*Supplementary Material*

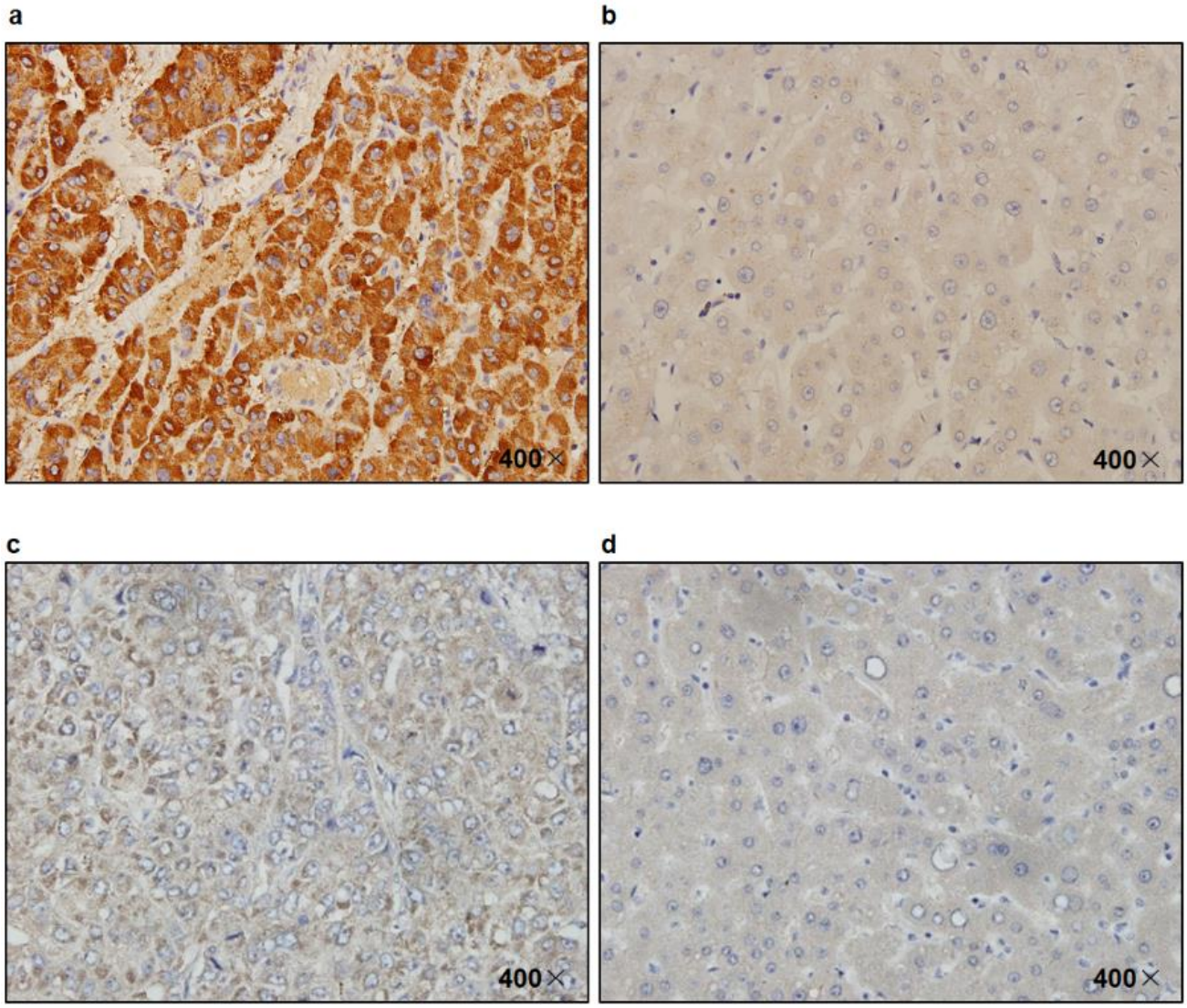
# SMAC mimetic APG-1387 targets the inhibitor of apoptosis proteins and sensitizes immune-mediated cell apoptosis in hepatocellular carcinoma

**Zide Chen[1], Jiehua Chen[1], Hongyan Liu[1], Wei Dong[2], Xuan Huang[1], Dajun Yang[3], Jinlin Hou[1], Xiaoyong Zhang[1,*]**

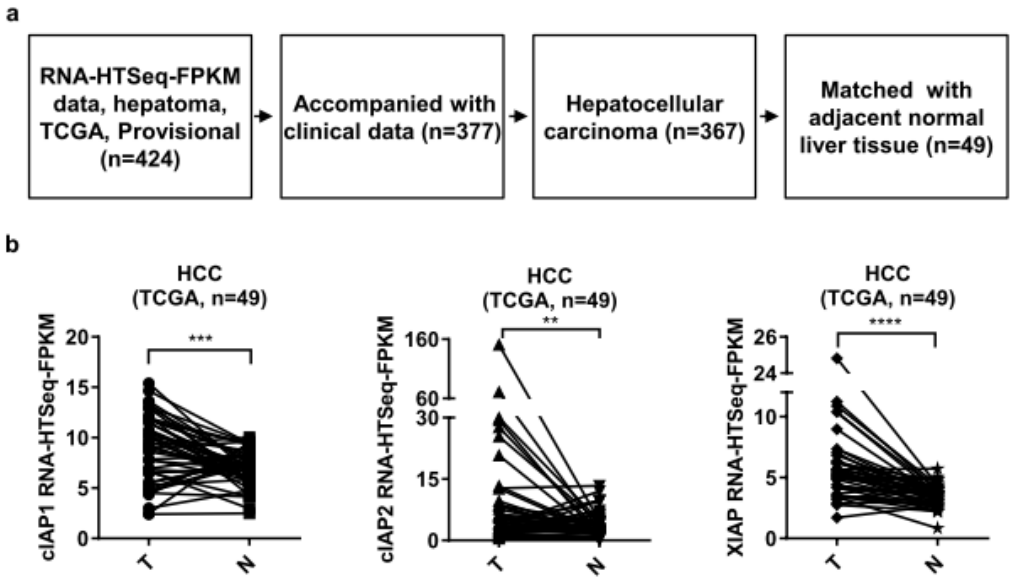
*** Correspondence:** Xiaoyong Zhang: xiaoyzhang@smu.edu.cn

## Supplementary Figures and Tables

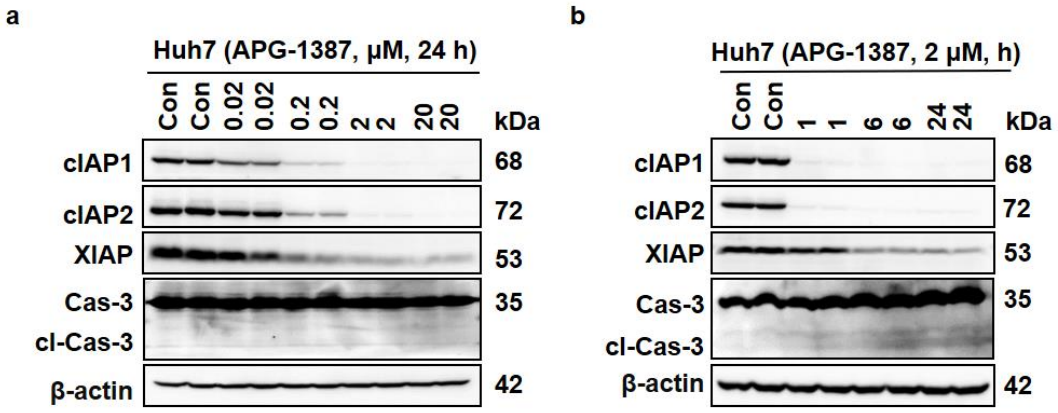
### 1 Supplementary Figures



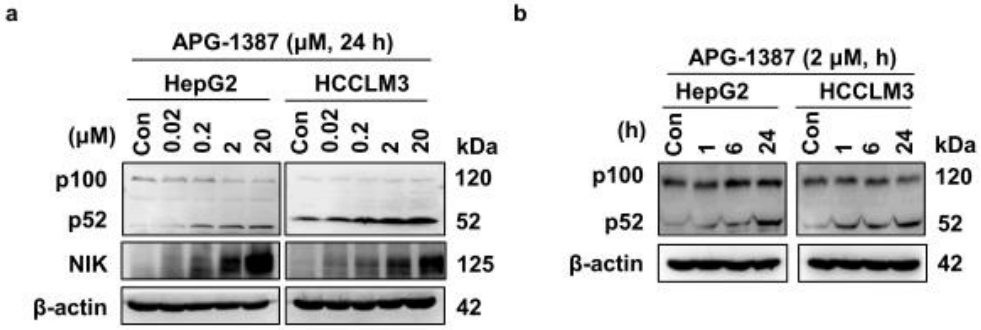
**Supplementary Figure 1.** The localization of cIAP1 and cIAP2 proteins in HCC tumor tissue. Immunohistochemical analysis of paraffin-embedded HCC (**a, c**) and adjacent normal liver tissue (**b, d**) showed that cIAP1 (**a, b**) and cIAP2 (**c, d**) were mainly located in the cytoplasm of hepatoma cells and normal hepatocytes. 400 × , 400 × magnification.



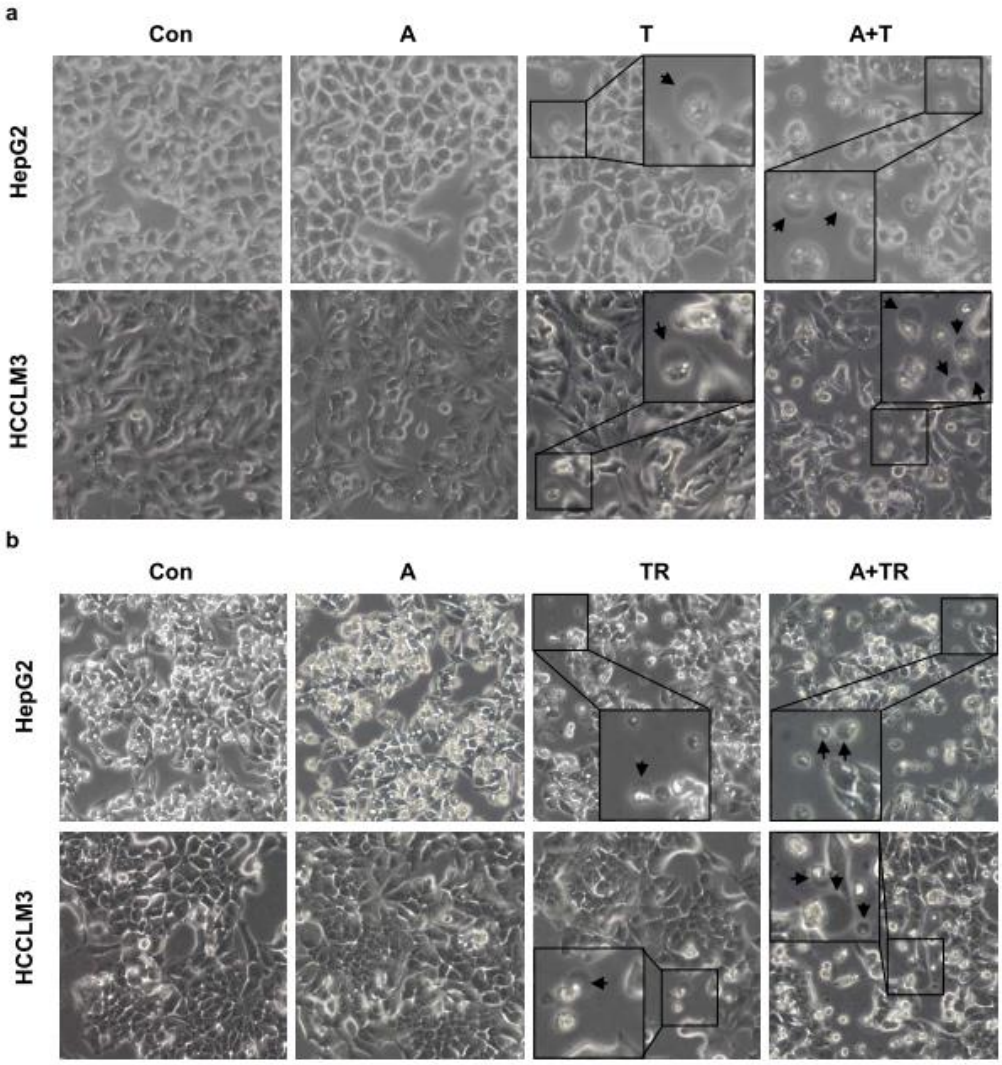

**Supplementary Figure 2.** Inhibitor of apoptosis protein (*IAP*) genes were highly expressed in HCC tissue. (**a**) Screening strategy of HCC patients from the cancer genome atlas (TCGA) database (updated until Jun 02, 2016; http://tcga-data.nci.nih.gov/). (**b**) The expressions of *cIAP1*, *cIAP2*, and *XIAP* mRNA in tumor and normal adjacent liver tissue from 49 HCCs were compared. RNA-HTSeq-FPKM, RNA-high throughput sequencing-fragments per kilobase of exon per million mapped reads; T, HCC tumor tissue; N, normal adjacent liver tissue. ** p <0.01, *** p <0.001, *** p <0.0001, by two-tailed pair t-test.



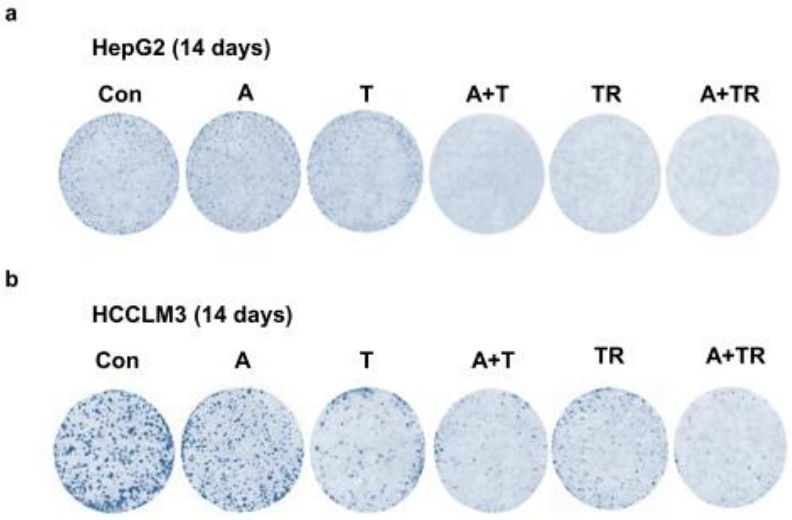

**Supplementary Figure 3.** The influence of APG-1387 on IAPs and caspase-3 in HCC cell line, Huh7, as a single drug. (**a, b**) Huh7 cells were seeded at $1.5 \times 10^6$ cells per well in 6-well plates for 12 hours. After 24 hours of stimulation with 0, 0.02, 0.2, 2, 20 μM APG-1387 respectively, or 0, 1, 6, 24 hours stimulation of 2 μM APG-1387, lysates of Huh7 cells were collected and analyzed by Western blot to show the changes in cIAP1, cIAP2, XIAP and caspase-3 protein levels. Con, control; Cas-3, caspase-3; kDa, kilodalton.



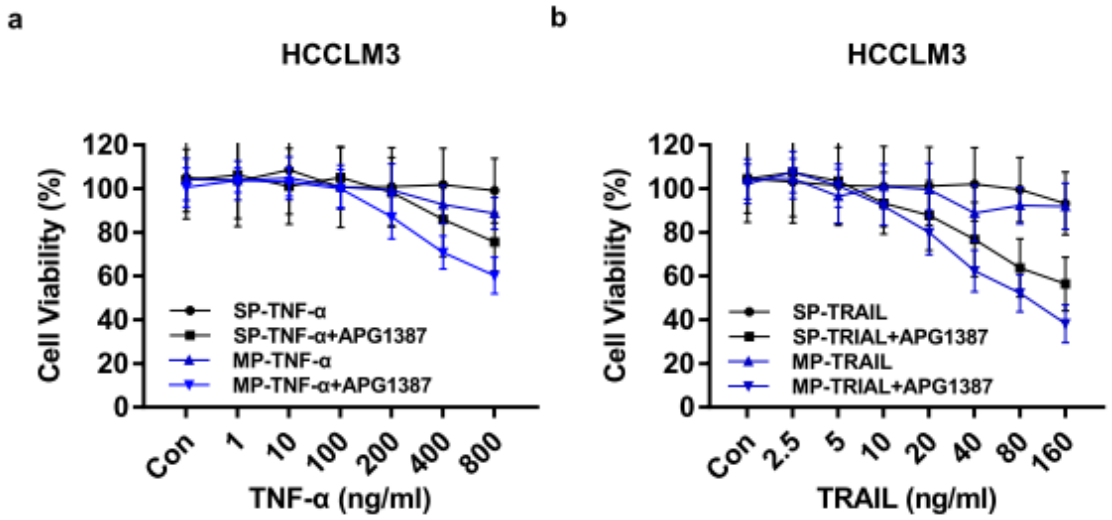

**Supplementary Figure 4.** APG-1387 activated non-classical NF-κB signaling of HCC cell lines. HepG2 and HCCLM3 cells were seeded at $1.5 \times 10^6$ cells per well in 6-well plates for 12 hours. (**a, b**) After stimulating with different concentrations of APG-1387 for specified times, lysates of HepG2 and HCCLM3 cells were collected. The protein levels of p100, p52 and NF-κB inducing kinase (NIK) were analyzed by Western blot. Con, control; kDa, kilodalton.



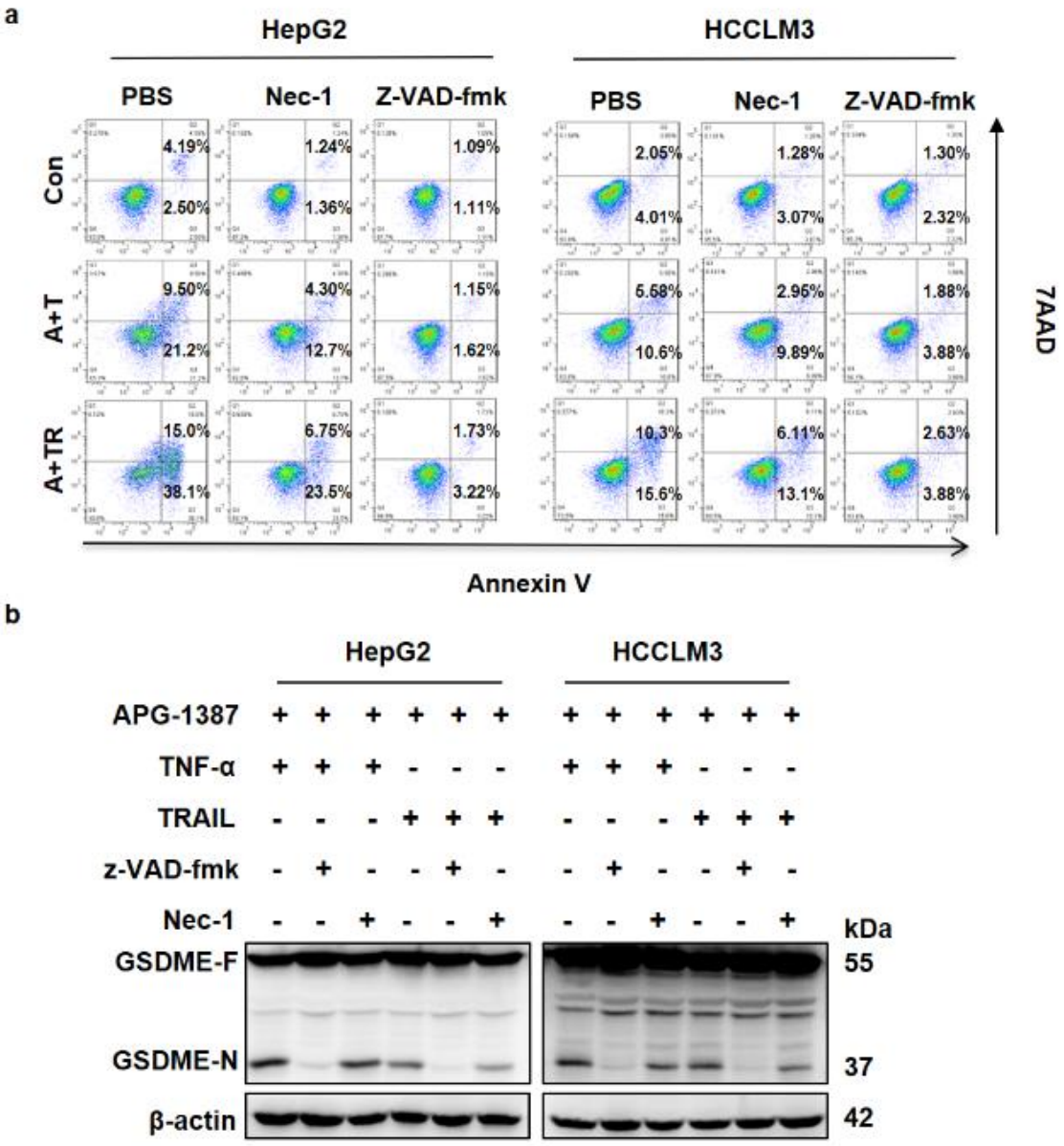

**Supplementary Figure 5.** The morphology changes of HCC cells, HepG2 and HCCLM3, after stimulation with APG-1387 and TNF-α or TRAIL. (**a**) HepG2 and (**b**) HCCLM3 cells were seeded at $1 \times 10^6$ cells per well in 6-well plates for 12 hours. After 24 hours of stimulation with APG-1387 and TNF-α or TRAIL, the morphologic alterations of HepG2 or HCCLM3 were captured under inverted optical microscope (magnification, 100×). The arrows in the enlarged part (magnification, 400×) of the picture showed the images of cell necrosis. Con, control; A, 2 μM APG-1387; T, 100 ng/ml TNFα; TR, 20 ng/ml TRAIL.



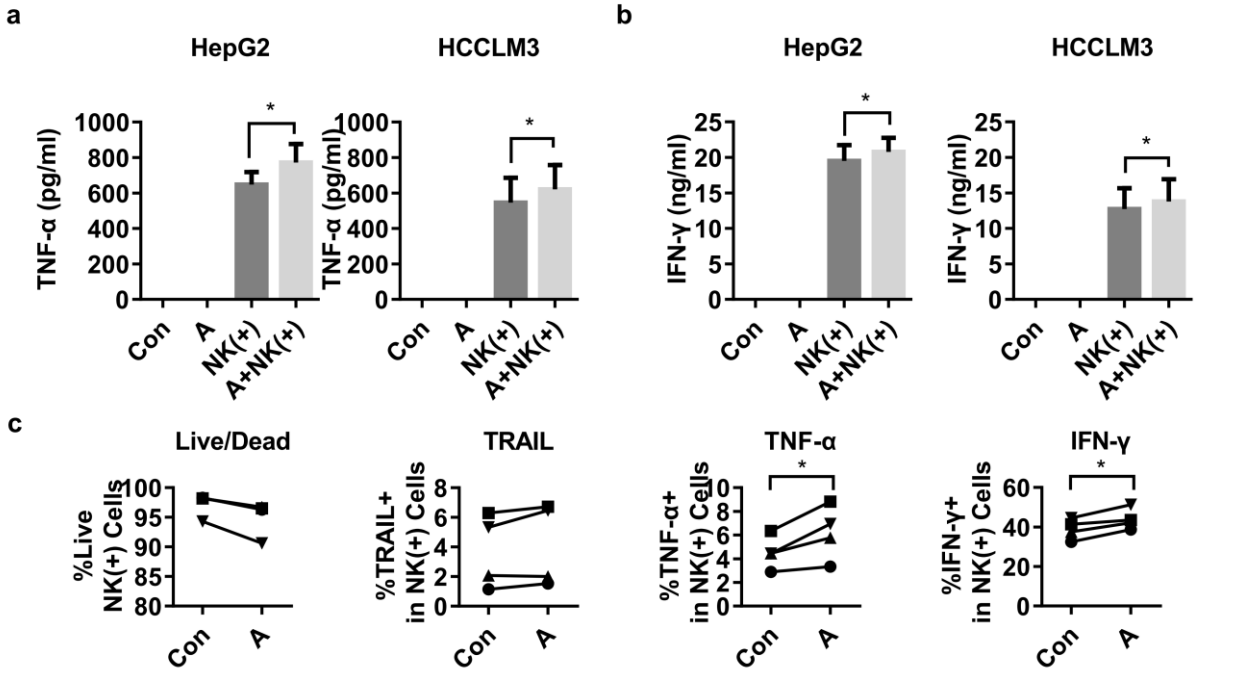

**Supplementary Figure 6.** APG-1387 treatment enhanced the inhibitory effect of TNF-α or TRAIL on the proliferation of HCC cells. (**a**) HepG2 and (**b**) HCCLM3 cells were pre-seeded in 6-well plates at 1,000 cells per well for 12 hours and then stimulated with 2 μM APG-1387, 100 ng/ml TNF-α, 20 ng/ml TRAIL or their combination. After incubating for 14 days, the colonies were stained with Giemsa dye and counted with Image J software.



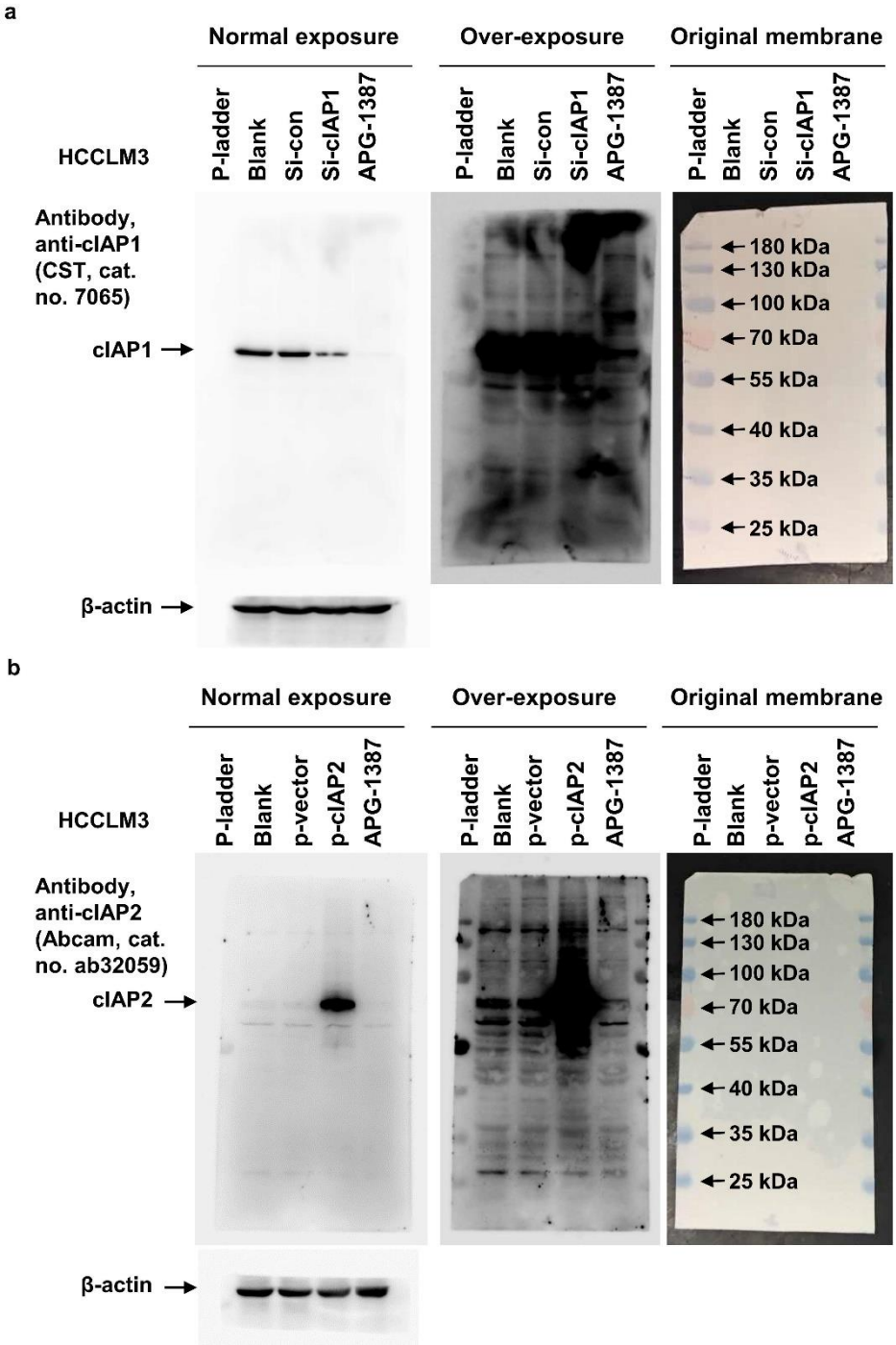

**Supplementary Figure 7.** The susceptibility of side population (SP) and major population (MP) cells in HCCLM3 cell line to the combination of APG-1387 and TNF-α/TRAIL. SP and MP cells were sorted from HCCLM3 cell line by flow cytometry and pre-inoculated in triplicate at 2,000 cells per well in 96-well plates for 12 hours. Cell viabilities were evaluated using a CCK-8 assay after 24 hours' stimulation with TNF-α (**a**) or TRAIL (**b**), or in combination with APG-1387. Con, control.




**Supplementary Figure 8.** APG-1387 treatment enhanced TNF-α- and TRAIL-induced cell death in HepG2 and HCCLM3 cells. HepG2 and HCCLM3 cells were pre-treated with a pan-caspase inhibitor (20 μM Z-VAD-fmk) or RIPK1 inhibitor (50 μM Nec-1) for 1 hour. Then the combination treatments involving 2 μM APG-1387 with 100 ng/ml TNF-α or 20 ng/ml TRAIL were used. Cell death were assessed by flow cytometry (**a**) and Western blot (**b**). Nec-1, necrostatin-1; GSDME-F, full-length GSDME; GSDME-N, N terminal fragment of GSDME.




**Supplementary Figure 9.** Changes in cytotoxic potential of interleukin (IL)-12, IL-15, and IL-18 activated NK cells after APG-1387 stimulation. (**a, b**) HepG2 or HCCLM3 cells were co-cultured with purified NK cells, which stimulated with 10 ng/ml IL-12, 10 ng/ml IL-15 and 100 ng/ml IL-18, either alone or in the presence of 2 μM APG-1387. After co-incubation for 24 hours, supernatants were collected for the examination of TNF-α and IFN-γ by ELISA. (**c**) NK cells stimulated with 10 ng/ml IL-12, 10 ng/ml IL-15 and 100 ng/ml IL-18, were co-cultured with 2 μM APG-1387 or not for 24 hours, then the percentage of living cells, TRAIL expression, TNF-α and IFN-γ in NK cells were analyzed by flow cytometry. Con, control; A, 2 μM APG-1387; NK(+), NK cells co-cultured with 10 ng/ml IL-12, 10 ng/ml IL-15 and 100 ng/ml IL-18; Live/Dead, a membrane and intracellular dye (ThermoFisher, cat. no. L34976) that can distinguish live cells from stained dead cells. Error bars represented the mean ± S.E.M. of triplicate representative experiments; * $P < 0.05$, by two-tailed paired t-test.




**Supplementary Figure 10.** Validation of cIAP1 and cIAP2 antibody. (**a**) For cIAP1 antibody, HCCLM3 cells were transfected with cIAP1 small interfering RNA (si-cIAP1), control small interfering RNA (si-con) or 2 μM APG-1387; (**b**) for cIAP2 antibody, HCCLM3 cells were transfected with cIAP2 plasmid (p-cIAP2), negative control plasmid vector (p-vector) or 2 μM APG-1387. After 48 hours treatment, cell lysates were collected. Western blot analysis was used to detect specificity of cIAP1 antibody (CST, cat. no. 7065) and cIAP2 antibody (Abcam, cat. no. ab32059). P-ladder, Pre-stained protein ladder (Life, cat. no. 26616); APG-1387, 2 μM APG-1387.

## 2 Supplementary Tables

**Supplementary Table 1.** Clinical characteristics of 12 HCC patients from Tongji hospital

| **Variable** | **Number/Mean±SD (range)** |
|---|---|
| Age (year) | 53.17 ± 3.657, (26-68) |
| Gender (male or female) | 10/2 |
| HBV (+/-) | 12/0 |
| HBsAg (>250/≤250 IU/mL) | 9/3 |
| HBsAb (>10/≤10 IU/L) | 2/10 |
| HBeAg (>1/≤1 S/CO) | 4/8 |
| HBeAb (>1/≤1 S/CO) | 5/7 |
| HBcAb (>1/≤1 S/CO) | 12/0 |
| HCV (+/-) | 0/12 |
| History of Schistosoma (+/-) | 0/12 |
| History of smoking (+/-) | 4/8 |
| History of drinking (+/-) | 3/9 |
| diabetes mellitus (+/-) | 7/5 |
| Hepatic encephalopathy (+/-) | 1/11 |
| Ascites (+/-) | 4/8 |
| Pathological grading (poorly/moderately/well differentiation) | 4/4/4 |
| Number of tumor nodules (>1/≤1) | 2/10 |

| | |
|---|---|
| Tumor size (>3/≤3 cm) | 11/1 |
| Portal vein tumor thrombus (+/-) | 4/8 |
| Distance metastasis (+/-) | 1/11 |
| Tumor encapsulation (+/-) | 6/6 |
| Cirrhosis (+/-) | 10/2 |
| Alanine aminotransferase (≥40/＜40 U/L) | 3/9 |
| alpha-Fetoproteins (≥20/＜20 μg/L) | 8/4 |

SD, standard deviation;

HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBsAb, antibody to HBsAg; HBeAg, HBV e Antigens; HBeAb, antibody to HBeAg; HBcAb, HBV core antibody; HCV, hepatitis C virus.

**Supplementary Table 2.** HCC patient characteristics data from TCGA public database

| **Characteristics** | **HCC matched with adjacent normal liver tissue (n=49)** |
|---|---|
| Age (year) | 61.4±16.2 |
| (range) | (20-81) |
| Gender (male/female) | 28/21 |
| TNM | |
| T1/T2/T3/T4 | 20/13/13/3 |
| N0/N1/Nx | 31/1/17 |
| M0/M1/Mx | 32/1/16 |
| Stage (I/II/III/IV/-) | 18/10/12/1/8 |
| Pathological grade (1/2/3/-) | 5/26/15/3 |

HCC, hepatocellular carcinoma;

T, local tumor; N, lymph node; M, metastases.