Supplemental Tables and Figures
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Synthesis and Evaluation of a Novel Deguelin Derivative, L80, which Disrupts ATP Binding to the C-terminal Domain of Heat Shock Protein 90

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Table S1. BBB permeability of deguelin, L80, progesteron, and theophylline as determined by the Parallel Artificial Membrane Permeability assay.

Compound	-log *Pe**	CNS+/- prediction
Degueline	4.78±0.20	+
L80	4.82±0.25	+
Progesteron†	4.62±0.11	+
Theophylline†	6.77±0.30	-

*Average −log *P*e values were calculated by the PAMPA Explorer software v. 3.5.
† Progesteron, and theophylline were selected for comparison based on their known CNS activity

Table S2. Histology and genetic background of used cell lines

	EGFR	K-Ras	p53	PTEN	PI3KCA	LKB1	B-Raf
A549	WT	Mut	WT	WT	WT	Mut	WT
H226B	WT	WT	WT	U	WT	WT	WT
H1299	WT	WT	Nu	Nu	WT	WT	WT
H460	WT	Mut	WT	WT	Mut	Mut	WT

* WT: wild type Mut: mutnat type Nu: null U: unknown

Supplemental Figure Legends

Figure S1. Toxicity of L80 compared to deguelin (A) Blood-Brain-Barrier permeability assay was conducted by using the PAMPA Explorer kit (*p*ION Inc.) Progesteron, and theophylline were used as positive, and negative control. (Pro: progesterone, Theo: theophylline, Deg: deguelin) (B-D) Mouse hppocampal cell (HT-22 cell), Human umbilical vein endothelial (HUVEC), retinal pigment epithelial (RPE), and normal immortalized lung epithelial cells (HBEC and BEAS-2B) were treated with increasing concentrations of L80 or deguelin for 3 days. Values represent mean ± SD of experiments conducted in triplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by one-way analysis of variance (ANOVA) or Student's t-test compared with control group.

Figure S2. Inhibitory effects of L80 on the proliferation and colony formation of human lung cancer cells. (A) H1299, A549, H460, and H226B cells; paclitaxel-resistant lung cancer cells (H460/R and H226B/R); and were treated with increasing concentrations of L80 or deguelin for 3 days. Cell viability was determined by the MTT assay. (B) H1299, A549, H460, H460/R, H226B, and H226B/R cells were seeded onto 6-well plates at a density of 300 cells/well and then treated with L80 (0, 0.1, 1, and 10 μM). After two weeks, colonies were stained with crystal violet and counted. (C) The anchorage-independent growth of cells treated with increasing concentrations of L80 was determined by soft agar colony formation assay. All values represent mean ± SD of experiments conducted in triplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by one-way analysis of variance (ANOVA) compared with control group.

Figure S3. Induction of apoptosis by treatment with L80. (A) Cells were treated with L80 for 24 h. The distribution of cells in each phase of the cell cycle was analyzed by flow cytometry.

Figure S4. L80 exerts its antiangiogenic potential through inhibiting the stability and transcriptional activity of HIF-1α. (A) The HIF-1α expression level in the indicated NSCLC cells were treated with increasing concentrations of L80 under hypoxia condition was analyzed by Western blot analysis. (B) The indicated NSCLC cells were co-transfected with pGL3-basic or pGL3-HRE-Luc and pRL-TK-Luc. After treatment with L80 under hypoxia condition, luciferase activity was monitored as described in the Materials and Methods section. Values represent mean ± SD of experiments conducted in triplicate. *$p < 0.05$ by Student's t-test compared with control group. (C, D) The indicated NSCLC cells treated with increasing concentrations of L80 (C) or H1299 treated with 1 μM of deguelin or L80 (D) were subjected to RT-PCR for the mRNA expression of VEGF and IGF2. (E) HUVECs were treated with CM obtained from H1299 cells treated with increasing concentrations of L80 and incubated under hypoxic conditions. The morphological changes of HUVECs were imaged and scored. Values represent mean ± SD of experiments conducted in triplicate. *$p < 0.05$, ***$p < 0.001$ by one-way analysis of variance (ANOVA) compared with control group (0μM of L80).

Figure S5. Inhibition of migration and invasion of lung cancer by treatment with L80. H1299, H226B, and H226B/R cells were seeded onto 96-well plates and incubated for 12h to evaluate proliferation effects by MTT assay (A). These cells also were seeded onto Transwells coated with either gelatin only (B) or gelatin and matrigel (C). After incubation for 18-19h, the migratory or invaded cells on the bottom of the membrane were stained and counted. All values represent mean ± SD of experiments conducted in triplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Student′s *t*-test compared with control group.

Figure S6. The effect of L80 on body weight in a tumor xenograft model. H1299 cells were inoculated into the right flank of NOD/SCID mice. Mice harboring H1299 xenograft tumors were treated with vehicle or L80 for 3 weeks. The body weight were monitored on 1day, and 20days. Body weight was analyzed by one-way analysis of variance (ANOVA) compared with control group (0mg/kg).

Figure S7. Downregulation of Hsp90 function by treatment with L80. (A) H1299 cells were treated with L80 under hypoxic conditions. Total cell lysates were prepared and immunoprecipitated with anti-HIF-1α antibodies. The interaction between HIF-1α and Hsp90 was analyzed by Western blot analysis. (B) H1299 lysates interacted with histidine-tagged recombinant HIF-1α in the presence or absence of L80. After pull-down with Ni-NTA agarose beads, the amount of Hsp90 bound to the recombinant HIF-1α was determined by Western blot analysis. (C) The expression levels of ErbB2 and Akt in the indiated NSCLC cells treated with increasing concentrations of L80 were analyzed by Western blot analysis. (D) Recombinant Hsp90 proteins containing the FL protein or the NM, M, and C domains interacted with ATP-agarose in the presence or absence of L80. The protein level bound to the ATP-agarose beads was determined by Western blot analysis.

Figure S8. Full blots of cleaved parp in NSCLC cells. H1299, H226B,H460, H226B/R, and H460/R cells were seeded and treated with 0, 1, and 10 μM L80 for 24 h. Cell lysates were obtained by RIPA buffer and determined by Western blot analysis.

Figure S1

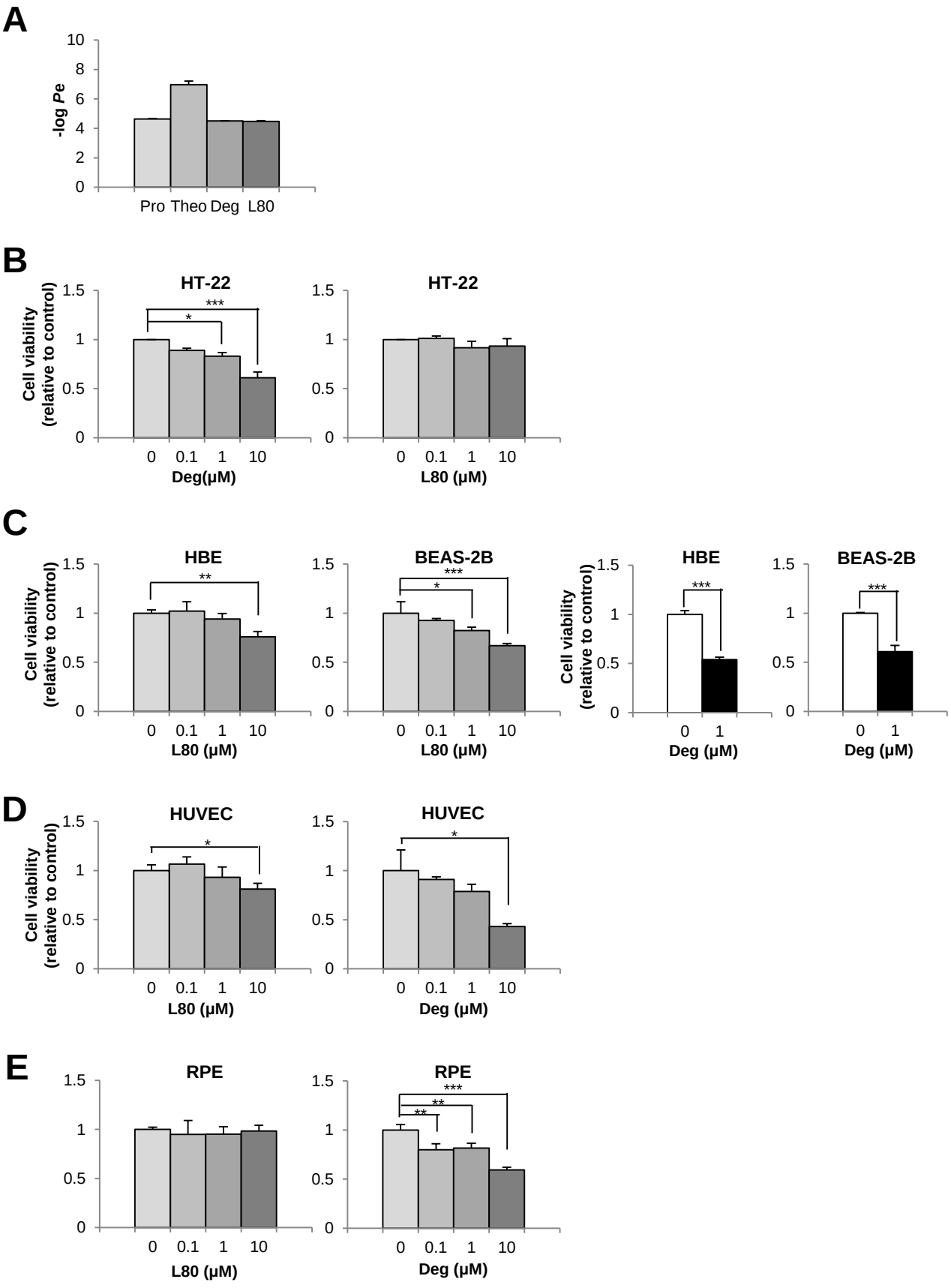

A
-log Pe
Pro Theo Deg L80
B
HT-22
HT-22
Cell viability (relative to control)
Deg(μM)
L80 (μM)
C
HBE
BEAS-2B
HBE
BEAS-2B
L80 (μM)
Deg (μM)
D
HUVEC
HUVEC
L80 (μM)
Deg (μM)
E
RPE
RPE
L80 (μM)
Deg (μM)

Figure S2

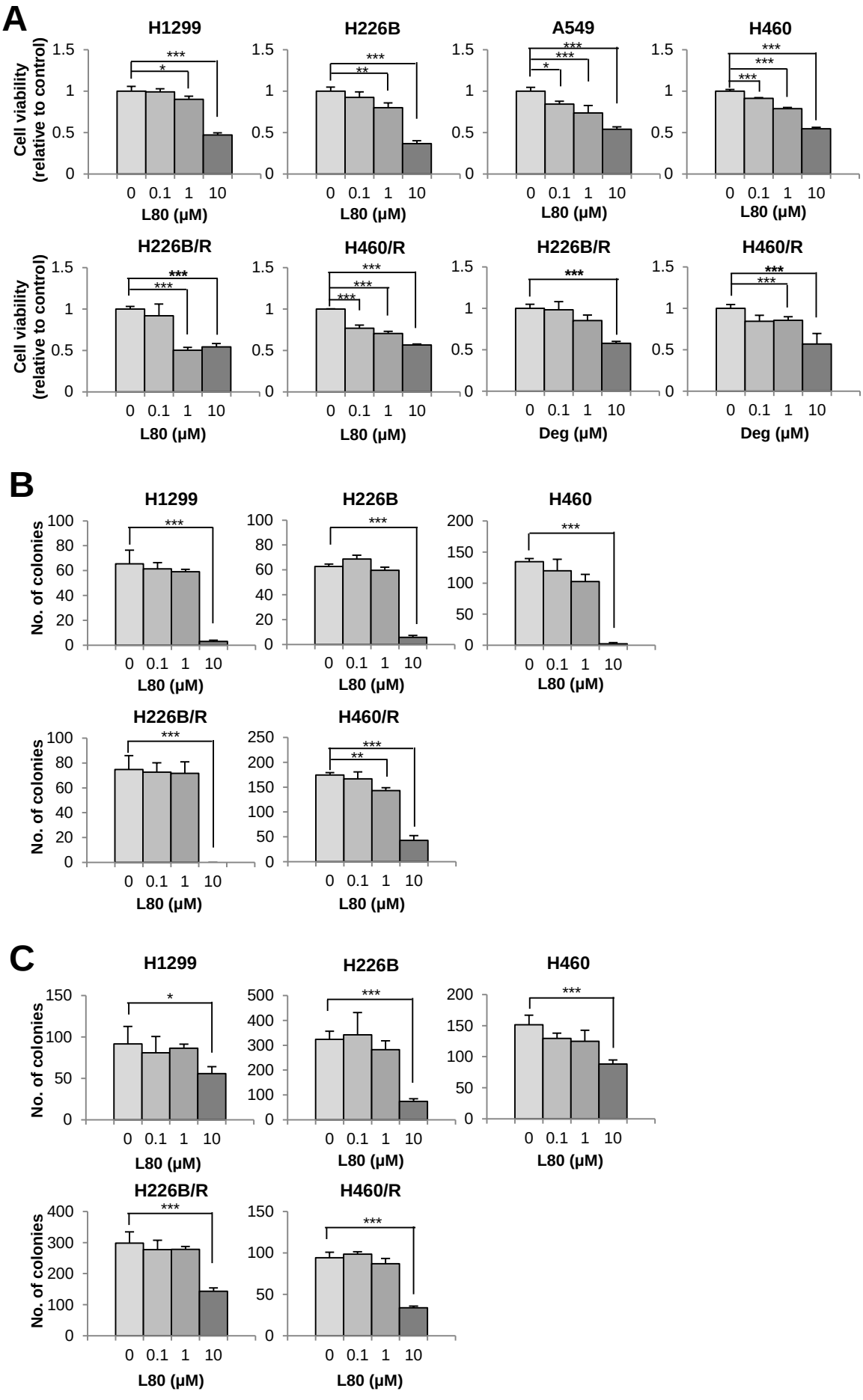

A
H1299
H226B
A549
H460
H226B/R
H460/R
Cell viability (relative to control)
L80 (μM)
Deg (μM)
B
No. of colonies
C

Figure S3

A

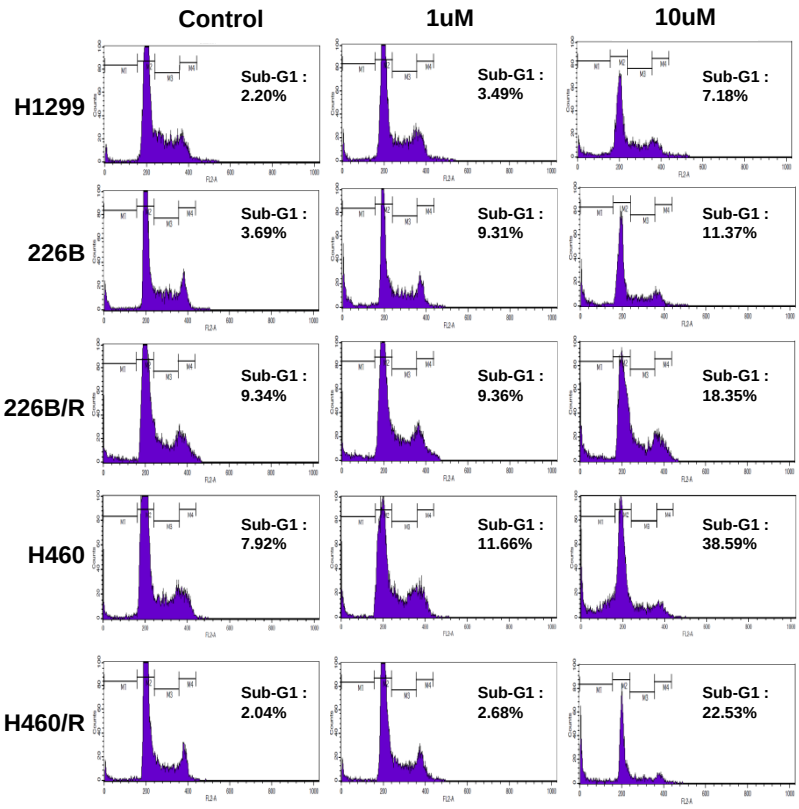

Control
1uM
10uM
H1299
Sub-G1 : 2.20%
Sub-G1 : 3.49%
Sub-G1 : 7.18%
226B
Sub-G1 : 3.69%
Sub-G1 : 9.31%
Sub-G1 : 11.37%
226B/R
Sub-G1 : 9.34%
Sub-G1 : 9.36%
Sub-G1 : 18.35%
H460
Sub-G1 : 7.92%
Sub-G1 : 11.66%
Sub-G1 : 38.59%
H460/R
Sub-G1 : 2.04%
Sub-G1 : 2.68%
Sub-G1 : 22.53%

Figure S4

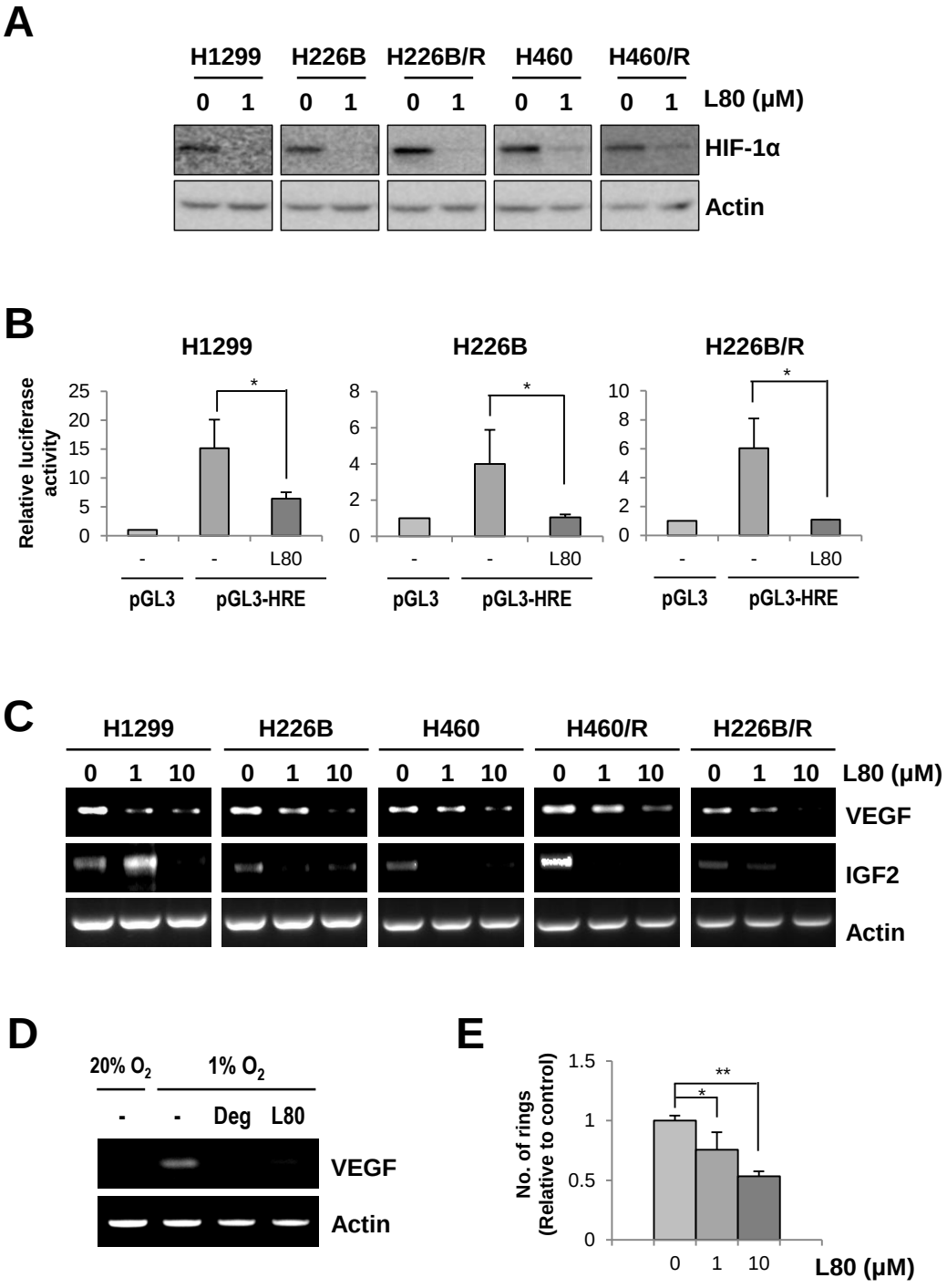

A
H1299
H226B
H226B/R
H460
H460/R
0 1 0 1 0 1 0 1 0 1
L80 (μM)
HIF-1α
Actin
B
H1299
H226B
H226B/R
Relative luciferase activity
25
20
15
10
5
0
8
6
4
2
0
10
8
6
4
2
0
*
- - L80
pGL3
pGL3-HRE
C
H1299
H226B
H460
H460/R
H226B/R
0 1 10 0 1 10 0 1 10 0 1 10 0 1 10
L80 (μM)
VEGF
IGF2
Actin
D
20% O2
1% O2
- - Deg L80
VEGF
Actin
E
No. of rings (Relative to control)
1.5
1
0.5
0
**
*
0 1 10
L80 (μM)

Figure S5

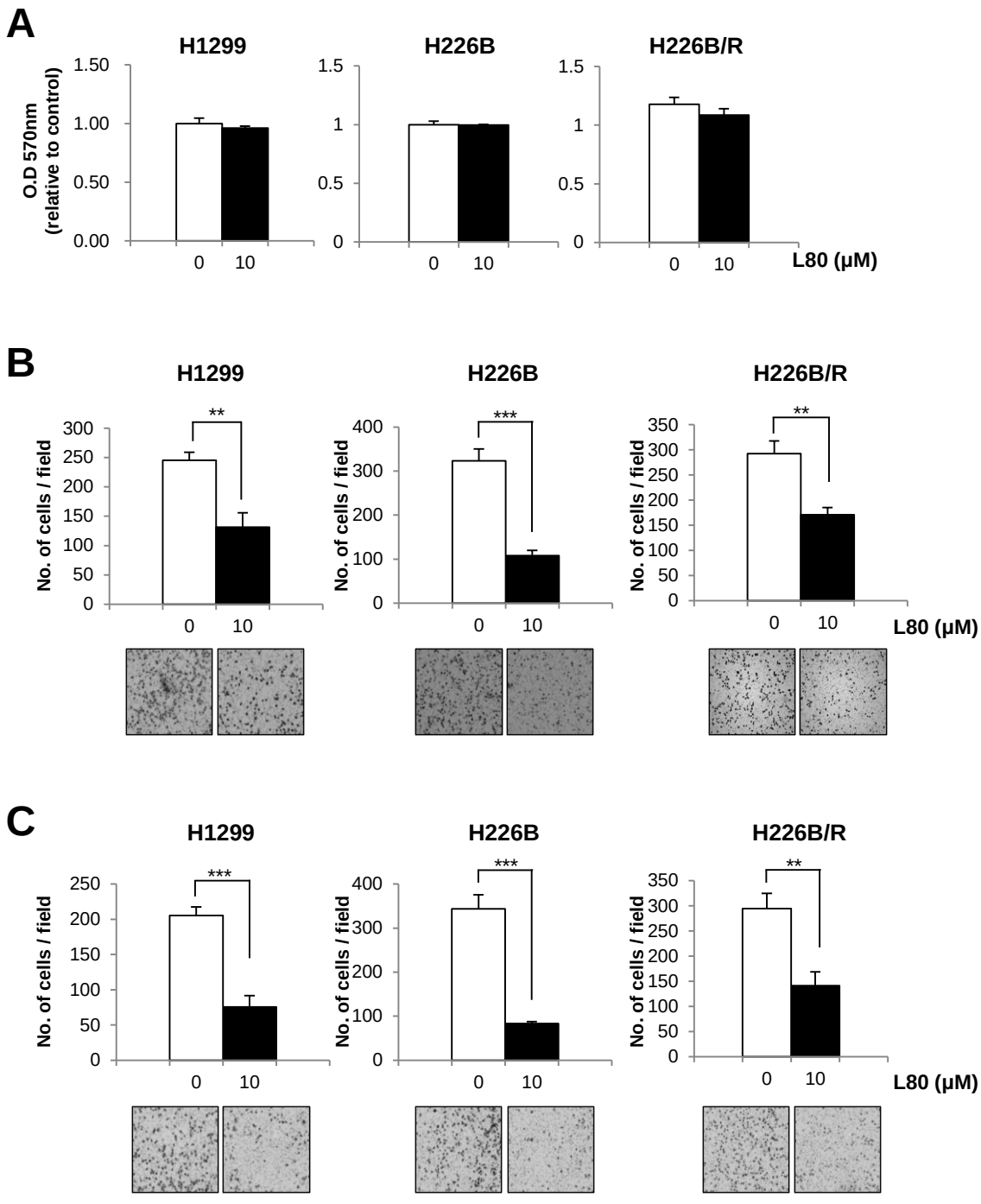
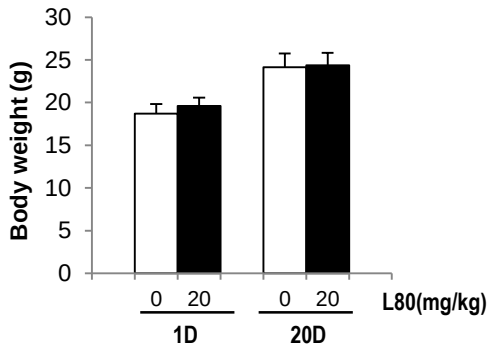

A
H1299
H226B
H226B/R
O.D 570nm
(relative to control)
1.50
1.00
0.50
0.00
1.5
1
0.5
0
0
10
L80 (μM)
B
H1299
H226B
H226B/R
No. of cells / field
**
***
**
300
250
200
150
100
50
0
400
350
0
10
L80 (μM)
C
H1299
H226B
H226B/R
No. of cells / field
***
***
**
250
200
150
100
50
0
400
350
300
0
10
L80 (μM)

Figure S6



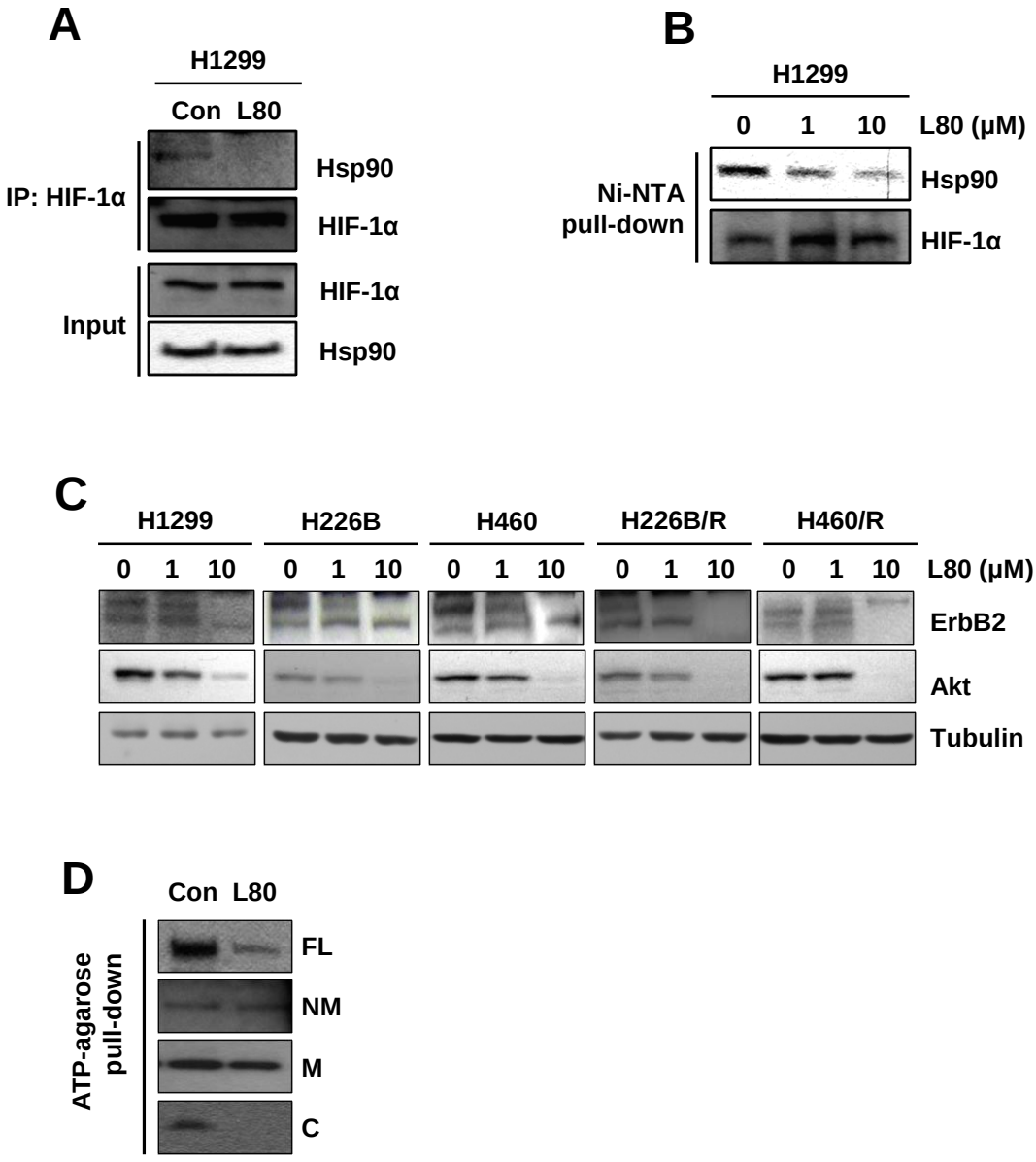
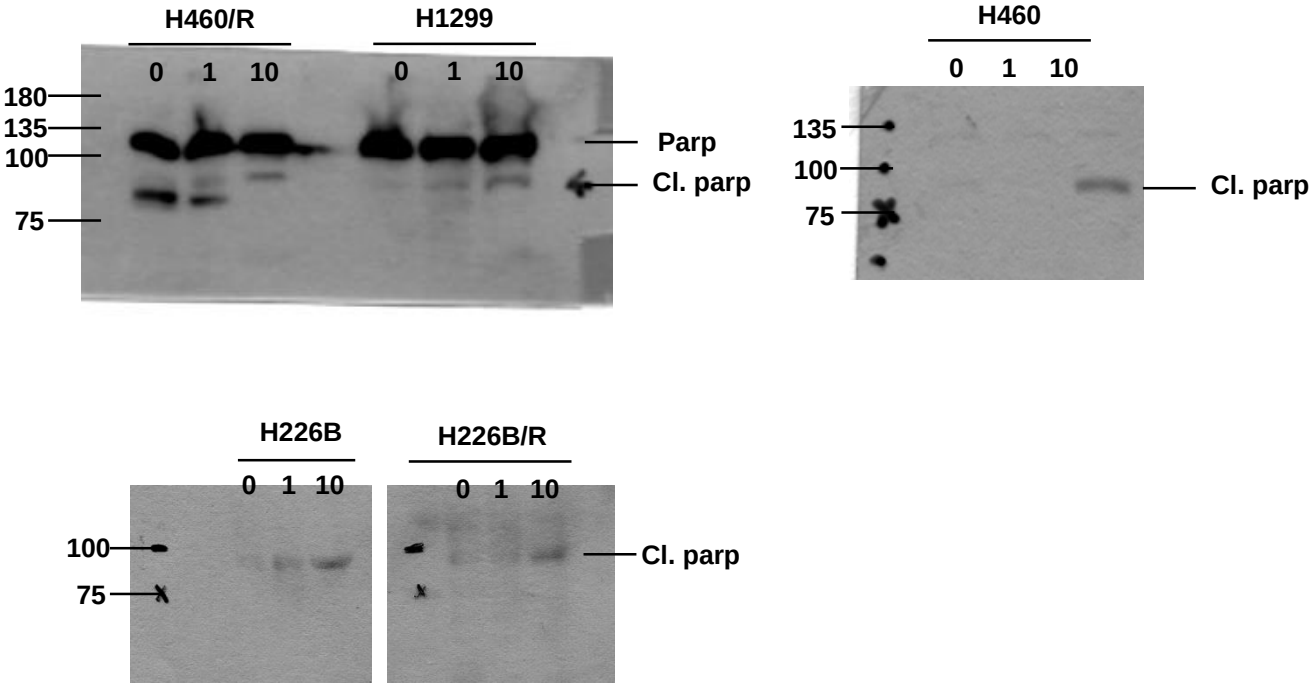

A
H1299
Con L80
IP: HIF-1α
Hsp90
HIF-1α
Input
HIF-1α
Hsp90
B
H1299
0 1 10 L80 (μM)
Ni-NTA pull-down
Hsp90
HIF-1α
C
H1299 H226B H460 H226B/R H460/R
0 1 10 0 1 10 0 1 10 0 1 10 0 1 10 L80 (μM)
ErbB2
Akt
Tubulin
D
Con L80
ATP-agarose pull-down
FL
NM
M
C

Figure S8


H460/R
H1299
0 1 10
0 1 10
180
135
100
75
Parp
Cl. parp
H460
0 1 10
135
100
75
Cl. parp
H226B
H226B/R
0 1 10
0 1 10
100
75
Cl. parp