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# Sustained antitumor activity by co-targeting mTOR and the microtubule with temsirolimus/vinblastine combination in hepatocellular carcinoma

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ABSTRACT

The mammalian target of rapamycin (mTOR) and the microtubules are prominent druggable targets for hepatocellular carcinoma (HCC). PI3K/Akt/mTOR activation is associated with resistance to microtubule inhibitors. Here, we hypothesized that co-targeting of mTOR (by mTOR inhibitor temsirolimus) and the microtubule (by microtubule-destabilizing agent vinblastine) would be more efficacious than single targeting in HCC models. *In vitro* studies showed that effective inhibition of mTOR signaling with temsirolimus alone was able to suppress HCC cell growth in a dose-dependent manner. Among five cell lines tested, Huh7 was the most temsirolimus-sensitive ($IC_{50}$ = 1.27 ± 0.06 μM), while Hep3B was the most temsirolimus-resistant ($IC_{50}$ = 52.95 ± 17.14 μM). We found that co-targeting of mTOR (by temsirolimus) and the microtubule (by vinblastine, at low nM) resulted in marked growth inhibition in Huh7 cells and synergistic growth inhibition in Hep3B cells (achieving maximal growth inhibition of 80–90%), demonstrating potent antitumor activity of this novel combination. *In vivo* studies showed that temsirolimus treatment alone for 1 week was able to inhibit the growth of Huh7 xenografts. Strikingly, the temsirolimus/vinblastine combination induced a significant and sustained antitumor activity (up to 27 days post-treatment), with effective reduction of tumor vessel density in both Huh7 and Hep3B xenograft models. Mechanistic investigation revealed that this marked antitumor effect was accompanied by specific and concerted down-regulation of several key anti-apoptotic/survival proteins (survivin, Bcl-2, and Mcl-1), which was not observed in single agent treatments. Our findings demonstrated that the potent anti-cancer activity of this co-targeting strategy was indeed mediated in parts by inhibition of these key survival/anti-apoptotic proteins.



## 1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide [1,2]. Despite technological advancement, only the minority of HCC patients (10–15%) can undergo surgical resection of the tumors due to late diagnosis of the disease in general. A significant proportion of HCC patients are not amenable to, or eventually failed, locoregional therapies, and have to be considered for systemic treatment. Chemotherapies are ineffective, and hence, until a few years ago, supportive/palliative treatments have been the main management modality for advanced diseases. There is an urgent need for the development of more effective therapies for HCC. Although sorafenib (a multi-kinase inhibitor of VEGFR, PDGFR, and Raf) has been approved for the treatment of HCC as the first line therapy for unresectable HCC, this clinically approved molecular targeting agent only offers limited clinical efficacy [3,4]. Hence, there is a continued need to identify better therapies for HCC patients.

The mammalian target of rapamycin, mTOR, is a prominent target for HCC therapy. Aberrant activation of mTOR is common in HCC (~45% cases) and its over-expression is associated with poor prognosis in HCC patients [5,6]. mTOR is a key effector in the PI3K/Akt/mTOR pathway and it plays a critical role in regulating cell proliferation and survival. The downstream targets of mTOR

*Abbreviations:* HCC, hepatocellular carcinoma; mTOR, mammalian target of rapamycin; 4E-BP1, eIF4E binding protein; MVD, microvessel density; MDR, multi-drug resistant gene.

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include ribosomal p70S6 kinase and the eukaryotic initiation factor eIF4E binding protein (4E-BP1), which regulates cell cycle, growth and protein synthesis. Inhibition of mTOR has been shown to be effective in suppressing tumor cell growth in several cancers, including renal cell carcinoma, gastric cancer, as well as mantle cell lymphoma in both preclinical and clinical settings [7–12]. In HCC, mTOR targeting by everolimus showed a modest antitumor effect with disease control rate of 44% in a phase I/II study [13]. It is believed that in most cancers, the effect of mTOR targeting will be largely cytostatic [14,15], which may limit its use as a single agent for anti-cancer treatment. It is anticipated that combination of an mTOR inhibitor with a cytotoxic agent may represent a more effective therapeutic regimen than mTOR targeting alone. Indeed, studies in breast cancer, prostate cancer and multiple myeloma supported this notion. However, it remains unclear what cytotoxic agent would be a rational choice for combination with mTOR targeting in HCC.

Recent gene expression profiling studies revealed that microtubule-related cellular assembly and organization is the most crucial cellular event in HCC development [16–18], suggesting microtubule to be an important target for therapeutic intervention in HCC. Microtubule-targeting agents are compounds which bind to soluble tubulin and/or directly to tubulin in the microtubules, thus affecting microtubule dynamics. These agents exert anti-mitotic activity and inhibit cell proliferation by inducing cell cycle arrest and apoptosis. Previous study from us showed that microtubule-targeting was effective in inhibiting HCC growth *in vitro* and *in vivo*, and demonstrated the crucial role of microtubule in HCC cell proliferation, which is potentially an important therapeutic target for HCC [18].

Recently, VanderWeele et al. showed that activation of the upstream signaling molecule of mTOR, namely Akt, resulted in marked increase in resistance against microtubule-directed agents, while rapamycin (an mTOR inhibitor) treatment was able to inhibit this PI3K/Akt/mTOR-associated resistance [19]. Due to the druggable nature of these prominent targets in HCC (namely mTOR and the microtubule), and the demonstrated involvement of the PI3K/Akt/mTOR pathway in resistance to microtubule-targeting chemotherapeutic agents [19,20], we hypothesized that rational co-targeting of mTOR and the microtubule in HCC may be more efficacious than single targeting alone. To test this, we investigated the antitumor efficacy of temsirolimus, an mTOR targeting agent currently undergoing Phase I/II clinical trials in HCC, either alone or in combination with vinblastine, a common microtubule-destabilizing agent, in both *in vitro* and *in vivo* models of HCC. Our findings demonstrated such a novel co-targeting strategy was able to elicit potent and sustained antitumor effects in both *in vitro* and *in vivo* models of HCC, partially via inhibition of several key survival/anti-apoptotic proteins.

## 2. Materials and methods

### 2.1. Reagents

Temsirolimus (Torisel®) was obtained from Wyeth (Monmouth Junction, NY, USA), and dissolved in DMSO at 100 mM stock concentration. Vinblastine sulfate (DBL) was obtained from Hospira (Wellington, New Zealand). The following antibodies were used in the study: anti-mTOR, anti-pi-mTOR (ser2448), anti-Akt, anti-pi-Akt (ser473), anti-p70S6k, anti-pi-p70S6k (Thr389), anti-S6, anti-pi-S6 (ser240/244), anti-4E-BP1, anti-pi-4E-BP1 (ser65), anti-survivin (all from Cell Signaling Technology, Beverly, MA, USA); anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Bcl-2 (Epitomics, Burlingame, CA, USA) and anti-Actin (Calbiochem, Nottingham, UK).

### 2.2. Cell culture

Human hepatocellular carcinoma cell lines Hep3B, HepG2, PLC/PRF/5 and SNU398 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Huh7 was obtained from Japanese Collection of Research Bioresources (JCRB, Japan). Hep3B, HepG2, Huh7, PLC/PRF/5 were cultured in Dulbecco's modified Eagle medium with Glutamax-1 (HycClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum, FBS (Hyclone). SNU398 was cultured in complete RPMI-1640 medium (HyClone) containing 10% FBS (HyClone). All cells were cultured under a humidified atmosphere of 5% $CO_2$ at 37 °C as previously described [18].

### 2.3. Cell viability assay

Cells (8000–18,000 cells per well) were treated with either vehicle (DMSO) or increasing concentrations of temsirolimus (ranging from 1 nM to 100 μM) for 24 and 48 h. For combination treatment, cells were treated with increasing concentrations of temsirolimus and low concentration of vinblastine (1 nM). Cell viability was determined by MTT assay as previously described [21]. The percentage growth inhibition was calculated as $(OD_{vehicle} - OD_{drug})/OD_{vehicle} \times 100\%$. The $IC_{50}$ value was determined as the drug concentration at which half of the maximal growth inhibition was observed.

### 2.4. Construction of expression plasmids and transfection

Full length human survivin was cloned into pcDNA3 vector with an HA-tag by RT-PCR and sequence was confirmed by DNA sequencing. Both the pcDNA3-Bcl-2 plasmid and pTOPO-Mcl-1 plasmid were obtained from Addgene (Addgene Inc, Cambridge, MA, USA). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as previously described [22].

### 2.5. Cell cycle analysis

Cell cycle analyses were performed at 12 or 16 h upon drug treatment by flow cytometry as previously described [18].

### 2.6. Western blotting

Protein lysates were obtained as previously described [21]. Protein lysates (25–50 μg) were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After primary and secondary antibody incubations, the signal was detected by autoradiography using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

### 2.7. HCC xenograft study

Six to eight weeks old male athymic nude mice (nu/nu) were used for the establishment of HCC xenografts. All experiments were conducted under license from the Department of Health and according to animal ethics approval from the University Animal Experimentation Ethics Committee, the Chinese University of Hong Kong. HCC cells ($2 \times 10^6$ of Huh7 cells or $3 \times 10^6$ of Hep3B cells suspended in 200 μl serum free medium) were inoculated into the dorsal flanks of mice by subcutaneous injection. Mice were randomized into four groups. Treatments were started on day 10 (for Huh7 xenografts), or day 24 (for Hep3B xenografts) post-inoculation. The 4 treatment groups were: (1) vehicle control, (2) temsirolimus alone (25 mg/kg, 3–4 times a week, i.p.), (3) vinblastine alone (0.4 mg/kg, 1–2 times a week, i.p.), (4) a

combination of temsirolimus and vinblastine (two agents administered in an alternating schedule, not as mixed solution, with same doses and schedules as single agents alone). Tumor growth was monitored twice weekly and tumor volume was calculated using the formula of [(Length × Width$^2$)/2] as previously published [23].

### 2.8. Immunohistochemistry and microvessel density (MVD) determination

Immunohistochemistry was performed as previously described [24]. IHC score approach initiated by McCarty et al. [25] was applied to assess both the intensity of staining and % of positive cells. The IHC score ranged from 0 to 300. Tumor microvessels were stained with a rabbit anti-CD34 antibody (1:100 dilution, Santa Cruz Biotechnology). MVD were assessed by counting 5 fields under light microscope at 100× magnification and the average MVD expressed as number of MVD per field was calculated for each xenograft specimen.

### 2.9. Statistical analysis

All data were presented as mean ± SEM. Student's *t*-test (with Welch's correction) was performed using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Results were considered as statistically significant if $P < 0.05$.

## 3. Results

### 3.1. Temsirolimus inhibited HCC cell proliferation with effective inhibition of mTOR signaling

To examine the effects of temsirolimus on HCC cell proliferation, five HCC cell lines (SNU398, Hep3B, HepG2, PLC/PRF/5 and Huh7) were treated with temsirolimus at increasing concentrations (1 nM–100 μM). As early as 24 h upon treatment, temsirolimus was able to induce dose-dependent growth inhibition in all five cell lines tested, with a maximal achievable growth inhibition of ~90–95% at 100 μM concentration. Among these HCC cell lines tested, Huh7 was the most temsirolimus-sensitive (average $IC_{50}$ = 1.27 ± 0.06 μM), while Hep3B was the most resistant one with a 42-fold higher $IC_{50}$ value (average $IC_{50}$ = 52.95 ± 17.14 μM) compared to that of Huh7. The remaining three cell lines, SNU398, HepG2 and PLC/5, had intermediate sensitivities (Fig. 1A and B).

Next, we examined the effects of temsirolimus on mTOR signaling in HCC cells. In Huh7, Hep3B and HepG2 cells, temsirolimus (1 μM) was able to elicit marked inhibition of mTOR signaling at 24 h, sustaining up to 48 h (Fig. 1C). This was indicated by significant inhibition of phospho-mTOR (ser2448), as well as effective inhibition of its downstream effectors, including phospho-p70S6k (Thr389), phospho-S6 (ser240/244), and phospho-4E-BP1 (ser65) (Fig. 1C). Our results showed that mTOR targeting by temsirolimus can abrogate mTOR activation in HCC cells. Note that a marked upregulation of phospho-Akt (ser473) was observed in Hep3B cells upon temsirolimus treatment (Supplementary Fig. 1A), implicating a possible feedback upregulation of p-Akt by temsirolimus in this temsirolimus-resistant cell line. In contrast, only a very mild feedback upregulation of p-Akt was observed in Huh7 and no prominent feedback was observed in HepG2 cells (Supplementary Fig. 1A).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2012.01.013.

### 3.2. Synergistic antitumor activity of temsirolimus/vinblastine combination in vitro

Studies in cervical and ovarian cancers revealed that activation of the PI3K/Akt/mTOR pathway associates with resistance to microtubule-targeting agents, implicating potential benefits of combined targeting of both the microtubule and the PI3K/Akt/mTOR pathway [20,26]. Vinblastine is a common chemotherapeutic agent targeting the microtubule. We examined the *in vitro* and *in vivo* antitumor activity of temsirolimus/vinblastine combination in both temsirolimus-sensitive (Huh7) and temsirolimus-resistant cells (Hep3B). As shown in Fig. 2A, the temsirolimus-resistant Hep3B cell line was only moderately sensitive to high dose of temsirolimus treatment at 24 h (with a maximal growth inhibition of only 31.41% even at 20 μM concentration). Vinblastine alone at low concentration (1 nM) only inhibited Hep3B proliferation by ~10%. Strikingly, combination of this low dose vinblastine with temsirolimus was able to elicit synergistic growth inhibition (77.67 ± 1.03%, $n = 3$, $P < 0.001$) in this temsirolimus-resistant HCC cell line as early as 24 h. Similar findings were observed in the temsirolimus-sensitive Huh7 cells, except that in this cell line, an additive to mild synergistic effect was observed in lower concentrations of temsirolimus (doses were chosen to show the best synergistic effect of the combination in this temsirolimus-sensitive cell line, data not shown). A growth inhibition of 65–70% was observed in Huh7 cells with low dose temsirolimus/vinblastine combination (Fig. 2A). An additive inhibition was also observed in HepG2 cells (with intermediate temsirolimus-sensitivity, Fig. 1B), achieving 91.23 ± 0.74% ($n = 3$, $P < 0.001$) maximal growth inhibition as early as 24 h (Supplementary Fig. 2A). Our findings in multiple HCC cell lines demonstrated marked therapeutic efficacy of such a combination in *in vitro* models of HCC.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2012.01.013.

### 3.3. Temsirolimus/vinblastine combination elicited potent and sustained antitumor activity in vivo

The striking *in vitro* anti-cancer activity of this temsirolimus/vinblastine combination compelled us to examine if this combination would be effective *in vivo*. Using established xenograft models of Hep3B (the most temsirolimus-resistant line, Fig. 1A and B above), we found that one week of temsirolimus treatment alone was not able to inhibit the growth of Hep3B tumors, when compared to vehicle alone (average tumor volume of 97.53 ± 29.36 mm$^3$ *vs* 105.64 ± 16.61 mm$^3$ in vehicle alone; $n = 10$, $P = 0.81$) (Fig. 3A and Table 1A). An additional week of temsirolimus treatment did not elicit any significant change in tumor volume (average tumor volume of 206.45 ± 42.01 mm$^3$ *vs* 269.98 ± 57.44 mm$^3$ in the vehicle-treated group; $n = 10$, $P = 0.40$), consistent with the *in vitro* observation that these cells are resistant to temsirolimus (Fig. 1A and B). Vinblastine alone seemed to achieve a moderate degree of growth inhibition. However, higher concentration of vinblastine was lethal to mice (data not shown), thus limiting high dose administration of vinblastine to mice. Consistent with the marked *in vitro* growth inhibitory activity of this combination, we found that this combination was able to significantly inhibit Hep3B tumor growth as early as 11 days after treatment (*i.e.* 35 days after tumor cell inoculation in Fig. 3A). The most remarkable observation was that with only 2 weeks of treatment, this combination was able to result in sustained and marked antitumor effects up to day 45 (*i.e.* 21 days after treatment initiation). The final tumor volume of the combination group was 155.66 ± 22.64 mm$^3$ *vs* 649.24 ± 148.65 mm$^3$ in the vehicle-treated group (~4-fold difference, $n = 10$, $P < 0.01$), and 328.88 ± 86.88 mm$^3$ in the temsirolimus-treated group and 300.22 ± 30.44 mm$^3$ in the vinblastine-treated group (Table 1A and Fig. 3C upper panel). The treatment was tolerable by all groups with no deaths (data not shown).

An even more marked and sustained antitumor effect of this temsirolimus/vinblastine combination was observed in the Huh7 model, in which 2 weeks of initial treatment was able to induce a long-term inhibition of tumor growth up to 27 days

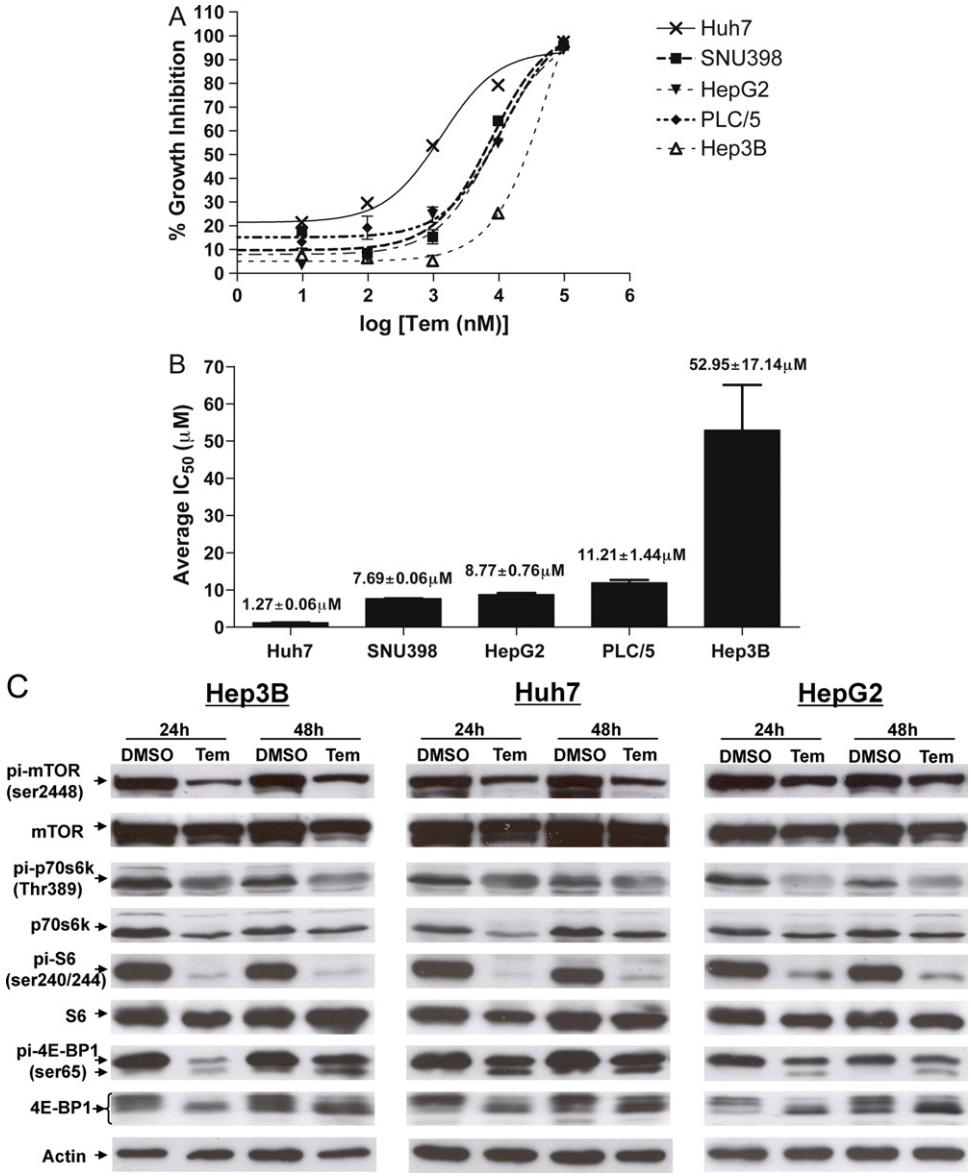


**Fig. 1.** Temsirolimus inhibited proliferation and mTOR signaling in HCC cell lines. (A) Dose-dependent inhibition of HCC cell proliferation by temsirolimus. The effect of temsirolimus on cell viability was assessed by MTT assay. Dose-response curves of temsirolimus for all HCC cell lines were shown. Similar results were observed in 3 independent experiments. (B) Average $IC_{50}$ values of temsirolimus in HCC cell lines. Cumulative results from 3 independent experiments were shown as mean ± SEM. (C) Temsirolimus inhibited the mTOR pathway in HCC cells. Hep3B, Huh7 and HepG2 Cells ($3 \times 10^5$) were treated with 1 μM temsirolimus (hereafter labeled as Tem) or DMSO control for 24 h and 48 h. The expression levels of the mTOR pathway components, pi-mTOR (ser2448), mTOR, pi-p70S6K (Thr389), p70S6K, pi-S6 (ser240/244), S6, pi-4E-BP1 (ser65), 4E-BP1, and actin were examined by Western blotting. Similar results were observed in 3 independent experiments.

post-treatment (*i.e.* 38 days after treatment initiation) (Fig. 3B and Table 1B). Although single agent alone (temsirolimus or vinblastine) seemed to inhibit Huh7 tumor growth during drug administration (in the first 2 weeks of injection), cessation of treatment was immediately followed by steady increase in tumor volumes (since day 25 in Fig. 3B, *i.e.* 4 days upon cessation of treatment), which was not observed in the combination treatment. In fact, the tumor volumes of the temsirolimus/vinblastine-treated group remained the same from 2 weeks of treatment ($11.74 \pm 1.67$ mm$^3$, $n = 10$) till the end of the experiment on day 48 ($10.45 \pm 2.61$ mm$^3$, $n = 10$), indicating no measurable growth in tumor size even upon long-term cessation of combination treatment, while during very same period, the tumor volumes of the vehicle-treated group have increased by ~3-fold (from $41.99 \pm 3.68$ mm$^3$ to $120.08 \pm 16.33$ mm$^3$, $n = 10$; $P < 0.01$). The experiment was terminated on day 48 due to the large tumor size of the vehicle-treated group. The final tumor volume of the temsirolimus/vinblastine-treated group was $10.45 \pm 2.61$ mm$^3$, *vs* $120.08 \pm 16.33$ mm$^3$ in the vehicle-treated group (~11-fold difference, $n = 10$, $P < 0.001$), $58.04 \pm 12.33$ mm$^3$ in the temsirolimus alone and $91.77 \pm 26.96$ mm$^3$ in the vinblastine-treated groups, respectively (Table 1B and Fig. 3C lower panel).

In addition to the observed potent antitumor activity in HCC xenografts, we also found that this combination was able to induce a significant reduction in microvessel density (MVD) in both Hep3B and Huh7 models as compared to vehicle control (Fig. 3D), suggesting potent anti-angiogenic activity of this combination in HCC models. As shown in Fig. 3D lower panel, administration of temsirolimus or vinblastine alone in Hep3B xenografts for 45 days was able to inhibit MVD by 66% and 52%, respectively, while the combination inhibited MVD by 81% ($n = 10$, $P < 0.001$). Similarly, the combination was the most anti-angiogenic regimen in Huh7 xenograft models (63% of MVD inhibition by the combination *vs*

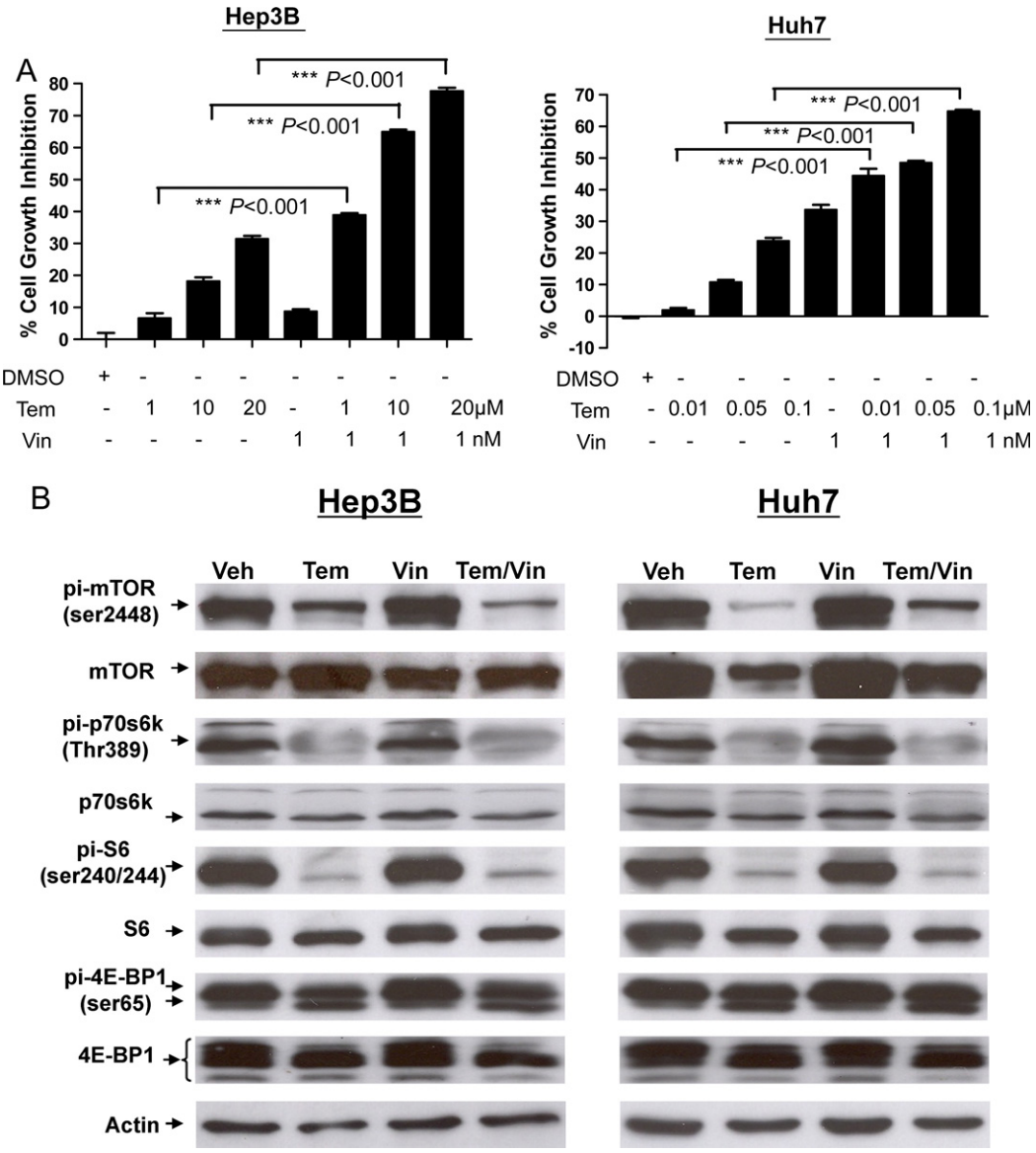


**Fig. 2.** Synergistic/additive antitumor activity of the temsirolimus/vinblastine combination in HCC cell lines. (A) Hep3B and Huh7 cells ($1 \times 10^4$) were treated with various concentrations of temsirolimus in combination with 1 nM vinblastine (Vin) for 24 h. Cell viability was assessed by MTT assay. Cumulative results from 3 independent experiments were shown as mean ± SEM (***$P < 0.001$ *vs* temsirolimus-treated group). (B) The mTOR signaling in HCC cells was not further suppressed by the temsirolimus/vinblastine combination treatment. Hep3B and Huh7 cells ($3 \times 10^5$) were treated with temsirolimus (1 μM for the temsirolimus-resistant Hep3B cells, 0.05 μM for the temsirolimus-sensitive Huh7 cells) and/or vinblastine (Vin) (1 nM) for 24 h. The temsirolimus/vinblastine combination is abbreviated as Tem/Vin hereafter. The expression levels of the mTOR pathway components, pi-mTOR (ser2448), mTOR, pi-p70S6K (Thr389), p70S6K, pi-S6 (ser240/244), S6, pi-4E-BP1 (ser65), 4E-BP1, and actin were examined by Western blotting. Similar results were observed in 3 independent experiments.

39% and 16% by temsirolimus or vinblastine alone, respectively). In summary, we demonstrated in both Hep3B and Huh7 xenograft models, the combination treatment was by far, the most effective regimen *in vivo*.

### 3.4. Temsirolimus/vinblastine combination induced $G_2$/M cell cycle arrest

Next, we examined the effect of the combination on HCC cell cycle progression. We found that the combination resulted in $G_2$/M arrest in Huh7 and HepG2 cells, and also to a lesser extent in Hep3B cells (Fig. 4 and Table 2). Interestingly, the observed $G_2$/M arrest was found to occur with concomitant reduction in S phase populations in all 3 HCC cell lines. In Huh7, the combination resulted in 16.76% increase in $G_2$/M population ($P < 0.01$) with a 26.02% drop in S population ($P < 0.01$) when compared to vehicle control. Similar findings were observed in HepG2 cells (14.41% increase in $G_2$/M, $P < 0.05$; 23.44% decrease in S population, $P < 0.001$ *vs* control) and to a lesser extent in Hep3B cells (6.01% increase in $G_2$/M, $P = 0.35$; 4.64% decrease in S population, $P = 0.07$ *vs* control). Thus, the combination induced $G_2$/M arrest with concomitant reduction of S phase population in Huh7 and HepG2 cells, with a similar trend in Hep3B cells.

### 3.5. Temsirolimus/vinblastine combination does not further suppress mTOR signaling in HCC cells

In order to examine the mechanism of such a strong antitumor activity of this combination, we first examined the effects of this temsirolimus/vinblastine combination on mTOR signaling in HCC cells. As shown in Fig. 2B, temsirolimus/vinblastine combination did not result in further suppression of mTOR signaling when

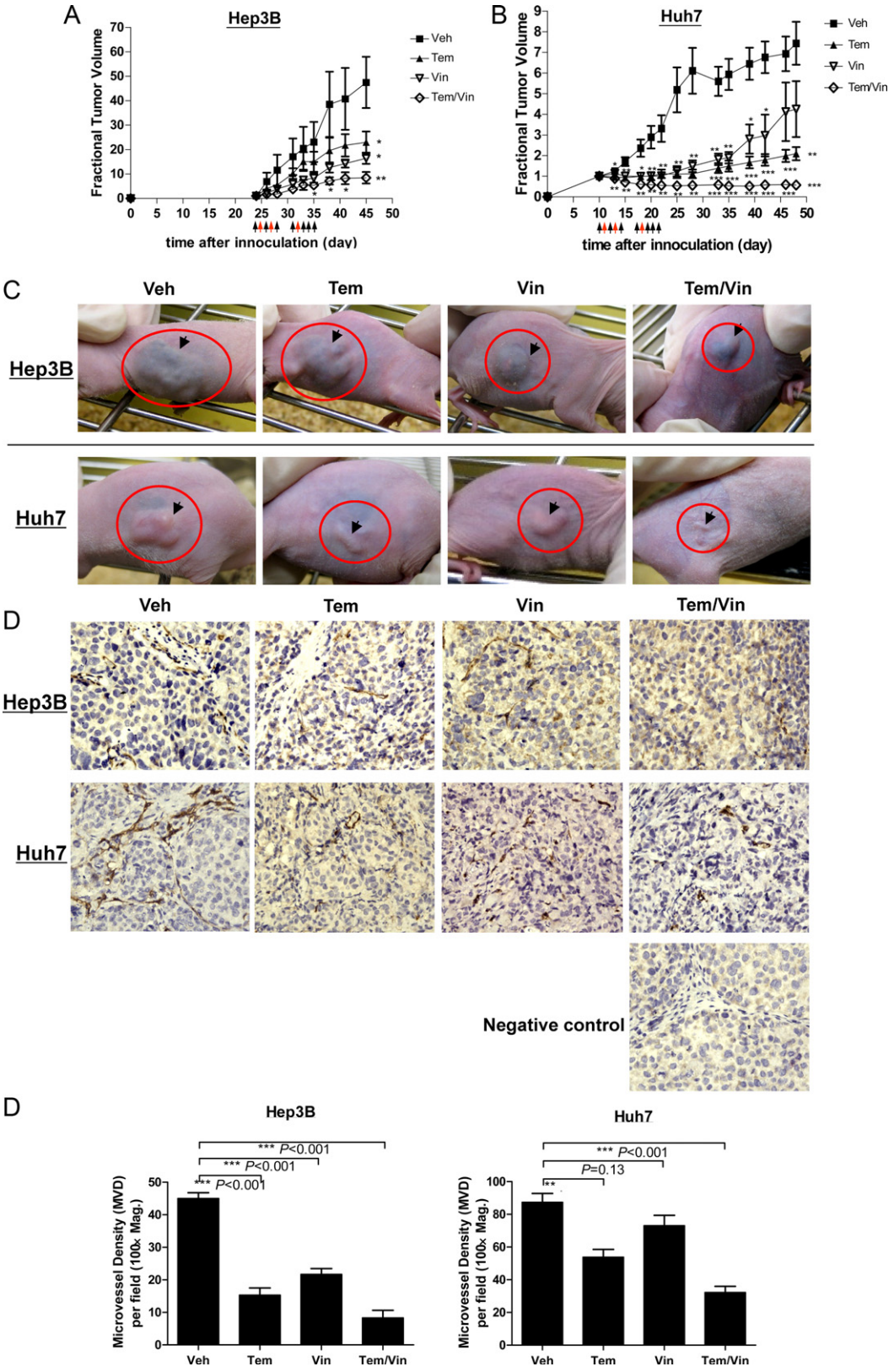


**Fig. 3.** Potent and sustained antitumor effects of the temsirolimus/vinblastine combination in *in vivo* models of HCC. HCC cells ($2 \times 10^6$ of Huh7 cells or $3 \times 10^6$ of Hep3B cells) were inoculated into nude mice by subcutaneous injection. Drug treatments were started on day 10 (for Huh7 xenografts), or day 24 (for Hep3B xenografts) post-inoculation. Mice received intraperitoneal injection of drugs for two weeks (black arrow: temsirolimus injection; red arrow: vinblastine injection). (A, B) Treatment of mice with temsirolimus, vinblastine or combination suppressed tumor growth in established xenografts of (A) Hep3B and (B) Huh7. Tumor growth was monitored twice weekly. Arrows indicated time of drug injection. The *in vivo* antitumor activity of temsirolimus/vinblastine combination was more significant and sustained than either agent alone ($n = 10$ per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle group). (C) Representative photographs showing Hep3B xenografts (upper panel) and Huh7 xenografts (lower panel) at the end of experiments. Arrows indicated the tumor xenografts. (D) Anti-angiogenic activity of the temsirolimus/vinblastine combination in *in vivo* models of HCC. Tumor xenografts were harvested, fixed, and stained for CD34 by immunohistochemistry. Representative images (400× magnification) were shown. Quantitation of CD34 staining (microvesssel density, MVD) was shown in lower panel ($n = 10$ per group, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle group).

**Table 1**
Average tumor volume during treatments in Hep3B xenograft models (A), and Huh7 xenograft models (B).

| (A) | | | |
|---|---|---|---|
| Hep3B xenografts | Average tumor volume ($mm^3$) | | |
| | Week 1 | Week 2 | End of Experiment |
| Veh | $105.64 \pm 16.61$ | $269.98 \pm 57.44$ | $649.24 \pm 148.65$ |
| Tem | $97.53 \pm 29.36$ ($P = 0.81$) | $206.45 \pm 42.01$ ($P = 0.40$) | $328.88 \pm 86.88$ ($P = 0.09$) |
| Vin | $89.97 \pm 5.30$ ($P = 0.48$) | $166.03 \pm 15.77$ ($P = 0.14$) | $300.22 \pm 30.44$ ($P = 0.06$) |
| Tem/Vin | $55.08 \pm 12.68$ ($^{*}P < 0.05$) | $131.35 \pm 23.31$ ($^{*}P < 0.05$) | $155.66 \pm 22.64$ ($^{**}P < 0.01$) |
| (B) | | | |
| Huh7 xenografts | Average tumor volume ($mm^3$) | | |
| | Week 1 | Week 2 | End of Experiment |
| Veh | $26.58 \pm 2.88$ | $41.99 \pm 3.68$ | $120.08 \pm 16.33$ |
| Tem | $21.19 \pm 2.50$ ($P = 0.18$) | $24.64 \pm 3.78$ ($^{**}P < 0.01$) | $58.04 \pm 12.33$ ($^{*}P < 0.05$) |
| Vin | $23.03 \pm 2.53$ ($P = 0.37$) | $25.74 \pm 4.59$ ($^{*}P < 0.05$) | $91.77 \pm 26.96$ ($P = 0.39$) |
| Tem/Vin | $14.97 \pm 1.86$ ($^{**}P < 0.01$) | $11.74 \pm 1.67$ ($^{***}P < 0.001$) | $10.45 \pm 2.61$ ($^{***}P < 0.001$) |

compared to temsirolimus treatment alone, while vinblastine alone did not alter mTOR signaling in Hep3B, Huh7 (Fig. 2B) nor in HepG2 cells (Supplementary Fig. 2B). These results indicate that the marked anti-proliferative effect of the temsirolimus/vinblastine combination is probably unrelated to further suppression of mTOR signaling in HCC cells. Note that the feedback activation of Akt still persisted with the temsirolimus/vinblastine combination treatment in both Huh7 and Hep3B cells (Supplementary Fig. 1B), suggesting the efficacy of this combination was probably not due to inhibition of this Akt feedback in HCC cells.

### 3.6. Temsirolimus/vinblastine combination downregulates key survival/anti-apoptotic proteins in HCC models

Next, we examined if the marked antitumor activity of the combination was due to possible induction of apoptosis in these HCC models, as the PI3K/Akt/mTOR signaling pathway is known to be crucial for cell survival. As shown in Fig. 5A, PARP cleavage was readily detected (by IHC) in both Hep3B and Huh7 tumors treated with either vinblastine alone or with the combination (Fig. 5A upper and lower panels), while temsirolimus did not increase cell apoptosis *vs* vehicle alone. These results implied that the observed apoptosis was mainly mediated by vinblastine in the combination.

The known involvement of the PI3K/Akt/mTOR signaling pathway in regulating cancer cell survival prompted us to look at the effect of this combination on a panel of survival or anti-apoptotic proteins, including survivin, Bcl-2, and Mcl-1 in these treated xenografts. Interestingly, we found that the combination treatment, but not the single agents, consistently and specifically inhibited the expression of survivin, Bcl-2, and to a lesser extent, Mcl-1 in both the Hep3B and Huh7 xenografts when compared to their respective vehicle treatments (Fig. 5B and C upper panel). Indeed, immunohistochemical scoring (IHC score) showed a specific and quantitative reduction of ~50% of survivin, ~60% reduction of Bcl-2 and ~40% reduction in Mcl-1 expression in both Hep3B and Huh7 xenografts treated with the combination as compared to vehicle control (Fig. 5B and C lower panel). In fact, these *in vivo* findings were also confirmed in the respective *in vitro* models as well (Fig. 5D). Taken together, our data suggested that concerted down-regulation of these important anti-apoptotic/survival proteins may mediate the marked anti-cancer effect of the combination in HCC cells.

### 3.7. Over-expression of survivin/Bcl-2/Mcl-1 rescued HCC cells from the growth-inhibitory activity of the combination

To further investigate if down-regulation of any of these anti-apoptotic/survival proteins (Bcl-2, survivin and Mcl-1) is mechanistically involved in the marked growth-inhibitory activity of the combination in HCC cells, we performed rescue experiments to see if over-expression of Bcl-2, survivin or Mcl-1 could reverse the growth-inhibitory action of this combination in HCC cells. As shown in Fig. 6A, transient transfection was able to induce overexpression of Bcl-2, survivin and Mcl-1 in all 3 HCC cell lines at 48 h. In the most temsirolimus-resistant HCC cell line, Hep3B, in which the temsirolimus/vinblastine combination resulted in synergistic growth inhibition (*vs* single agent alone), transient overexpression of Bcl-2 was able to rescue these cells from the synergistic growth-inhibitory activity of the combination to a level comparable to that of temsirolimus or vinblastine alone (Fig. 6B); whereas overexpression of survivin or Mcl-1 alone resulted in partial rescue of the growth-inhibitory effect of the combination in this cell line (Fig. 6B). Note that in all these experiments, overexpression of Bcl-2, survivin, or Mcl-1 did not result in any rescue from the growth inhibitory action of either agents alone (either temsirolimus or vinblastine alone), demonstrating the specificity of such a rescue effect for the combination only. Our results demonstrated that in this temsirolimus-resistant HCC cell line, Hep3B, the combination-induced growth inhibition was mainly mediated by downregulation of Bcl-2, and probably to a lesser extent, by downregulation of survivin or Mcl-1. Similarly, in HepG2 cells (intermediate sensitivity to temsirolimus; Fig. 1A and B), Bcl-2 and Mcl-1 overexpression partially mitigated the growth inhibitory activity of the combination, and survivin overexpression was able to rescue HepG2 cells from the combination-induced growth inhibition to a level that was similar to that of temsirolimus treatment alone (Fig. 6C). Lastly, in the most temsirolimus-sensitive cell line, Huh7, over-expression of Bcl-2, survivin or Mcl-1 alone all resulted in partial rescue from the growth inhibitory effect of the combination treatment (although the degree of rescue in this temsirolimus-sensitive cell line was less than that observed in Hep3B and HepG2 cells) (Fig. 6D). Taken together, our results showed direct mechanistic involvement of these key survival/anti-apoptotic proteins in the growth-inhibitory action of this combination in HCC cells.

## 4. Discussion

Here, we reported for the first time strong and sustained antitumor activity of co-targeting of mTOR (by temsirolimus) and the microtubule (by vinblastine) in both *in vivo* and *in vitro* models of HCC, with concerted downregulation of several key survival/anti-apoptotic proteins, Bcl-2, survivin and Mcl-1. With only two cycles of temsirolimus/vinblastine treatment, a sustained antitumor effect (and potent anti-angiogenic activity) was observed up

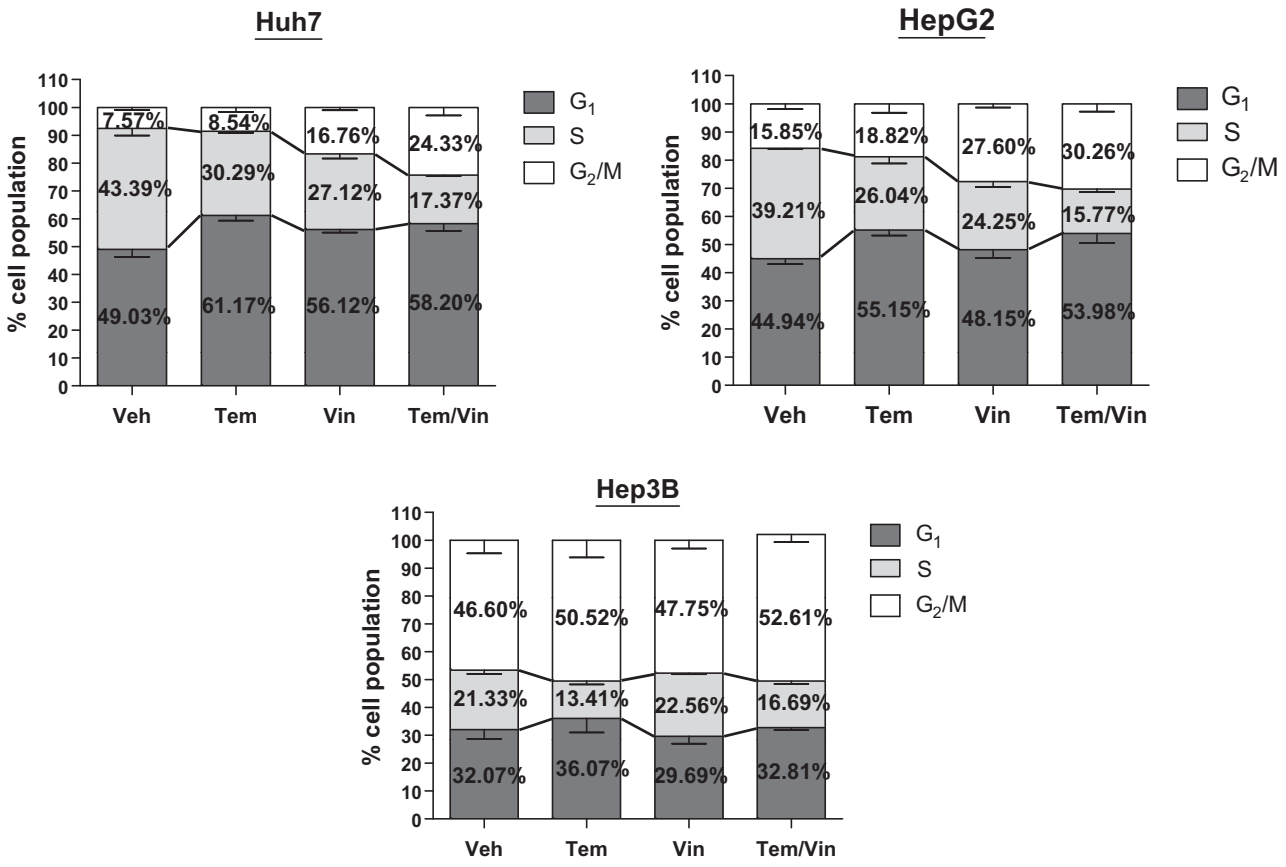


**Fig. 4.** Temsirolimus/vinblastine combination induced cell cycle arrest at $G_2$/M phase. Huh7, Hep3B and HepG2 Cells ($5 \times 10^5$) treated with temsirolimus (0.05 μM for Huh7, 1 μM for Hep3B and HepG2) and/or vinblastine (Vin) (1 nM) for 12 h or 16 h were harvested, and fixed in 70% ethanol at 4 °C overnight. Then cells were stained with propidium iodide, and analyzed by flow cytometry. Cumulative results from 3 independent experiments were shown as mean ± SEM.

to 21–38 days post-treatment in Hep3B and Huh7 xenograft models (Fig. 3), demonstrating a potent antitumor activity of this combination against HCC. This was in agreement with our observed additive to synergistic inhibitory effects of this combination on HCC cell growth in multiple models of HCC *in vitro* (Fig. 2A and Supplementary Fig. 2A). Further mechanistic studies demonstrated that this marked antitumor effect was, at least in part, mediated by Bcl-2, survivin, or Mcl-1 in HCC cells (Figs. 5 and 6). Our results indicate that combination of temsirolimus with vinblastine could be a highly effective regimen for HCC treatment, which may worth further clinical investigations in HCC patients.

**Table 2**
Temsirolimus/vinblastine combination-induced cell cycle effects in HCC cell lines. Cumulative results of 3 independent experiments were shown.

(A) Cumulative analysis of drug treatments on Huh7 cell cycle distribution.

| % cell population | Huh7 | | | |
|---|---|---|---|---|
| | Veh | Tem | Vin | Tem/Vin |
| $G_1$ | 49.03 ± 2.73 | 61.17 ± 1.79 (*$P < 0.05$) | 56.12 ± 1.06 | 58.30 ± 2.60 |
| S | 43.39 ± 2.50 | 30.29 ± 0.59 (**$P < 0.01$) | 27.12 ± 1.63 (**$P < 0.01$) | 17.37 ± 0.31 (**$P < 0.01$) |
| $G_2$/M | 7.57 ± 0.88 | 8.54 ± 1.58 | 16.76 ± 1.09 (**$P < 0.01$) | 24.33 ± 2.90 (**$P < 0.01$) |

(B) Cumulative analysis of drug treatments on HepG2 cell cycle distribution.

| % cell population | HepG2 | | | |
|---|---|---|---|---|
| | Veh | Tem | Vin | Tem/Vin |
| $G_1$ | 44.94 ± 1.92 | 55.15 ± 1.93 (*$P < 0.05$) | 48.15 ± 2.93 | 53.98 ± 3.42 |
| S | 39.21 ± 0.13 | 26.04 ± 2.44 (**$P < 0.01$) | 24.25 ± 1.95 (**$P < 0.01$) | 15.77 ± 1.13 (***$P < 0.001$) |
| $G_2$/M | 15.85 ± 1.90 | 18.82 ± 3.18 | 27.60 ± 1.32 (**$P < 0.01$) | 30.26 ± 2.74 (*$P < 0.05$) |

(C) Cumulative analysis of drug treatments on Hep3B cell cycle distribution.

| % cell population | Hep3B | | | |
|---|---|---|---|---|
| | Veh | Tem | Vin | Tem/Vin |
| $G_1$ | 32.07 ± 3.33 | 36.07 ± 5.02 | 29.69 ± 2.72 | 32.81 ± 0.88 |
| S | 21.33 ± 1.37 | 13.41 ± 1.86 (*$P < 0.05$) | 22.56 ± 0.34 | 16.69 ± 1.02 ($P = 0.07$) |
| $G_2$/M | 46.60 ± 4.69 | 50.52 ± 6.19 | 47.75 ± 2.99 | 52.61 ± 2.81 ($P = 0.35$) |

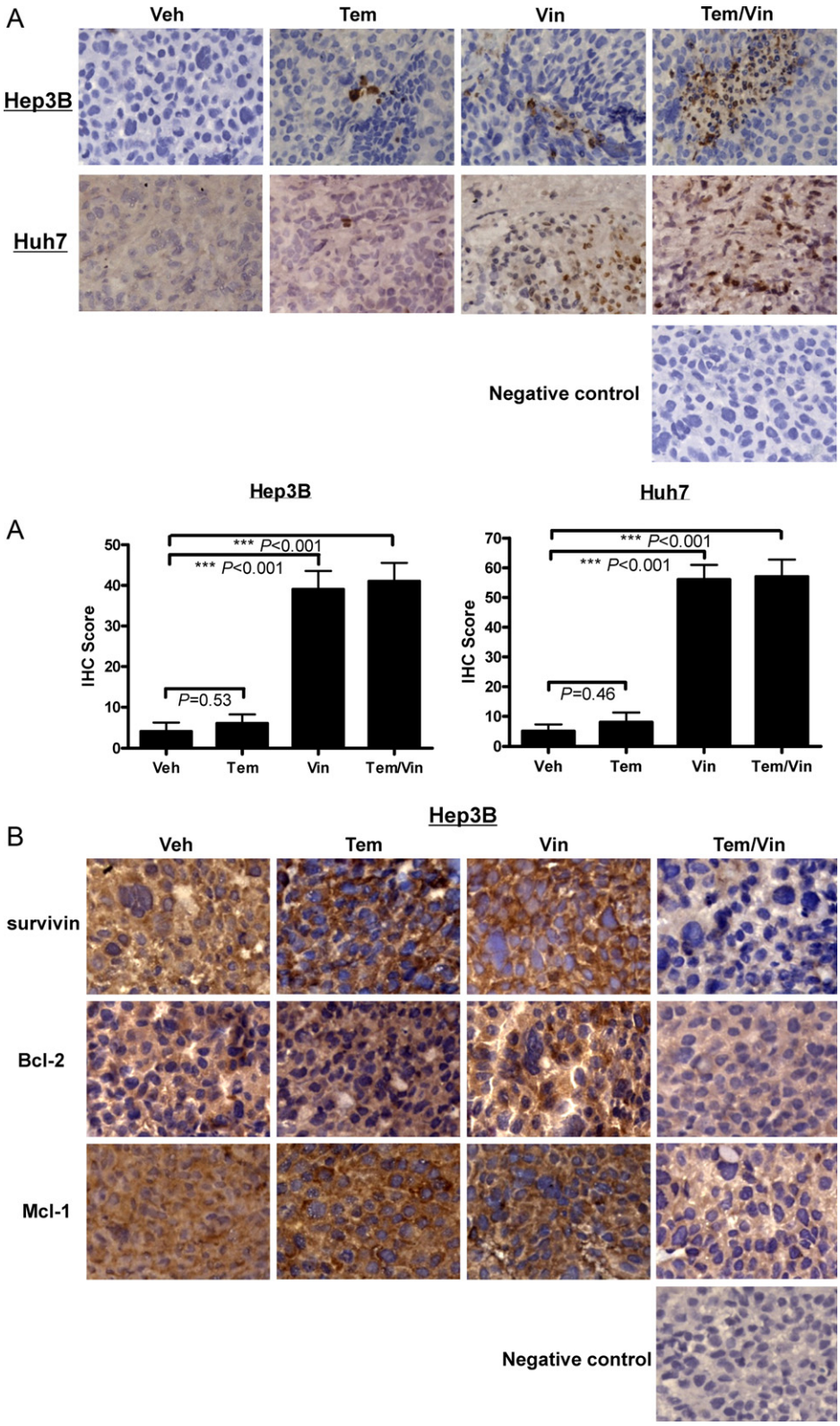


**Fig. 5.** Temsirolimus/vinblastine combination downregulates key survival/anti-apoptotic proteins in HCC models. (A) PARP cleavage (hallmark of apoptosis) was observed in HCC xenografts upon vinblastine treatment (Vin) or the temsirolimus/vinblastine combination treatment in HCC xenografts. Tumor xenografts were harvested, fixed, and stained for cleaved-PARP by immunohistochemistry. Representative images (400× magnification) were shown. Quantitation of cleaved-PARP staining using immunohistochemistry scoring (IHC score) was shown in lower panel ($n = 10$ per group, $^{***}P < 0.001$ *vs* vehicle group). (B, C) Survivin, Bcl-2 and Mcl-1 were down-regulated by temsirolimus/vinblastine combination treatment in Hep3B (B) and Huh7 (C) xenografts. Tumor xenografts were harvested, fixed, and stained for survivin, Bcl-2 and Mcl-1 by immunohistochemistry. Representative images (400× magnification) were shown. Quantitation of survivin, Bcl-2 and Mcl-1 staining using immunohistochemistry scoring was shown in lower panel ($n = 10$ per group, $^{***}P < 0.001$ *vs* Tem/Vin-treated group). (D) Survivin, Bcl-2 and Mcl-1 were downregulated by temsirolimus/vinblastine combination treatment in HCC cell lines. Hep3B and Huh7 cells ($3 \times 10^5$) were treated with temsirolimus (1 μM for Hep3B, 0.05 μM for Huh7) and/or vinblastine (1 nM) for 24 h. The expression levels of Mcl-1, Bcl-2, survivin and actin were assessed by Western blotting. Similar results were observed in 3 independent experiments.

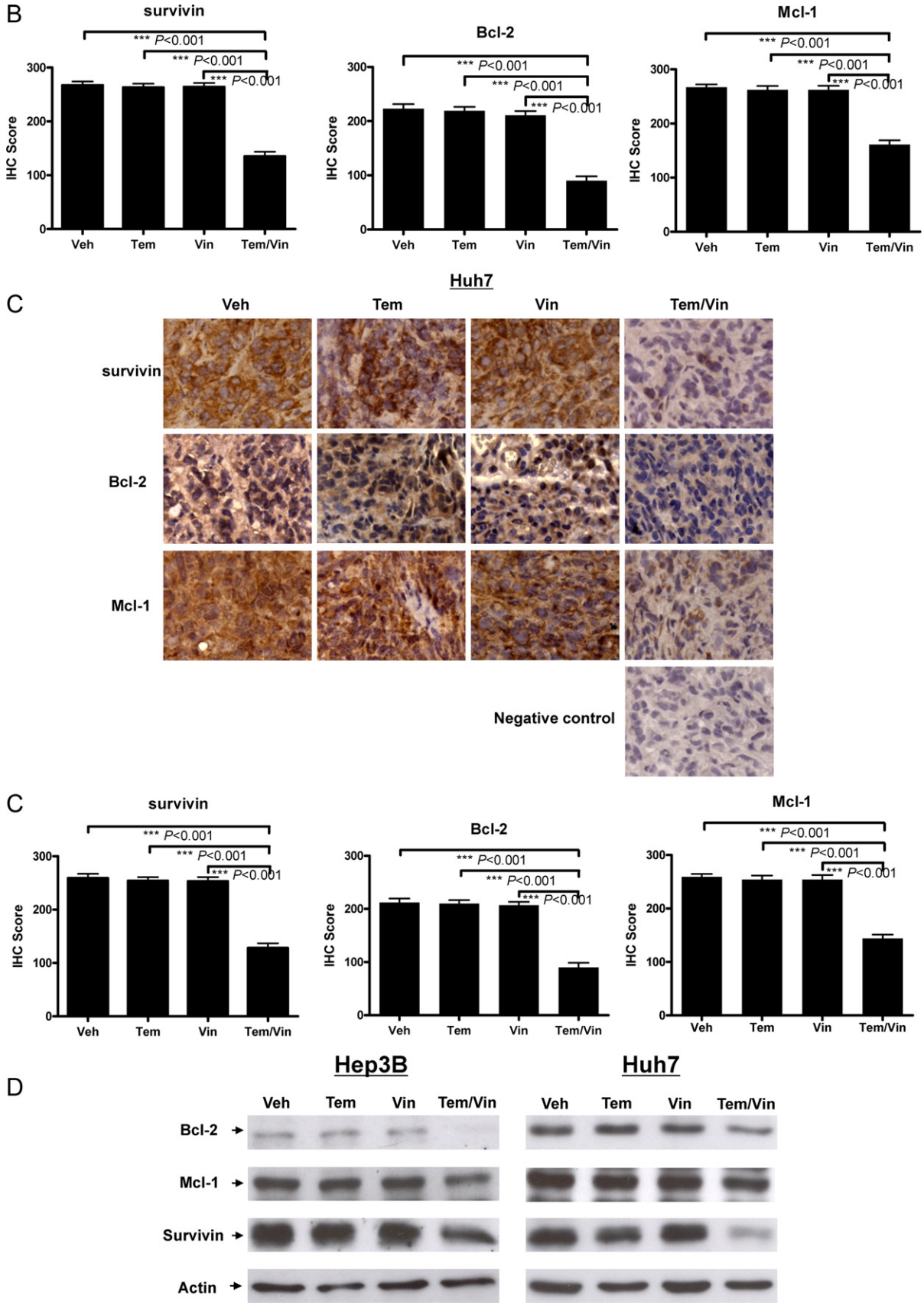


Fig. 5. (*Continued*).

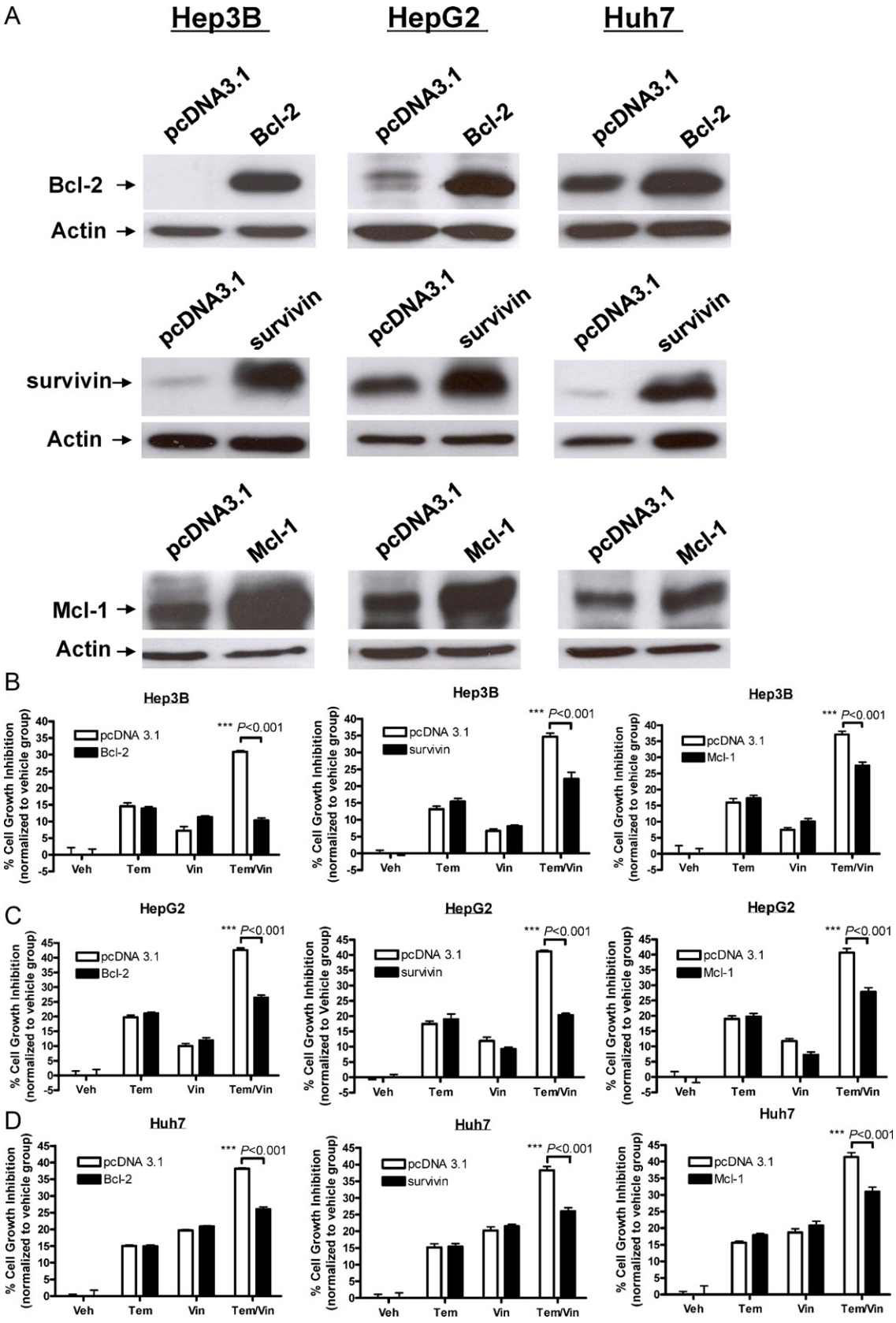


**Fig. 6.** Over-expression of survivin/Bcl-2/Mcl-1 rescued HCC cells from the growth-inhibitory activity of the temsirolimus/vinblastine combination. (A) Hep3B, HepG2 and Huh7 cells ($1.5 \times 10^5$) were transfected with 1 μg of expression plasmids (survivin, Bcl-2 and Mcl-1) as well as pcDNA3.1 vector control. Expression levels of survivin, Bcl-2 and Mcl-1 at 48 h after transfection were determined by Western blotting. (B, C, D) Over-expression of survivin, Bcl-2, Mcl-1 reversed the anti-proliferative effect of temsirolimus/vinblastine combination. HCC cells Hep3B (B), HepG2 (C) and Huh7 (D) transfected with survivin, Bcl-2 or Mcl-1 plasmid (for 48 h) were treated with temsirolimus (1 μM for Hep3B and HepG2, 0.05 μM for Huh7) and/or vinblastine (1 nM) for 24 h. Cumulative results from 3 independent experiments were shown as mean ± SEM (***$P < 0.001$ *vs* pcDNA3.1 transfectants).

We found that HCC cell lines demonstrated a wide-range of sensitivity toward mTOR targeting by temsirolimus alone (Fig. 1A and B), with Huh7 being the most sensitive cell line tested, and Hep3B being the most resistant cell line, with their respective $IC_{50}$ differing by 42-fold (Fig. 1B). Such a differential sensitivity was also observed in *in vivo* xenograft models of Huh7 and Hep3B (Fig. 3A and B). Among the five cell lines tested, Hep3B is p53 deleted, and others harbor p53 wildtype gene or mutant. Some reports indicated that p53 status may be related to PI3K pathway activation since p53 transcriptionally regulates PTEN [27,28], therefore p53 deletion may lead to over-activation of PI3K pathway and contributes to the observed resistance of Hep3B cells to mTOR inhibitors. Nevertheless, further investigations are warranted. Previous studies in other cancers indicated that mTOR targeting may elicit cytostatic effects rather than effective eradication of tumor cells [29,30], suggesting the potential advantage for combination of mTOR targeting with cytotoxic agents. Therefore, in search for a rational combination with temsirolimus, we decided to choose a combination with a microtubule-targeting agent, vinblastine, based on the facts that: (1) microtubule-targeting is believed to be a prominent druggable target in HCC [16–18], (2) targeting of the microtubule in HCC has recently been shown to be highly effective against HCC *in vivo* [18], more importantly, (3) several studies have demonstrated the link between PI3K/mTOR/Akt signaling with cancer cell resistance to microtubule-targeting agents [19,20], and lastly, (4) vinblastine is a clinically effective chemotherapeutic agent for a number of cancer types with well-established microtubule-targeting activity. Indeed, we found that when temsirolimus was combined with very low dose of vinblastine (1 nM), marked synergistic effect was observed in HCC cell lines with a maximal achievable growth inhibition of about 80–90%. More interestingly, we found that even though mTOR targeting by temsirolimus alone was effective in suppressing mTOR signaling and growth of HCC cells, combination with vinblastine has exhibited superior antitumor activity in HCC models without further suppressing mTOR signaling (*vs* temsirolimus alone; Figs. 2 and 3), implicating an mTOR-independent effects on growth inhibition by this combination in HCC models.

Mechanistic investigation revealed that the combination treatment significantly decreased the expression of several key anti-apoptotic and survival proteins, including Bcl-2, survivin and Mcl-1 in HCC models, suggesting the downregulation of these proteins to be a likely mechanism contributing to the synergistic growth inhibition in HCC models. Indeed, our rescue experiments demonstrated that overexpression of Bcl-2, survivin or Mcl-1, was able to reverse the potent anti-cancer effects of this combination in multiple HCC models (Fig. 6), confirming their direct mechanistic involvement in the anti-cancer activity of this combination in HCC. Several recent studies have shown that survivin, Bcl-2 and Mcl-1 are important proteins/targets in HCC [31–35]. Survivin is overexpressed in ~70% of HCC cases and is significantly associated with pathological grading of HCC, with high expression of survivin being an independent prognostic factor for poor survival in HCC patients [31,35]. Bcl-2 is expressed in ~20% of HCC tumors, but not in normal livers [34–36], suggesting that expression of Bcl-2 may be involved in HCC development in a subset of patients. Interestingly, in the study reported by Yang et al. [35], Bcl-2 was highly expressed in 67% of the adjacent non-tumor tissues of HCC tumors, suggesting its potential dysregulation during HCC development. Mcl-1, an anti-apoptotic member of the Bcl-2 family, which regulates intrinsic apoptosis induction at the mitochondrial level, is overexpressed in 51% of HCC tissues [33]. Given the widespread overexpression of these survival/anti-apoptotic proteins in HCC patients, this highly effective combination of temsirolimus/vinblastine may prove to be particularly relevant in clinical settings of HCC as a rational treatment option.

Temsirolimus is currently undergoing Phase I/II clinical trials either as a single agent alone or in combination with sorafenib, the only clinically approved targeting agent for HCC. In anticipation of the clinical trial results, our study revealed for the first time that combination with vinblastine, and possibly, other microtubule-targeting agents could be tested in preclinical, as well as clinical studies in HCC. Similarly, vinblastine combination with other mTOR targeting agents may also be advantageous in treating HCC, and possibly other cancers, and this would definitely worth further investigation. In fact, Marimpietri et al. recently demonstrated that combination of rapamycin and vinblastine enhanced the therapeutic effect on human neuroblastoma growth, apoptosis and angiogenesis [37]. Moreover, rapamycin/vinblastine combination was found to exert anti-angiogenic effects in an endothelial cell line, EA.hy926 [38], suggesting potential anti-angiogenic activity of mTOR/vinblastine targeting, which is consistent with our findings in the current study (Fig. 3D).

Vinblastine is a microtubule-destabilizing agent, widely used as conventional chemotherapeutic drug for the treatment of breast cancer, lymphoma, bladder cancer and lung cancer. It binds with high affinity to tubulin and prevents the formation of cytoskeleton microtubules, and subsequently cause cell cycle arrest [39]. Several studies have shown inherent or acquired resistance to vinblastine in HCC cells due to high intrinsic activity of multi-drug resistance gene (MDR) in HCC [40,41]. Based on data from the present study, we were able to show for the first time that combination of a very low dose of vinblastine with temsirolimus was able to result in a much stronger and sustained antitumor effect when compared to single agent alone in HCC xenograft models (in both the temsirolimus-sensitive as well as temsirolimus-resistant HCC models, Huh7 and Hep3B).

In conclusion, our study demonstrated that combination of temsirolimus with low dose of vinblastine could be a highly effective regimen for the treatment of HCC. Clinical investigations in the role of such combination in HCC patients are warranted.

## Conflict of interest

All authors declare no financial/commercial conflict of interest regarding the study.

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