_ILE84	-12.937332	-5.639400	-7.297932
	3_ARG86	-51.251030	-1.543698	-49.707333
	3_TYR272	-0.513667	0.790376	-1.304042
	3_ARG273	-94.616402	-6.316353	-88.300049
	3_ASP274	-8.820487	-3.979322	-4.841166
	3_GLY294	-39.505688	-1.613553	-37.892136
	3_LEU295	-26.351521	-0.321499	-26.030020
	3_CYS296	-32.409126	-2.410020	-29.999105
	3_LYS297	-106.833504	-1.890340	-104.943161
	3_GLU298	37.690502	1.080339	36.610165
	W_TIP1209	-6.077793	-0.863999	-5.213795
	W_TIP1817	-1.599456	-0.786087	-0.813368
	W_TIP4017	-4.126231	-0.852174	-3.274057
	W_TIP4019	-26.651405	-0.246958	-26.404446
	W_TIP4281	-6.561630	-1.043406	-5.518224
	W_TIP4293	-19.429232	0.078444	-19.507675
	W_TIP6149	-34.492458	-0.572381	-33.920074
	W_TIP7839	-10.957253	-0.399127	-10.558125

*3 is the abbreviation of AKT1 (3O96) and OPC represents procyanidine. In 3_XXX123, 3 represents AKT1(3O96), XXX represents the abbreviation of amino acid molecule, 123 represents the serial number of this amino acid molecule on AKT1.

Table S3: Reagents used in this study, related to Figure 1-5 and Figure S1-6.

Reagents	Source	Cat
MK-2206	Selleck	S1078
His-AKT1 Recombinant protein	Merck	14-276-D
cell counting Kit-8 (CCK8)	Bimake	B34304
Enhanced BCA Protein Assay Kit	Beyotime Biotechnology	P0009
Endotoxin-free plasmid DNA small-lifting medium kit	LifeFeng	DK312-01
TIANpure Mini Plasmid Kit	TIANGEN	DP1-7-02
[U-$^{13}C_6$]-glucose	Cambridge Isotope Laboratory	PR-28579
MG132	TOCRIS Bioscience	1748
Trypsin 0.25% EDTA	Invitrogen	25200056
LIPOFECTAMINE 2000	Invitrogen	11668019
Opti-MEM® Reduced	Invitrogen	11058-021
Proanthocyanidin B2	sigma	42157-1MG-F
D-Glucose Kit	R-Biopharm	10716251035
Cell Cycle and Apoptosis Analysis Kit	Beyotime	C1052
O-Isobutylhydroxylamine Hydrochloride	TCI	6084-58-8
N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide	Sigma	00942
FITC Annexin V Apoptosis Detection Kit I	BD Pharmingen	556547

Table S4: Antibodies used in this study, related to Figure 2-5 and Figure S2-4.

Antibodies	Source	Cat	Dilution ratio
Phosphor-AKT(Ser473)	cell signaling	13038	1:1000
phosphor-AKT (Thr308)	cell signaling	4060	1:1000
AKT	cell signaling	4685	1:1000
Phosphor-p44/42MAPK(ERK1/2) (Thr202/Tyr204)	cell signaling	4370	1:1000
p44/42MAPK(ERK1/2)	cell signaling	4695	1:1000
Phosphor-AMPKα (Thr172)	cell signaling	2535	1:1000
AMPKα	cell signaling	2532	1:1000
cyclin D1	Abcam	ab134175	1:1000
Rb	cell signaling	9313	1:1000
phosphor-Rb (Ser780)	cell signaling	8180	1:1000
phosphor-CDK4 (Thr172)	SAB	12403	1:500
CDK4	Proteintech	11026-1-AP	1:1000
Phosphor-GSK3β (Ser9)	cell signaling	5558	1:1000
GSK3β	cell signaling	12456	1:1000
Hexokinase 1 (HK1)	Proteintech	19662-1-AP	1:1000
HA-Tag (C29F4) Rabbit mAb	cell signaling	3724	1:1000
phosphor-mTOR(Ser2448)	cell signaling	5536	1:1000
mTOR	cell signaling	2972	1:1000
Rictor	cell signaling	9476	1:1000
PI3K	Proteintech	20584-1-AP	1:1000
phosphor-PDPK1	cell signaling	3438	1:1000
PDPK1	cell signaling	5652	1:1000
GAPDH	Proteintech	60004-1-AP	1:5000
Raptor	Proteintech	20984-1-AP	1:1000
phosphor-S6K(Thr398)	cell signaling	9209	1:1000
S6K	cell signaling	2708	1:1000
phosphor-S6(S235/236)	cell signaling	4858	1:1000
BAX	Proteintech	50599-2-lg	1:1000
BCL2	Proteintech	12789-1-AP	1:1000
Caspase 8	Proteintech	13423-1-AP	1:1000
Caspase 3	Proteintech	19677-1-AP	1:1000
α-tublin	Proteintech	11224-1-AP	1:1000
β-actin	Proteintech	60008-1-AP	1:5000
HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L)	Proteintech	SA00001-2	1:5000
Goat anti-mouse IgG (H+L), HRP conjugated	Proteintech	SA00001-1	1:5000
DYKDDDDK (Flag)-Tag Mouse	Abmart	M20008	1:1000
IPKine™ HRP conjugated Goat Anti-Mouse IgG light chain specific secondary antibody	Abbkine	A25012	1:5000