


Fingolimod exerts *in vitro* anticancer activity against hepatocellular carcinoma cell lines *via* YAP/TAZ suppression

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ABSTRACT

Hepatocellular carcinoma (HCC) remains a notably global health challenge with high mortality rates and poor prognosis. The deregulation of the Hippo signaling pathway, especially the overexpression and activation of downstream effector Yes-associated protein (YAP), has been demonstrated to result in the rapid malignant evolution of HCC. In this context, multiple efforts have been dedicated to targeting YAP for HCC therapy, but effective YAP inhibitors are still lacking. In this study, through a YAP-TEAD (8×GTIC) luciferase reporter assay, we identified fingolimod, an immunomodulatory drug approved for the treatment of multiple sclerosis, as a novel YAP inhibitor. Fingolimod suppressed the proliferation of HCC cell lines by downregulating the protein levels as well as the transactivating function of YAP. Overall, our current study not only identifies fingolimod as a novel YAP-targeting inhibitor, but also indicates that this clinically-approved drug could be utilized as a potential and feasible therapeutic drug for HCC.

Keywords: fingolimod, hepatocellular carcinoma, hippo signalling, YAP/TAZ

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Liver cancers rank third in the highest mortality rate and are the sixth most prevalent cancer worldwide (1). Hepatocellular carcinoma (HCC) is the most common histologic type of liver cancer, accounting for the vast majority of diagnoses and deaths (2). Patients with advanced HCC mainly rely on systemic drug therapy. Sorafenib is the standard of care for frontline therapy, but the drug response rate remains to be improved (3, 4). Therefore, it is urgent to explore drug candidates that could improve the current clinical outcomes of HCC patients.

Recently, mounting evidence has shown that the Hippo signalling pathway is closely involved in the carcinogenesis and development of HCC (5, 6). The Hippo pathway has been identified as a critical tumour suppressor pathway, which exerts the function through a series of kinase cascade reactions by MST1/2 and LATS1/2. As the downstream effectors, transcriptional coactivators YAP (Yes-associated protein) and its paralogue TAZ (Transcriptional coactivator with PDZ-binding motif) are phosphorylated by MST1/2 and LATS1/2, then retained in the cytosol and subjected to proteasomal degradation (7). On the other hand, dysregulation of the Hippo pathway (Hippo off) will result in the aberrant activation of YAP/TAZ, and ultimately lead to malignant cell proliferation and tumour outgrowth (8, 9).

A recent study shows that ~60 % of HCC patients harbour hyperactivated YAP/TAZ, and 5–10 % of HCC patients have genomic amplification of YAP-containing genomic locus (10, 11). The overexpression of YAP rapidly induces hepatomegaly and tumorigenesis. Moreover, the higher levels of YAP/TAZ are generally associated with decreased overall survival and poor disease-free survival times of HCC patients (10). To verify the essential roles of YAP/TAZ in HCC development, we analyzed the clinical significance of YAP in HCC patients using The Cancer Genome Atlas (TCGA) database (<http://ualcan.path.uab.edu/>). As shown in Fig. S1A, most HCC primary tumour samples exhibited remarkably higher mRNA levels of YAP than that of nontumorous tissues ($p < 0.001$). In addition, we also knocked down TAZ to evaluate the influence of TAZ depletion in HCC cell line proliferation, and found that TAZ silence greatly inhibited the colony formation of Bel-7402 cells (Fig. S1B). As such, the Hippo-YAP signalling cascade is an appealing therapeutic target for the treatment of HCC and other cancer types with hyperactivated YAP/TAZ.

Fingolimod (FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]propan-1,3-diol hydrochloride) is an FDA approved immunomodulator for multiple sclerosis treatment (12). Fingolimod would be phosphorylated by sphingosine kinase (SphK) into fingolimod-phosphate *in vivo*, then the fingolimod-P mimics sphingosine 1-phosphate (S1P) binding to sphingosine 1-phosphate receptors (S1PRs), acting as a partial agonist of S1PRs and simultaneously as a selective antagonist of S1PR1 (13). Fingolimod causes peripheral lymphopenia, particularly T cell subtypes and generates immunosuppression through its regulation of S1PR signaling. In addition to its effects on immunomodulation, mounting evidence has also revealed that fingolimod exhibits antitumor effects in a variety of cancer types, including glioblastoma multiforme, lymphoma, breast cancer, ovarian cancer *etc.*, through the S1P-dependent or independent mechanisms (14–17). However, its influence against HCC cells is not clear, and the mechanisms of action of fingolimod are yet remained to be elucidated.

In this study, we found that fingolimod was a potential YAP inhibitor through the YAP/TAZ activity-based 8×GTIIc luciferase reporter system. Further studies showed that fingolimod impeded the survival of HCC cell lines by down-regulating the protein level of YAP.

EXPERIMENTAL

Chemicals

Clinically-used drug library was purchased from TargetMol (USA) as powder and dissolved in DMSO to 10 mmol L⁻¹. Another 71 compounds with new chemical structures were from our in-house compound library (Supplementary Table I). Fingolimod hydrochloride was purchased from Aladdin (China) with the purity > 99 % and dissolved in DMSO to 40 mmol L⁻¹ as stock solution. In the following, fingolimod will be used to refer to fingolimod hydrochloride. They were diluted with the corresponding cell culture medium to obtain the final concentrations for dosing.

Cell culture

Human hepatocellular carcinoma cell lines HepG2 and Bel-7402, and human embryonic kidney HEK293 cell line were purchased from the Cell Bank of China Science Academy in 2016. The Bel-7402 cell line was maintained in RPMI-1640 media. The HepG2 and HEK293 were cultured in Dulbecco's modified Eagle's medium. Both media were supplemented with 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin sulfates and 10 % FBS (Hyclone) at 37 °C, in a 5 % CO₂ humid atmosphere.

8×GTIIC luciferase reporter activity assay

The 8×GTIIC (Addgene, catalogue: 34615) luciferase reporter, harbouring eight discontinuous repeating TEADs-DNA binding sequences, was applied to investigate the transcriptional activity of TEADs. HEK293 cells were plated in 96-well plates at a density of 30,000 per well. When reaching 70–80 % confluency, the cells were transfected with 0.2 µg 8×GTIIC firefly luciferase and 0.01 µg *Renilla* (internal control) luciferase plasmid each well using jetPrime and incubated for 4–6 h. After overnight incubation, these cells were exposed to tested compounds for the next 24 h. The corresponding luminescence number was obtained by the dual-luciferase reporter gene assay kit (Promega E1960), and the ratio of firefly and *Renilla* was normalized to the DMSO control, the inhibition ratio was calculated as:

$$[1 - (\text{firefly}/\text{Renilla})_{\text{treated}} / (\text{firefly}/\text{Renilla})_{\text{control}}] \times 100 \%$$

CT-707 was introduced as a positive control of YAP inhibitor as it exhibited potent suppressive effects on the YAP pathway (18).

Immunoblotting analysis

Immunoblotting analysis was generally performed using a standard protocol as previously described (19). The information of primary antibodies applied for immunoblotting were as follows: YAP (#A1002) antibody was purchased from ABclonal (China) and GAPDH (#db106) antibody was purchased from Diabgio (China). The quantification results of the bands were analyzed in ImageJ.

Sulforhodamine B (SRB) assay

HepG2 or Bel-7402 cells suspended in a medium containing 10 % FBS were seeded 3000/well in 96-well plates for 12 h. Different concentration gradients of fingolimod or

DMSO were added to each dish for the treatment of another 72 h. When harvesting cells, 10 % trichloroacetic acid was utilized to fix cell monolayers for 1 h at 4 °C. Then cells were stained with SRB dye for 20 min and the plates were washed with 1 % acetic acid. After drying, the dye bound to protein was dissolved in 60 μL 10 mmol L^{-1} Tris base for OD determination at 515 nm. The cell proliferation fraction was calculated as A_{515} treated cells/ A_{515} control cells \times 100 %. Every experiment was repeated three times.

Lentiviral production and infection

For stably overexpress the protein of YAP, the human YAP coding region was amplified from the HCC cell line HepG2 cDNA library and was subcloned into the pCDH plasmid. Using this carrier vector plasmid with lentiviral packaging plasmids pCMV-p8.9 and pMDG-VSVG to produce lentivirus using a polyethyleneimine (PEI) transfection protocol. For 10 mL of HEK-293T culture plates, add a mix media with 5 μg target plasmid, 1 μg pMDG-VSVG, 5 μg pCMV-p8.9 and 50 μL PEI for 18 h. Replace the media with 10 mL DMEM with 10 mmol L^{-1} pyruvate to treat the cells for 24 h, then filter and harvest the lentiviral through a 0.45 μm PES filter. The HCC cells were infected by appropriate lentivirus and adding 1:1000 polybrene (6 mg mL^{-1}) for 24 h.

Statistical analysis

All statistical analyses were performed by GraphPad Prism 8. The data in experiments were presented as mean \pm SD and comparison between two groups was conducted by Student's *t*-tests ($p < 0.05$ means statistically significant).

RESULTS AND DISCUSSION

Fingolimod is identified as a potential inhibitor of YAP signalling

In order to establish a YAP inhibitor screening model, we overexpressed 8 \times GTIIC-luciferase reporter to monitor the YAP/TAZ transactivation activity, as well as a *Renilla* luciferase reporter which served as an internal control (Fig. 1a). Subsequently, a library of 120 compounds including 49 clinically-used drugs and 71 in-house stocks of compounds with new chemical structures was screened at a concentration of 20 $\mu\text{mol L}^{-1}$ (Supplementary information). Among these compounds, fingolimod (Fig. 1c), stood out as the most potent YAP/TAZ inhibitor, with an inhibitory ratio of 75.34 % against YAP/TAZ-induced 8 \times GTIIC-luciferase activity (Fig. 1b).

Fingolimod suppressed YAP signalling by downregulating the expression

In order to further evaluate the inhibitory effect of fingolimod on the Hippo-YAP signalling, we detected YAP/TAZ transcriptional activity in cells exposed to serial concentrations of fingolimod. Fig. 2a showed that the inhibition rate of YAP/TAZ transcriptional activity increased from 15.54 % at 0.31 $\mu\text{mol L}^{-1}$ to 81.16 % at 10 $\mu\text{mol L}^{-1}$ in a concentration-dependent way. We further evaluated the protein expression levels of YAP in HCC cell lines after fingolimod treatment for 48 h using Western Blot analyses. As shown in Fig. 2b,

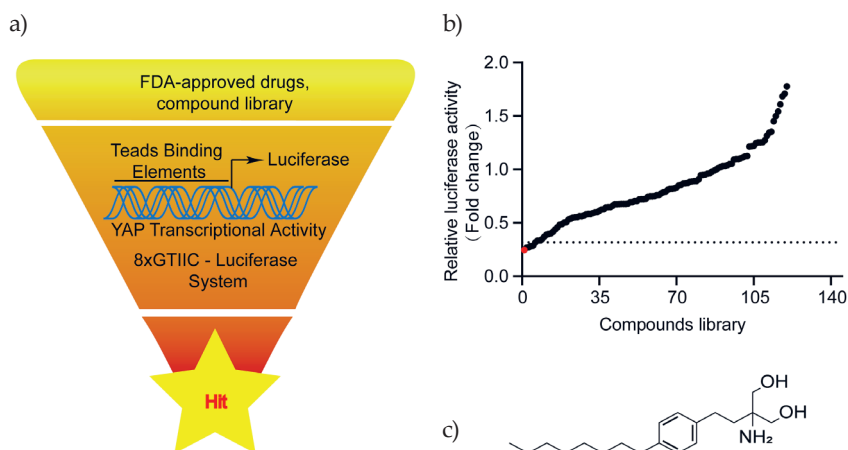


Fig. 1. Fingolimod is identified as a potential inhibitor of Hippo-YAP signalling: a) schematic diagram of the 8xGTIIC luciferase reporter activity assay; b) YAP transcriptional activity screening results of 120 compounds library displayed by the reporter system (listed in Supplementary information; dotted in red is fingolimod). Screening results are based on a single experiment; c) chemical structure of fingolimod.

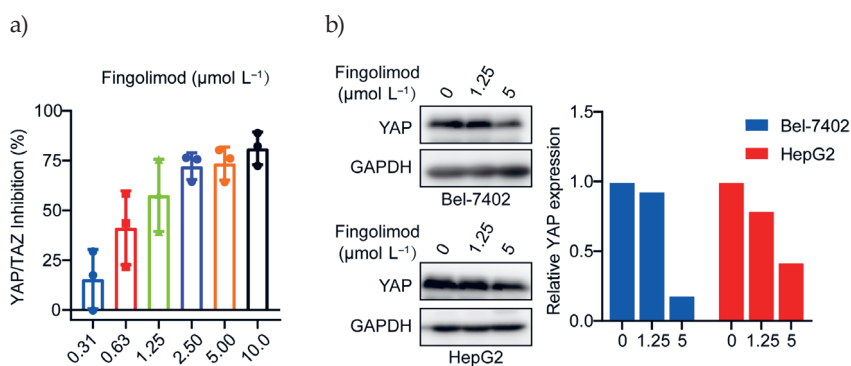


Fig. 2. Fingolimod suppressed YAP signalling by downregulating the protein expression: a) fingolimod inhibited the transcriptional activity of YAP/TAZ in 0.31, 0.63, 1.25, 2.5, 5.0, 10 $\mu\text{mol L}^{-1}$, the inhibition ratio was 15.54, 41.25, 57.57, 72.17, 73.51 and 81.16 % respectively; b) Western-bolt analyses showed that fingolimod decreased YAP protein in a concentration-dependent manner in both Bel-7402 and HepG2, then the value of protein level was quantified by ImageJ. The data represent the mean \pm SD of three independent experiments.

the protein level of YAP was significantly decreased along with the increasing concentrations of fingolimod in both Bel-7402 and HepG2 (1.25 and 5 $\mu\text{mol L}^{-1}$, respectively). These data confirmed our screening results and manifested that fingolimod may interfere with YAP signalling by reducing protein levels.

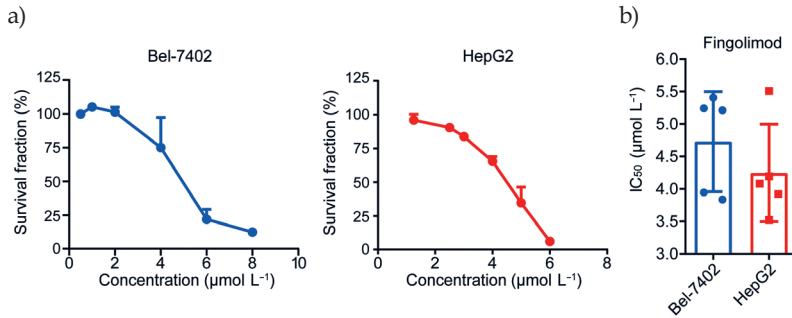


Fig. 3. Fingolimod exerted antitumor activity against hepatocellular carcinoma cell lines: a) the cytotoxicity of fingolimod was determined by the SRB assay in Bel-7402 and HepG2 cell lines treated at different concentrations. Each experiment was performed in triplicate in three independent experiments and the data are expressed as mean \pm SD; b) IC_{50} of fingolimod in Bel-7402 and HepG2. The results show the mean \pm SD of five independent experiments.

Fingolimod exerted antitumor activity against hepatocellular carcinoma cell lines

YAP/TAZ facilitates cancer cell proliferation by transactivating a lot of proliferation-related target genes, and the interruption of YAP/TAZ would arrest the cell growth in different cancer types. In this context, we were inspired to assess whether the survival of HCC cells would be influenced by fingolimod. As expected, the proliferation of Bel-7402 and HepG2 cells treated with fingolimod for 72 h was significantly suppressed in a concentration-dependent manner as detected by SRB assay (Fig. 3a). The survival ratio of fingolimod-treated in Bel-7402 was 12.35 % at 8 $\mu\text{mol L}^{-1}$, and 5.98 % at 6 $\mu\text{mol L}^{-1}$ in HepG2. The IC_{50} (half maximal inhibitory concentration) was calculated, and the value was 4.43 $\mu\text{mol L}^{-1}$ in Bel-7402 and 4.25 $\mu\text{mol L}^{-1}$ in HepG2 cells for fingolimod, respectively (Fig. 3b). These data showed that fingolimod exerted significant anticancer activities against HCC cell lines.

Fingolimod arrested HCC cell proliferation by suppressing the YAP pathway

YAP is a pivotal downstream effector of the Hippo pathway which plays a key role in tumorigenesis and progression in many cancers, especially in hepatocellular carcinoma (18). Activated YAP could enter the nucleus and act as a transcriptional co-activator promoting tumour cell proliferation (20). YAP-5SA, which lacks five serine residues, simulates activated YAP to locate the nucleus predominantly (21). We used lentiviral infection on Bel-7402 to overexpress YAP and YAP-5SA to confirm whether YAP plays a key role in the suppression of HCC cell lines caused by fingolimod (Fig. 4a). Inhibition ratios of infected cells were calculated after treatment for 72 h. In Bel-7402 cells, YAP-5SA overexpression significantly rescued the loss of viability exposed to fingolimod (the inhibition ratio in 5 $\mu\text{mol L}^{-1}$ from 85.47 to 72.86 %), however, YAP overexpression was ineffective (Fig. 4b). These results suggested that the YAP-inhibiting effect is crucial for fingolimod to impede the HCC cell proliferation. And this agent could antagonize the tumour-promoting effect of YAP overexpression, possibly by down-regulating the cytoplasmic YAP protein without affecting the function of YAP in the nucleus.

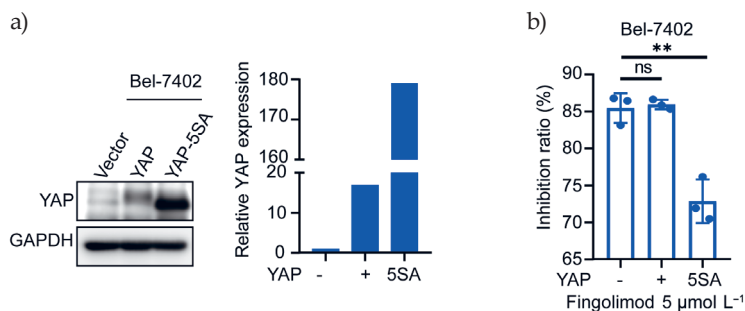


Fig. 4. YAP was the key to the anti-HCC cell lines effect of fingolimod: a) YAP and YAP-5SA overexpression successfully showed by Western-blot; b) the effect of YAP and YAP-5SA overexpression on Bel-7402 inhibition at different concentrations of fingolimod. The results correspond to the mean \pm SD of three independent experiments.

Given the high positive correlation between YAP expression and the malignant progression of liver cancer patients, extensive efforts have been devoted to developing YAP-targeted therapies. Currently, inhibitors targeting Hippo-YAP can be classified into three categories (22). For Class I, drugs regulate the transcriptional function of YAP-TEADs indirectly through the upstream crosstalk pathways. For example, statins act as mevalonate pathway inhibitors to interfere with the activation of Rho GTPases by inhibiting the HMGCR cascades, ultimately leading to YAP phosphorylation and cytoplasmic retention (23). In Class II, compounds directly target the binding surface of YAP-TEADs or affect the palmitoylation sites of TEADs to disrupt interaction, which ultimately prevents the normal output of the YAP-TEADs transcription complex. Verteporfin (VP) is recognized as a representation, but its photosensitivity and severe proteotoxic effects limit further clinical application (24, 25). Class III modalities focus on neutralizing or antagonizing the oncogenic downstream targets of Hippo-YAP including metabolic enzymes, kinases and ligands (26). However, among the many inhibitors mentioned above, there are still a few clinical trials using YAP as a therapeutic target. Therefore, finding fingolimod to be an effective YAP inhibitor in the clinically approved compounds library not only enriches the functions of the drug, but also bypasses the long streamline of the drug discovery process to finish drug repurposing.

Several antitumor mechanisms of fingolimod have been revealed, apart from classical function as an S1PR ligand, it also affects other signalling pathways to produce beneficial off-target effects. Fingolimod directly interferes with SET-PP2A (Protein phosphatase 2A) complexes and ultimately contributes to the reactivation of PP2A which is widely approved as a tumour suppressor (27). Fingolimod may also increase PTEN expression and inactivate PI3K/Akt through S1P-dependent and independent mechanisms (28). Woodcock *et al.* also demonstrated that 14-3-3 proteins could be directly modulated by fingolimod leading to increase phosphorylation and activation, then activated 14-3-3 proteins can bind with various substrate proteins to participate in related signal transduction (29). Interestingly, 14-3-3 proteins also bind YAP/TAZ in the Hippo signalling pathway, hinder its nuclear localization and promote degradation by ubiquitination (30, 31). This may explain why fingolimod couldn't change the survival fraction after YAP overexpression, but YAP-5SA stabilized into the nucleus could reduce the inhibitory rate. Of course, this hypothesis still

needs follow-up experiments for further validation. Besides, we have confirmed that fingolimod exerted the effect of inhibiting HCC cell lines *in vitro*, whether it has an antitumor effect *in vivo* remains to be confirmed on the animal model.

CONCLUSIONS

The aim of this study was to search for a potent YAP inhibitor. Here we used an 8×GTIIIC luciferase reporter system to filter to fingolimod, which showed a YAP inhibitory activity by downregulating the protein level as well as the transactivating function, ultimately leading to the anticancer effects against HCC cell lines (IC_{50} was $4.43 \mu\text{mol L}^{-1}$ in Bel-7402 and $4.25 \mu\text{mol L}^{-1}$ in HepG2). Therefore, in addition to the clinical utilization to treat multiple sclerosis, fingolimod might also be used as a novel YAP inhibitor for cancer therapy in HCC patients or the other cancer types with high activity of the YAP/TAZ pathway.

Supplementary materials available upon request.

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Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, H.Z. and S.Y.; methodology, Q.J.H. and B.Y.; analysis T.Y. and B.Z.; investigation, X.Q.C. and N.A.; writing, original draft preparation, J.M.D.; writing, review and editing, J.M.D., M.J.Q. and H.Z. All authors have read and agreed to the published version of the manuscript.

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