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The calcimimetic agent cinacalcet inhibits hepatocellular carcinoma via YAP/TAZ suppression

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The lack of effective strategies remains a pivotal challenge for hepatocellular carcinoma (HCC) treatment. YAP/TAZ is a promising target for effective drugs against HCC. In this study, we profiled the regulatory effect of 98 drugs on transcriptional activity of YAP/TAZ and identified the calcimimetic agent cinacalcet as a potent YAP inhibitor. Cinacalcet inhibited YAP expression in HCC models at both transcriptional and protein levels, and ultimately arrested cell proliferation of HCC. Overexpression of YAP weakened the anticancer efficacy of cinacalcet, indicating that YAP was responsible for the antineoplastic activity of cinacalcet. Collectively, this study suggested cinacalcet as a feasible anticancer drug for HCC via its inhibition on YAP/TAZ.

1. Introduction

Hepatocellular carcinoma (HCC) remains a global health challenge, with an estimated annually incidence of more than 1 million cases by 2025, which is the sixth most common cancer and the third leading cause of cancer-related death worldwide (Forner et al. 2018; Llovet et al. 2021). Since approved in 2007, sorafenib used to be the only available standard of strategy for advanced HCC for a decade (Llovet et al. 2018; Llovet et al. 2008). Although new tyrosine kinase inhibitors (eg. regorafenib) and immunotherapy strategies, such as the combination of atezolizumab (anti-PDL1 antibody) and bevacizumab (anti-VEGF antibody), have been developed, the overall outcome of HCC remains unsatisfactory with the median survival of 6-9 months in early and 1-2 months in advanced HCC (Ren et al. 2020). Due to the poor prognosis and limited effective therapeutic options, it is very important to explore new mechanisms of HCC development and seek for new drug candidates.

The Hippo signaling pathway plays an important role in organ size, cell proliferation, cell cycle, cell differentiation and apoptosis, while Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are the principal downstream effectors of Hippo cascade (Juan and Hong 2016; Moya and Halder 2019; Yimlamai et al. 2014; Zhao et al. 2010). It was reported that dysregulation of the Hippo pathway and alteration in YAP expression drove carcinogenesis in a broad range of human tumor types including HCC (Hayashi et al. 2015; Juan and Hong 2016; Yimlamai et al. 2014). YAP activation promotes cancer cell proliferation via TEAD-dependent transcription of CTGF that is a cell proliferation gene (Hayashi et al. 2015). Furthermore, YAP/TAZ were responsible for chemoresistance and HCC prognosis (Sorrentino et al. 2017; Sun et al. 2021; Zhang and Zhou 2019). It was reported that aberrant expression of YAP/TAZ is an efficient target to alleviate cancer malignant progression (Bradner et al. 2017). Therefore, discovering novel YAP/TAZ inhibitors or identifying potential YAP/TAZ inhibitors from existing drugs is a promising approach for cancer therapy.

Cinacalcet is a calcimimetic agent that directly lowers PTH levels by acting allosterically on calcium-sensing receptor, which is used to treat hyperparathyroidism and high calcium levels in patients with parathyroid disease (Dong 2005). It has also been assessed as targeted therapy for some cancers such as neuroblastoma, suggesting the anticancer potential of cinacalcet (Rodriguez-Hernandez et al. 2016).

For the first time, we found that cinacalcet potentially inhibited the expression of YAP/TAZ in hepatocellular carcinoma cells *via* screening drug libraries. Based on this hypothesis, we confirmed that cinacalcet downregulated protein level of YAP and then inhibited cell survival in HepG2 and Bel-7402 cells. This study suggested cinacalcet as a feasible anticancer drug for hepatocellular carcinoma, for which there is a lack of suitable therapeutic strategies.

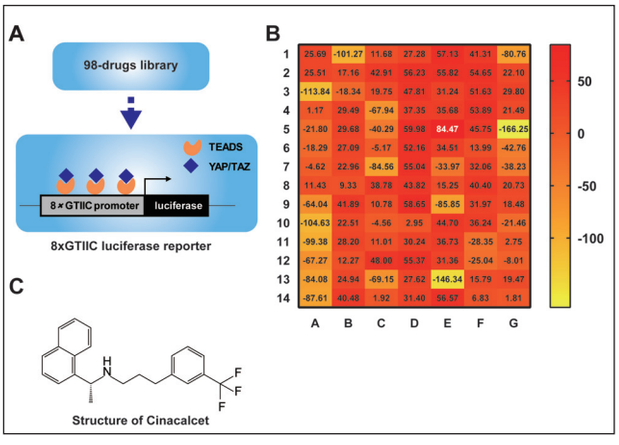

Fig. 1: Cinacalcet is a novel YAP/TAZ inhibitor. A) The schematic diagram of 8 x GTIIC luciferase reporter system that was used to screen YAP/TAZ inhibitors. 8 x GTIIC contains 8 TEAD-binding sites, which reflects the YAP/TAZ transcriptional activity. B) Inhibition ratio (%) against YAP/TAZ transcriptional activity of 98 drugs (listed in Supplementary information). Line 5 Column E which is highlighted as white is the inhibition ratio of cinacalcet. C) Structure of Cinacalcet.

2. Investigations and results

2.1. *Identification of cinacalcet as a potential YAP/TAZ inhibitor*

To search for potential YAP/TAZ inhibitors, A YAP/TAZ dependent transcriptional luciferase reporter (8 x GTIIC, Fig. 1A) was applied for screen a 98-drugs library. Among those tested, cinacalcet exerted potent inhibitory effect on YAP/TAZ-TEADs luciferase activity, with an inhibition ratio of 84.47% (Figs. 1B, 1C).

2.2. *Inhibitory effect of cinacalcet on the expression of YAP*

To further confirm the regulatory effect of cinacalcet on YAP/TAZ signaling, transcriptional activity of YAP expression in cells was detected by luciferase assay after 2.5, 5 and 10 μM cinacalcet treatment. As shown in Fig. 2A, 2.5, 5 and 10 μM cinacalcet significantly induced YAP inhibition in a dose-dependent way, with inhibition ratio of 49.41%, 59.55% and 68.47%, respectively. In addition of the regulation on the transcriptional level, protein levels of hepatocellular carcinoma cells after cinacalcet treatment are also investigated by Western Blot. Cinacalcet significantly decreased the expression of YAP protein in both HepG2 and Bel-7402 cells.

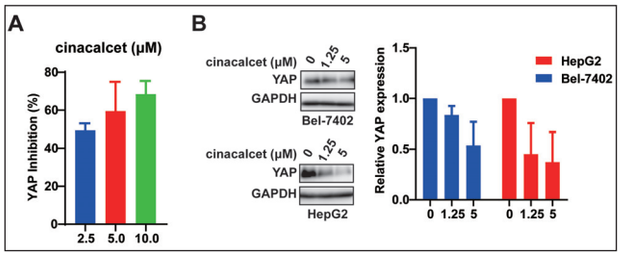

Fig. 2: Cinacalcet inhibited YAP expression. A) Inhibition ratio of 2.5, 5.0 and 10.0 μM cinacalcet on YAP/TAZ-TEADs luciferase activity. Data are shown as mean ± SD derived from three independent biological replicates. B) Bel-7402 and HepG2 cells were treated with 0, 1.25 or 5 μM cinacalcet for 48 h, after which immunoblotting analysis of YAP protein expression was performed. Semi-quantitative analysis with the immunoblotting results of three independent experiments are shown as histograms.

2.3. *Anticancer efficacy of cinacalcet against human hepatocellular carcinoma*

YAP plays an important oncogenic role in human hepatocellular carcinoma. In consideration of the inhibitory effect of cinacalcet on YAP expression, we then studied whether cinacalcet could suppress human hepatocellular carcinoma. Bel-7402 cells were treated by cinacalcet for 48 h at concentrations of 1.25 and 5 μM, after which cells were observed by microscope. As shown in Fig. 3A, the anchorage-dependent growth of Bel-7402 cells was dramatically decreased and cell survival was inhibited. Furthermore, Bel-7402 and HepG2 cells were treated with a series of concentrations of cinacalcet for 72 h, after which cell survival was detected by SRB assay and IC50 was calculated. In both Bel-7402 and HepG2 cells, cinacalcet induced cell death in a dose-dependent way with IC50 of 7.95 μM in Bel-7402 cells and 6.39 μM in HepG2 cells respectively, indicating that cinacalcet was a potential anticancer candidate against human hepatocellular carcinoma (Fig. 3B-3D).

2.4. *Role of YAP in anticancer efficacy of cinacalcet*

In order to confirm whether YAP expression plays a role in the anticancer efficacy of cinacalcet, Bel-7402 and HepG2 cells were transfected with a YAP plasmid for YAP overexpression (Fig. 4A). Cells transfected with YAP or PCCL (empty vector) were then treated with cinacalcet for 72 h. The results showed that YAP overexpression weakened the anticancer efficacy of cinacalcet.

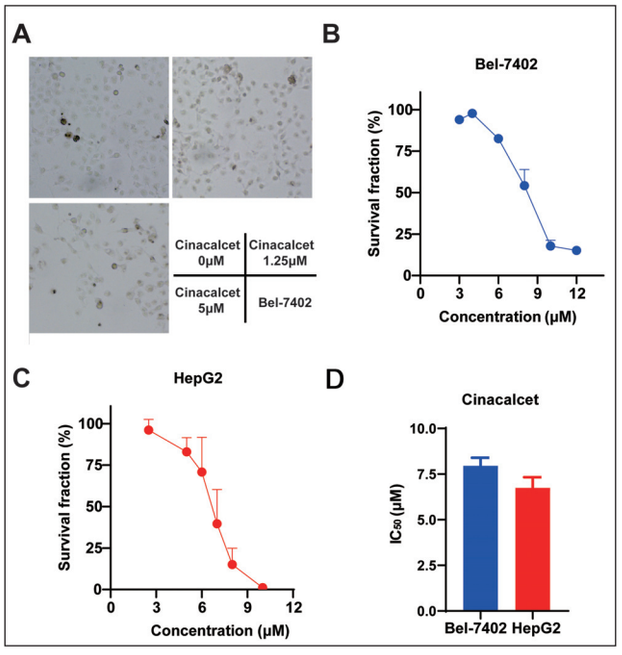

Fig. 3: The anticancer efficacy against human HCC of the novel YAP inhibitor cinacalcet. A) Bel-7402 cells were treated with 0, 1.25 or 5 μM cinacalcet for 48 h, after which the cell morphology was observed by microscope and shown as representative photos. The proliferation of Bel-7402 (B) and HepG2 (C) cells after treatment with indicated concentrations of cinacalcet were determined using SRB assay. D) The IC50 values of cinacalcet in Bel-7402 and HepG2 cells. Data are shown as mean ± SD derived from three independent biological replicates.

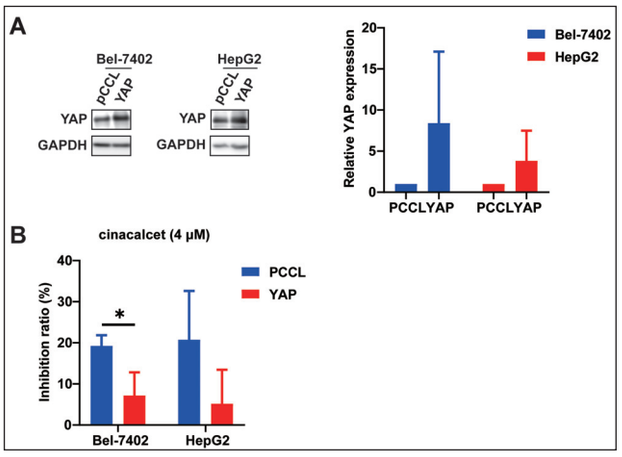

Fig. 4: YAP participated in the anticancer efficacy of cinacalcet. A) Immunoblotting analysis of YAP protein expression in Bel-7402 cells (left) and HepG2 cells (right) transfected with empty vector or YAP plasmid. Semi-quantitative analysis with the immunoblotting results of three independent experiments are shown as histograms. B) The inhibition ratio on cell proliferation of 4 μM cinacalcet in Bel-7402 and HepG2 cells. Data are shown as mean ± SD derived from three independent biological replicates. *, $p<0.05$.

In Bel-7402 cells, YAP overexpression decreased the inhibition ratio after cinacalcet treatment (4 μM cinacalcet, from 19.24% to 7.18%). Similar results were observed in HepG2 cells (4 μM cinacalcet, from 20.74% to 5.20%), suggesting that YAP was responsible for the anticancer activity of cinacalcet (Fig. 4B).

3. Discussion

HCC remains a global health challenge due to the lack of effective drugs in clinical application (Forner et al. 2018; Llovet et al. 2021). In consideration of the crucial role of YAP/TAZ in HCC, exploration of YAP inhibitors is regarded as an efficient approach to develop novel therapeutic strategies. In this study, an *in vitro*

functional assay on YAP/TAZ transcriptional levels was performed and the calcimimetic agent cinacalcet was identified as a novel inhibitor of YAP/TAZ, with inhibitory effect on hepatocellular carcinoma cell proliferation.

YAP is a key component of the Hippo signaling pathway, whose expression and localization are related to the cancer progression (Zhang and Zhou 2019; Zhao et al. 2010). Recent studies have implicated that YAP as an oncoprotein is involved in HCC (Zhou et al. 2018). The expression of YAP is elevated in HCC tissues compared with normal tissues and the aberrant expression of YAP is tightly correlated with decreased overall survival (Zhang et al. 2018; Zhu et al. 2020). Therefore, YAP has emerged as an attractive target for HCC therapeutics.

This study demonstrated that cinacalcet was able to inhibit the expression of YAP/TAZ *via* a gain-of-function screen of a drug library by monitoring YAP/TAZ-dependent transcriptional luciferase reporter. Moreover, cinacalcet led to significant cell proliferation inhibition in both HepG2 and Bel-7402 cells. We also provided evidence showing the overexpression of YAP reduced the anticancer efficacy of cinacalcet against HCC, further indicating that YAP suppression was involved in the anticancer effect of this drug.

Cinacalcet is a traditional oral calcimimetic agent that can reduce parathyroid hormone levels (Dong 2005). The association between Hippo pathway including YAP expression and parathyroid hormone remains unknown. It was reported that the key parathyroid oncosuppressor multiple endocrine neoplasia type 1 (MEN1) negatively regulated YAP1 expression and YAP1 was a potential calcium sensing receptor target in human parathyroid tumors (Tavanti et al. 2021). However, further study is needed to clarify the precise mechanisms underlying the regulatory effect on YAP of cinacalcet.

In conclusion, our study identified cinacalcet as a novel potential anticancer drug to intervene HCC cell growth and its anticancer efficacy was related to its function as a YAP inhibitor, suggesting a novel therapeutic candidate for hepatocellular carcinoma therapy.

4. Experimental

4.1. Cell culture

Human HCC cell lines HepG2, Bel-7402 and human embryonic kidney HEK293 cells were purchased from Cell Bank of China Science Academy in 2016. Cells were maintained in DMEM or RPMI1640 medium (Gibco) containing 10% FBS (Gibco) at 37, in 5% CO_2 humid atmosphere. "STR" (short tandem repeat) analysis was performed every 6 months for cell line authentication. Cells used for this study were passaged up to 10 times since thawed.

4.2. Luciferase reporter activity assay

The 8 x GTIIC (Addgene) luciferase reporter was applied to detect the transactivation of YAP/TAZ. After 293T cells were treated by drug candidate in the drug library for 24 h, the corresponding luminescence number was obtained, while a construct containing Renilla luciferase (Promega) was used as internal control. The ratios of firefly and Renilla luciferase were normalized to the no drug control groups (Zhu et al. 2020).

4.3. Sulforhodamine B (SRB) assay

Cells were plated in 96-well plate and incubated for 24 h before treatment. Different concentrations of drugs were added for treatment. After an incubation period, cell were fixed with 10% trichloroacetic acid and stained with SRB dye for 20 min. Then the plates were washed repeatedly with 1% acetic acid. The dye bound with protein was dissolved in 10 mM Tris base solution for OD determination at 515 nm.

4.4. Plasmid transfection

The human YAP plasmid was amplified from the HepG2 cDNA library and was subsequently subcloned into the pCDNA3.0 plasmid. The transfection was performed using jetPrime (Polyplus) and plasmid according to the manufacturer's recommendations.

4.5. Western blot

Westerrn blot analysis was generally performed as previously described (Zheng et al. 2014). Briefly, cells extracts were lysed in 2×SDS gel loading buffer, which contains 4% SDS, 24 mmol/L Tris-HCl (pH 6.8), 0.02% mercaptoethanol, 0.4% bromphenol blue, 20% glycerol. Cell lysates were separated on 8%-10% SDS-PAGE gels and bloted onto PVDF membranes, after which the membranes were incubated in 5% skim milk as blocking agent. Subsequently, the membranes were incubated with primary antibodies in 4 °C overnight and then the suitable secondary antibodies conjugated with horseradish peroxidase (HRP). Primary antibodies used for immunoblotting were as follows: YAP (#4912) and GAPDH (db106) antibodies were purchased from Cell Signaling Technology and Diagbio, respectively.

4.6. Statistical analysis

Two-tailed unpaired Student t tests were used for statistical analysis. Results were considered significant when $P < 0.05$ (*, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$).

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Conflicts of interest: No potential conflicts of interest were disclosed.

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Supplementary information

The 98-drugs library is listed below:

	A	B	C	D	E	F	G
1	Vigabatrin Hydrochloride	Ticagrelor	Medrysone	Erdosteine	Rimonabant Hydrochloride	Trelagliptin succinate	Obeticholic acid
2	LCZ696	Prednisolone disodium phosphate	Ergotamine bitartrate	Tanshinone I	Sodium phenylbutyrate	Indacaterol	Ataluren
3	Tyloxapol	Amlodipine besylate	Choline chloride	Isoliquiritigenin	Montelukast	Cilastatin	Macitentan
4	Pargyline	Amcinonide	Risedronate sodium	Allopurinol	Varenicline Tartrate	Etonogestrel	Perampanel
5	Zolpidem	Dimenhydrinate	Capsaicin	Aptal	Cinacalcet hydrochloride	Pitavastatin Calcium	Ivacaftor
6	Risedronic acid	Methylprednisolone sodium succinate	Ketanserin	Benfluorex hydrochloride	Desvenlafaxine	Aclidinium Bromide	MK 4305
7	Ethamsylate	Canrenone	Tolnaftate	Sulfadoxine	Acetohydroxamic acid	Nafamostat mesylate	Tranilast (trans-)
8	Adiphenine hydrochloride	Aminoguanidine hydrochloride	2-Pyridinealdoxime	Methimazole	Ezetimibe	Fimasartan	Conivaptan hydrochloride
9	Procainamide hydrochloride	Leucobasal	Rasagiline	L-Thyroxine	Fasudil	Danazol	Maraviroc
10	Molsidomine	Diammonium Glycyrrhizinate	Betamethasone 17-valerate	Amprolium	Rivastigmine tartrate	Clinofibrate	Selexipag
11	Brinzolamide	Metyrapone	Papaverine hydrochloride	Torsemide	Amitraz	Nicorandil	Valsartan
12	Roxatidine Acetate Hydrochloride	2-Aminobenzenesulfonamide	Clopidogrel bisulfate	Memantine hydrochloride	Edoxaban tosylate monohydrate	Azatadine dimaleate	Piribedil
13	Pranoprofen	Pioglitazone hydrochloride	Racecadotril	Captopril	Montelukast sodium	Aprepitant	Delapril hydrochloride
14	Nylidrin hydrochloride	Pyridoxine	Milnacipran hydrochloride	Indapamide	Pirinixic acid	Canagliflozin	Istradefylline