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Supplemental information

miR-199a-3p increases the anti-tumor activity of palbociclib in liver cancer models

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL TABLES

Table S1. Anti-cancer activity of palbociclib, MK-2206 and their combination

	Number of mice	Tumor Volume difference between post and pre-treatment (mean ± SD, mm^3)
CTRL	13	9,80 ± 28,14
PB	6	0,55 ± 4,62
MK	6	4,63 ± 10,73
PB+MK	5	-0,01 ± 0,48

CTRL = untreated; PB= palbociclib; MK=MK-2206

Table S2. Gene Set Enrichment Analysis (GSEA) of hallmark gene sets (MSigDB collection) and canonical pathways gene sets (Reactome pathway database) in treated mice versus CTRL group

Table S3. Mean tumor size at various time points

	Tumor volume (Mean ± SD, mm^3)		
	T=0	T=42	T=63
SF (2 cycles) + STOP SF	2,44 ± 1,8	1,08 ± 0,8	5,52 ± 7,4
SF (2 cycles) + SF 3rd cycle	7,65 ±7,0	2,50 ± 2,3	0,98 ± 0,8
SF (2 cycles) + PB + miR-199	4,86 ± 7,5	2,61 ± 5,2	1,91 ± 4,2

SF=sorafenib; PB=palbociclib
Tumor volume measurement (Mean ± SD).
(T=0) day 0, before any treatment
(T=42) day 42, end of two 21-days cycles of sorafenib
(T=63) day 63, end of 3rd cycle regimen

SUPPLEMENTAL FIGURES

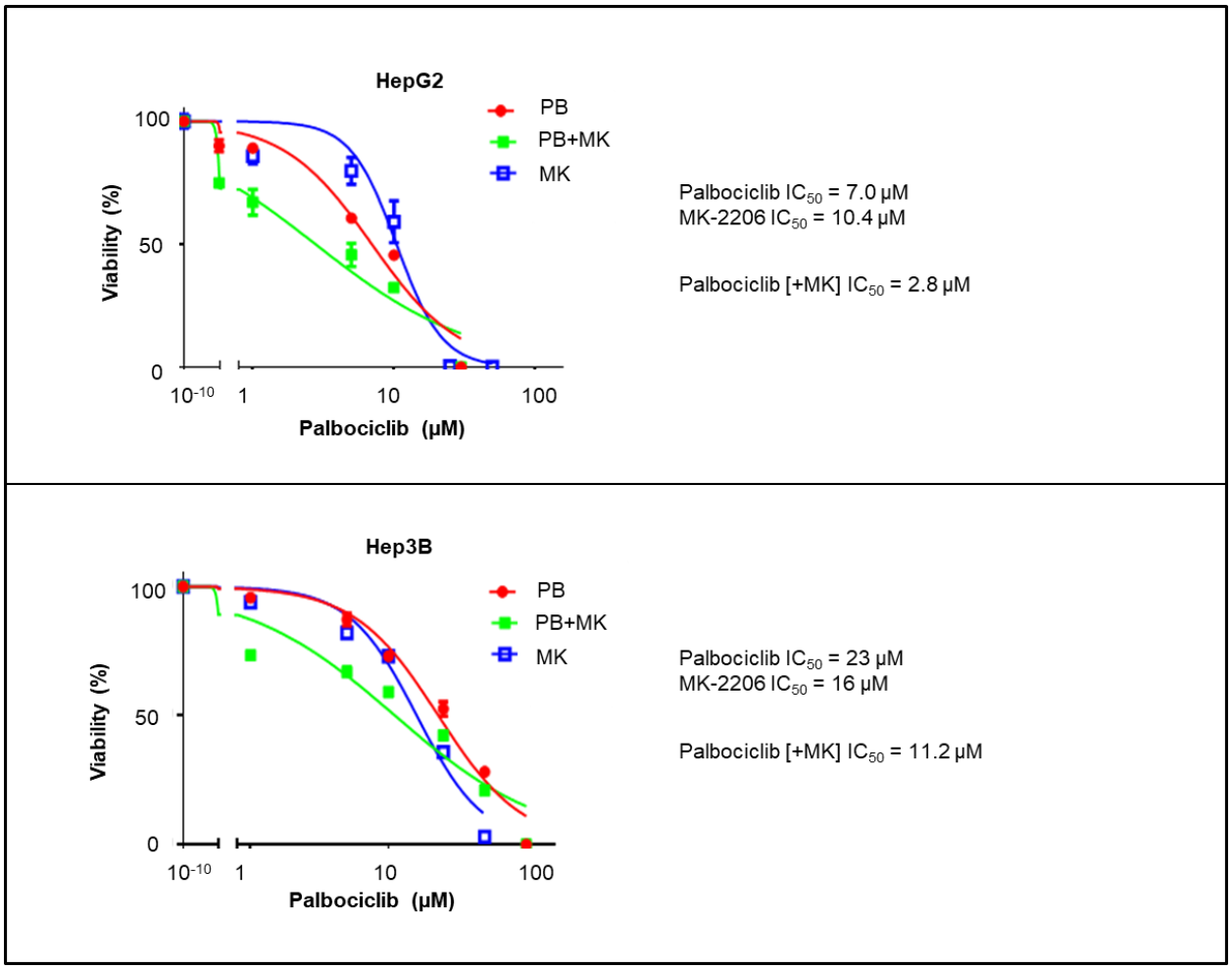

Figure S1: IC50 of palbociclib and MK-2206 IC50 as single agents and in combination. Increasing concentrations of palbociclib (PB) or MK-2206 (MK) were tested to evaluate their IC50 in Hep3B and HepG2 cells. Considering that cell viability of both cell lines was 80% at 5µM MK-2206, we assessed drugs combination using increasing concentrations of palbociclib at a fixed concentration of MK-2206 (5µM). In Hep3B, the combination led to a reduction of palbociclib IC50 to 11.15 µM; in HepG2, to 5 µM. Viability of treated cells were normalized on the average viability of untreated cells. The concentration of drugs [µM] is expressed as a log10 scale. The points represent the average percentage of the viability at each drug concentration and the error bars represent the SD.

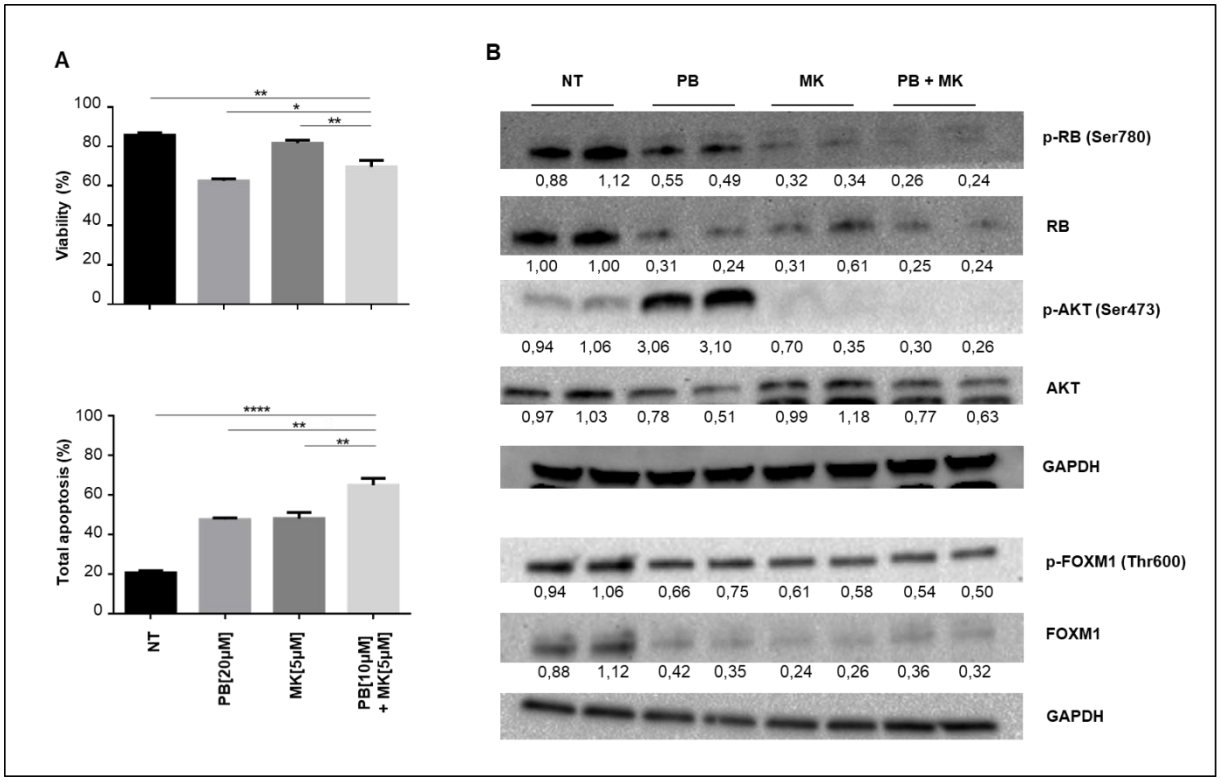

Figure S2. Biological and molecular effects of palbocicib and the AKT inhibitor MK-2206 on Hep3B cells. (A) Hep3B cells were treated with palbocilib (PB) (20µM) or MK-2206 (MK) (5µM) as single agents and with the combination of the two drugs (palbociclib 10uM and MK-2206 5µM). No treatment (NT) was a negative control. Viability and apoptosis were evaluated 72 hours after start of treatment. Data are represented as mean + SD. **(B)** Western blot analysis for quantification of RB1, AKT and FOXM1 proteins and their phosphorylated forms. The values are normalized on GAPDH protein levels and to the average protein levels of the untreated cells (NT). Because Hep3B cells exhibit low level of full length protein, digital images of RB1 and p-RB1 were acquired with an exposure time of 300 seconds instead of 30 sec. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001

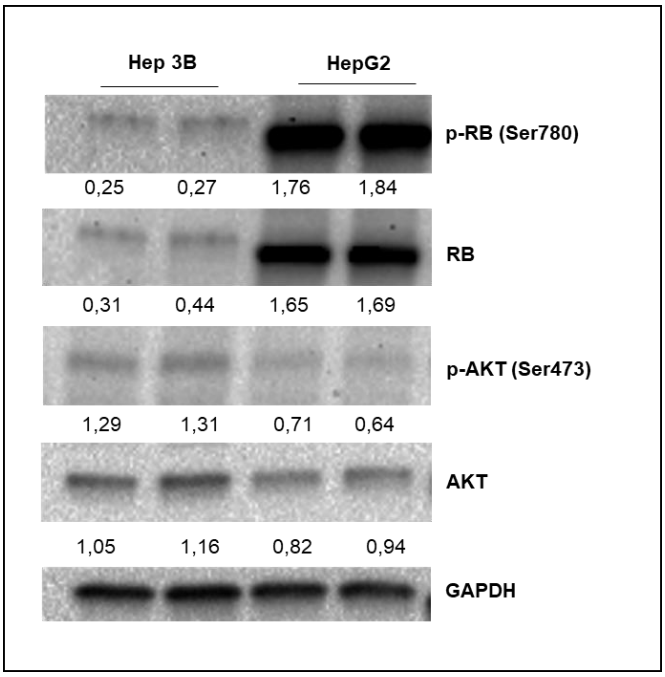

Figure S3. **Basal expression of RB1, AKT proteins in Hep3B and HepG2 cell lines**. Expression of RB1, AKT proteins and their phosphorylated forms was assessed by western blot analysis. The values were normalized on the GAPDH protein levels

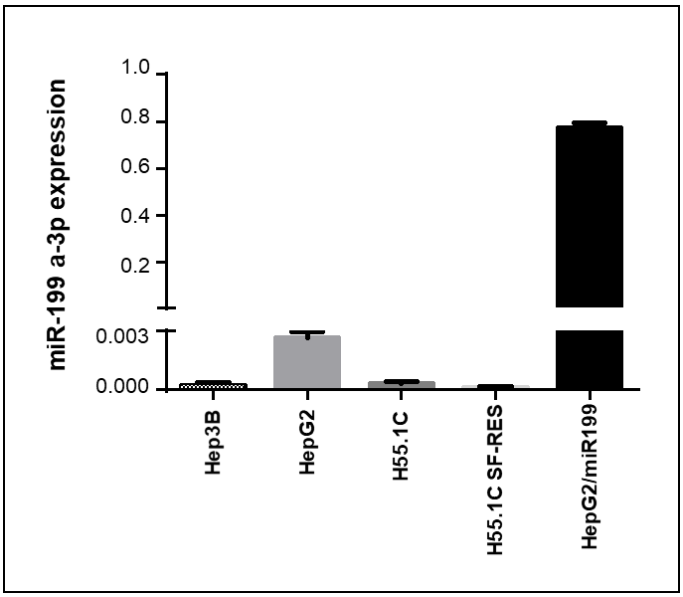

Figure S4. miR-199a-3p expression levels in human and mouse cell lines. Basal levels of miR-199 expression levels were assessed in all the human and mouse cell lines employed in the present study**.** As a positive control for miRNA expression, RNA extracted from HepG2/miR199 cells, a stable cell clone over-expressing miR-199a-3p[1], was included in the analysis. Data are represented as mean + SD.

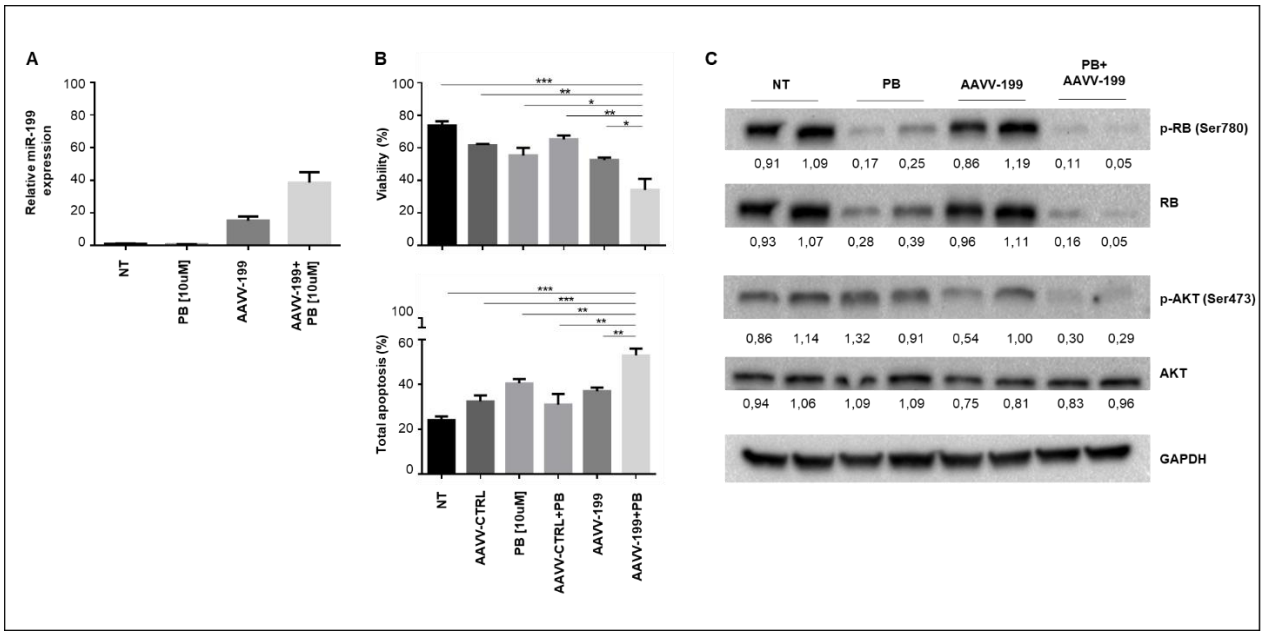

Figure S5. Biological and molecular effects of palbocicib and miR-199a-3p on HepG2 cells. (A) miR-199 expression was measured in HepG2 cells in various conditions: (NT) untreated; (PB 10 uM) palbociclib; (AAVV-199) cells transduced with an Adeno Associated Viral vector expressing miR-199a-3p (MOI=200); (AAVV-199 + PB) combination of AAVV-199 with palbociclib. **(B)** Cells viability and apoptosis levels were evaluated 120h after transduction. Data are represented as mean + SD. **(C)** Western blot analysis and quantification of RB1, AKT proteins by Western blot analysis. The values are normalized on the GAPDH protein and compared to the average levels detected in the untreated cells. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

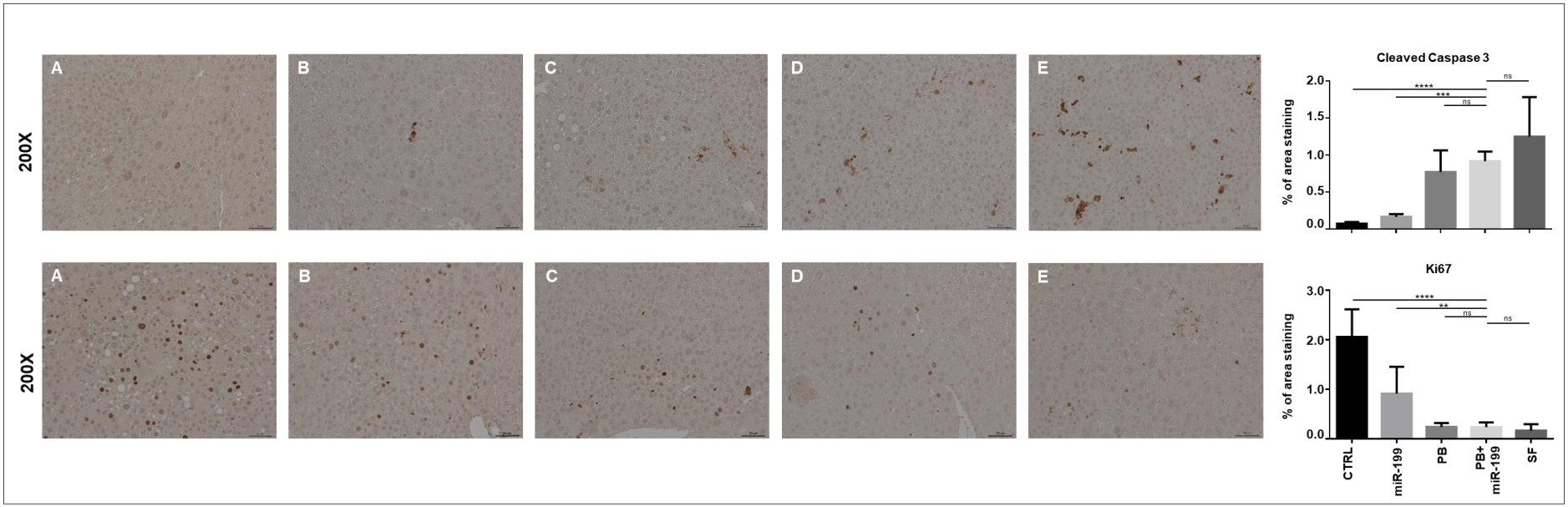

Figure S6: Immunohistochemical analysis of cleaved Caspase-3 (upper panel) and Ki67 (lower panel) in TG221-derived HCC. Each panel is representative of the following experimental conditions: **(A)** CTRL, **(B)** miR-199a-3p, **(C)** Palbociclib, **(D)** Palbociclib+ miR199a-3p, **(E)** Sorafenib. The graphs show the percentage of stained areas for cleaved Caspase-3 or Ki-67 in tissue regions (n = 6) selected for each condition. Magnification 200X, scale bar = 50µm. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

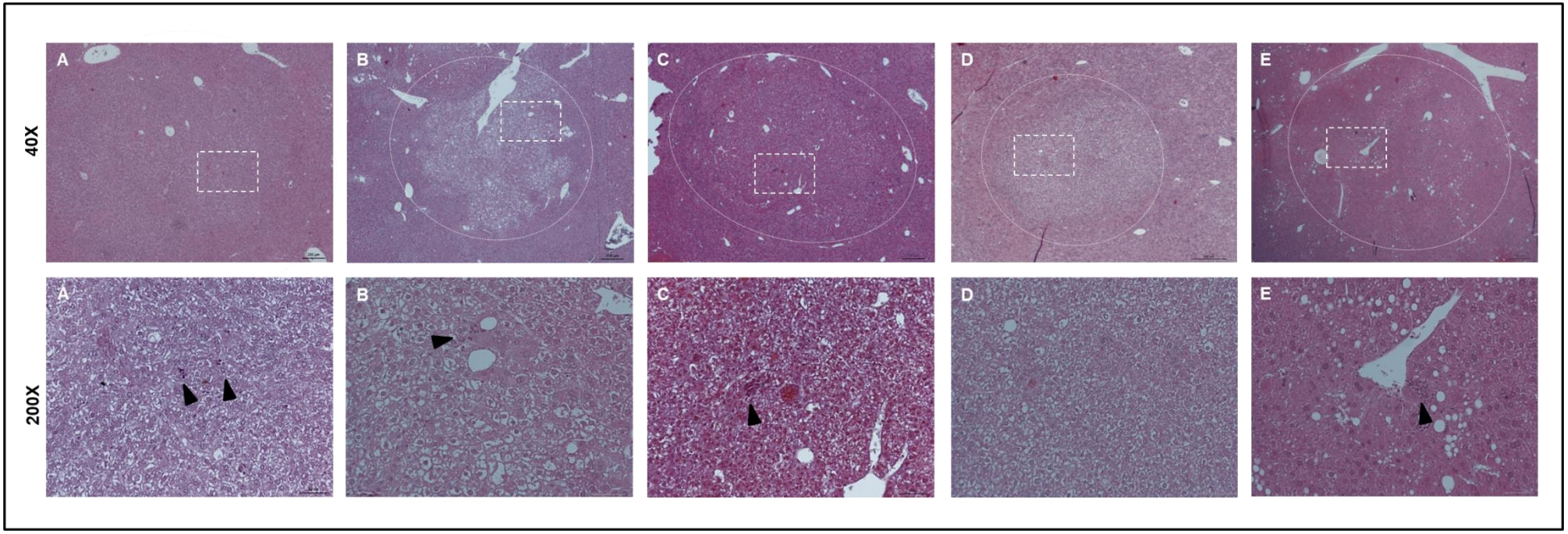

Figure S7. Single drugs or combination treatments do not have a significant impact on the immune response against tumor. Sections derived from the following mice groups: **(A)** CTRL, **(B)** miR-199, **(C)** Palbociclib, **(D)** Palbociclib+ miR199, **(E)** Sorafenib, were stained with hematoxylin-eosin and analysed for the presence of immune and flogistic cell infiltration. Few spots of tumor-infiltrating lymphocytes are indicated by black arrows. Within the 40X magnification images (scale bar = 200µm), circles indicate the tumor nodules and rectangles refers to tissue sections shown in the 200X magnification images (scale bar = 50µm).

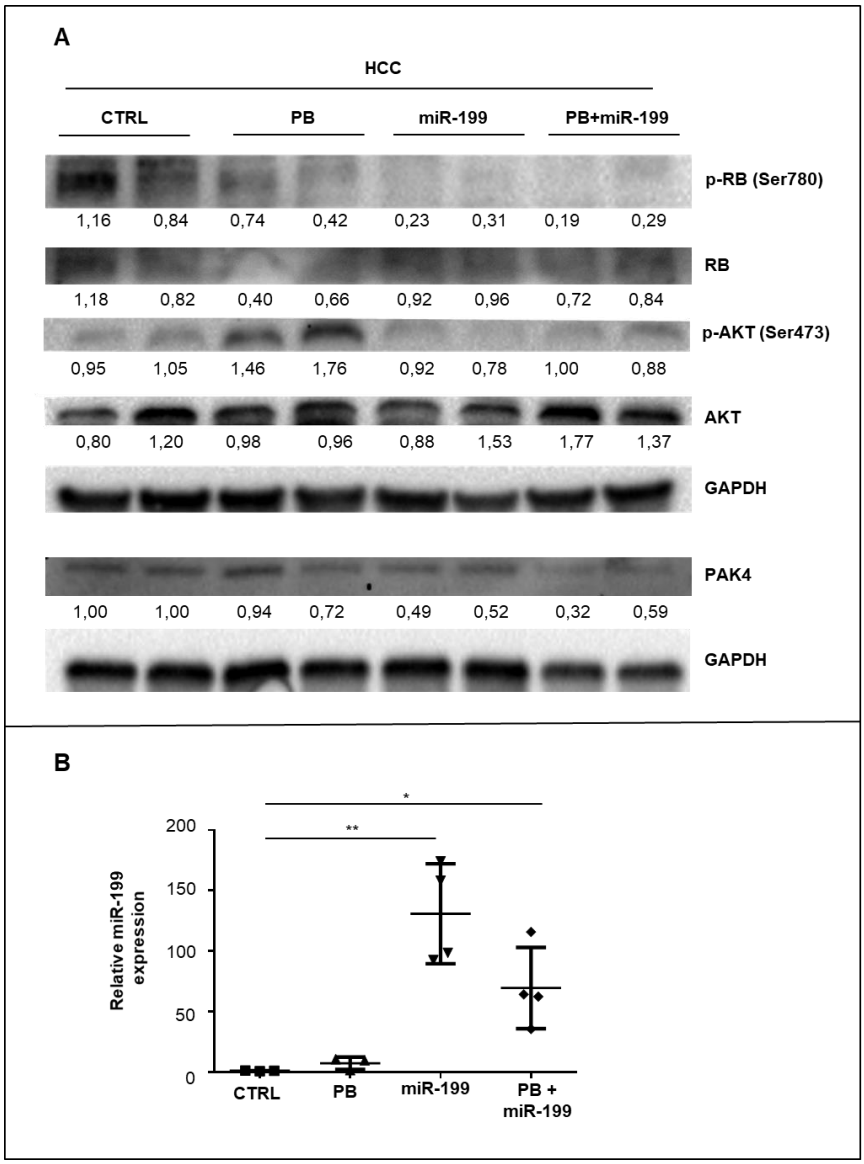

Figure S8. Modulation of palbociclib and miR-199a-3p molecular targets *in vivo*. (A) The expression levels of molecular targets of palbociclib and miR-199a-3p were assessed in HCC samples by Western blot analysis. Each value was normalized on the GAPDH and compared to the average of the untreated (CTRL) samples. **(B)** miR-199 expression levels were evaluated in HCC samples, *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

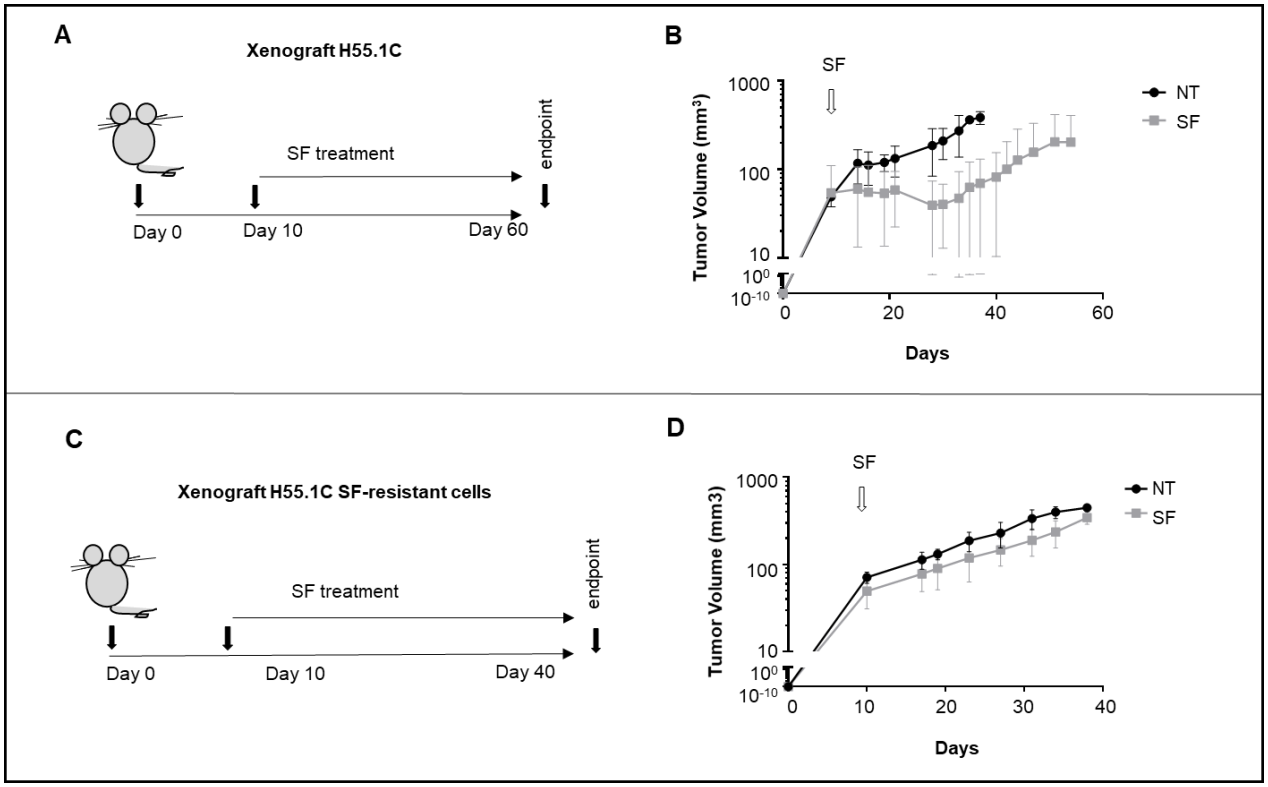

Figure S9. *In vivo* selection of mouse cells resistant to sorafenib. (**A**) H55.1C mouse hepatoma cells were injected subcutaneously into the lateral backside of 8 mice. When tumor reached a volume of ~50 mm^3 (10 days after cells injection), mice were randomly divided into two groups: a group was treated with sorafenib (SF) (n=4), while a second group was not treated (NT) (n=4). **(B**) Size of tumor nodules was measured with a caliper every two days. **(C)** *In vivo* selected SF-resistant cells were injected subcutaneously into the lateral backside of 8 mice. When tumor reached a volume of ~50 mm^3 (10 days after cells injection), mice were randomly divided into two groups: a group was treated with sorafenib (SF) (n=4) for 21 days, while a second group was not treated (NT) (n=4). **(D)** Size of tumor nodules was measured with a caliper every two days. Data are represented as mean ± SD.

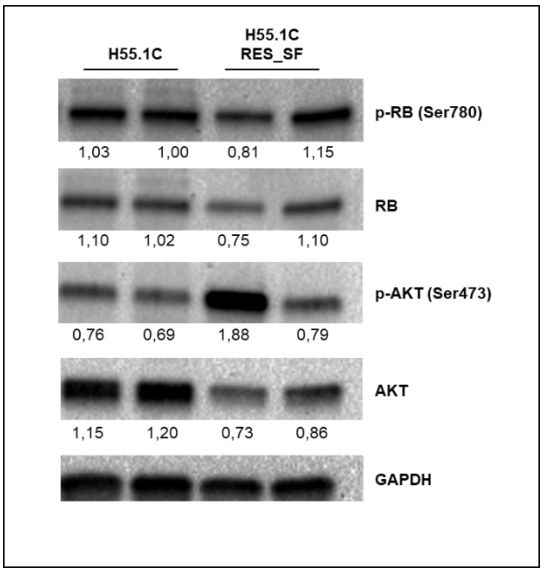

Figure S10. Basal expression of RB1, AKT proteins in H55.1C and H55.1C sorafenib resistant cells. Expression of RB1, AKT proteins and their phosphorylated forms was assessed by western blot analysis. The values were normalized on the GAPDH protein levels

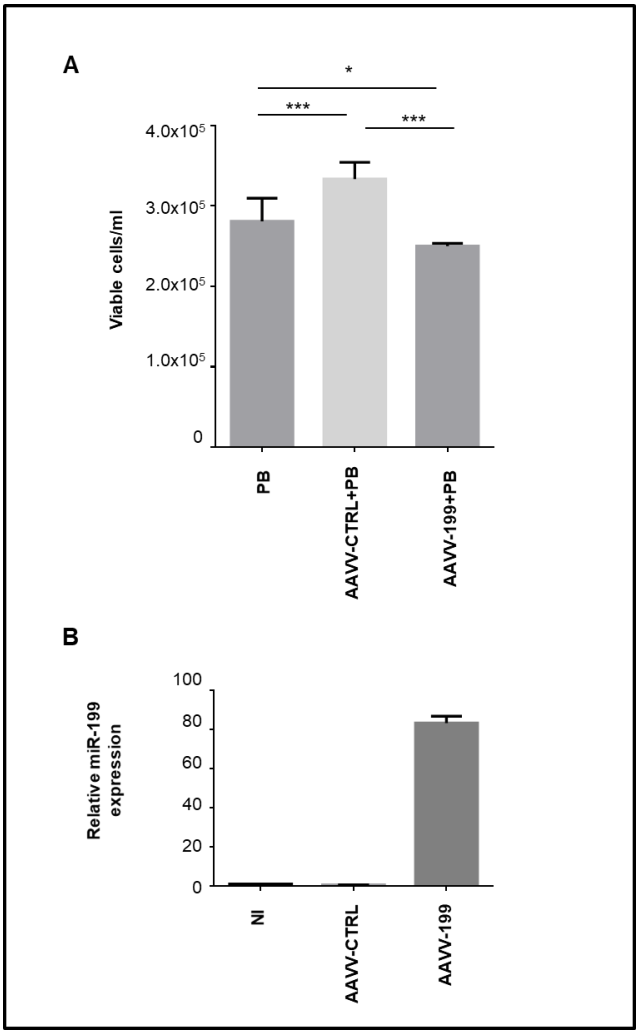

Figure S11. Enforced expression of miR-199a-3p increases palbociclib growth inhibitory effects on sorafenib-resistant HCC mouse cells *in vitro*. Mouse cell lines derived from a SF-resistant tumor (see Figure S9) were infected with an Adeno Associated Virus expressing miR-199a-3p (AAVV-199) or with a control AAVV (AAVV-CTRL) (MOI= 500) and treated with 10 µM palbociclib. At 120h after infection, cells were collected and analyzed. **(A)** Cells treated with miR-199a-3p in combination with palbociclib showed a significant decrease in cell viability compared to single agent treatment. **(B)** miR-199 expression levels were assessed to confirm effective viral vector transduction. Data are represented as mean + SD. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

SUPPLEMENTAL REFERENCES

1. Callegari E, Elamin BK, D'Abundo L, Falzoni S, Donvito G, Moshiri F, Milazzo M, Altavilla G, Giacomelli L, Fornari F, et al. Anti-Tumor Activity of a miR-199-dependent Oncolytic Adenovirus. PLoS One. 2013;8(9):e73964.