ORIGINAL ARTICLE

Identification of two novel inhibitors of mTOR signaling pathway based on high content screening

Juming Yan · Hongyu Zhou · Lingmei Kong · Jing Zhang · Qinshi Zhao · Yan Li

Abstract

Purpose Mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in regulating cell growth, proliferation and survival. Dysregulation of mTOR signaling pathway is closely involved in cancer development and chemotherapy resistance. Inhibitors of mTOR signaling pathway have been demonstrated to be attractive therapeutics for cancer therapy. In the present study, we aim to discover novel mTOR signaling pathway inhibitors from a natural compound library.

Methods Inhibitors of mTOR signaling pathway were discovered via high content screen assay based on the subcellular localization of eukaryotic initiation factor 4E (eIF4E) in mouse embryonic fibroblast cells. Candidate compounds were further assessed in cancer cells. Phosphorylation levels of mTOR complexes downstream targets were analyzed using Western blot. Cell cytotoxicity and apoptosis were evaluated using MTS assay and flow cytometry, respectively.

Results Two compounds, 1,4-O-diferuloylsecoisolariciresinol (IM-1) and Pierreione B (IM-2), were identified which induced significant nuclear translocation of eIF4E in a panel of cancer cells. Both of the compounds decreased the phosphorylation levels of p70 ribosomal protein S6 kinase (S6K) and eIF4E binding protein 1 (4E-BP1), resulting in cancer cell cytotoxicity and apoptosis.

Conclusions Via high content screen assay, two novel inhibitors of mTOR signaling, IM-1 and IM-2, were identified with strong anticancer activity. IM-1 and IM-2 could be potential candidates for anticancer therapeutics by targeting mTOR signaling pathway and as such warrants further exploration.

Keywords mTOR signaling · Inhibitor · High content screening · Cancer · Apoptosis

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Introduction

Mammalian target of rapamycin (mTOR) is a highly conserved serine–threonine kinase belonging to phosphoinositide-3 kinase (PI3K)-related kinase family. Cumulative evidence indicates that mTOR integrates diverse signals to regulate cell growth, proliferation, survival, protein translation and autophagy [1–4]. mTOR functions as two structurally and functionally distinct multiprotein complexes: mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2) [5]. mTORC1 regulates cell growth, proliferation and survival by sensing mitogen, energy and nutrient signals [5]. mTORC2 modulates the actin cytoskeleton and regulates cell survival by phosphorylating its downstream effector Akt, also known as protein kinase B, at the hydrophobic motif site Ser473 responding to growth factors [6, 7].

p70 ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) are two best-characterized effectors of mTORC1 signaling pathway. Activated mTORC1 phosphorylates S6K at Thr389 and activates S6K, which phosphorylates ribosomal protein S6 (S6), subsequently promoting translation initiation [8]. In response to sufficient growth factors and nutrients stimulation, mTORC1 hierarchically phosphorylates

4E-BP1 at multiple sites which regulate the interaction between 4E-BP1 and eIF4E. Hyperphosphorylated 4E-BP1 dissociates from eIF4E and releases the inhibitory effect on eIF4E [5, 9–12]. At the molecular level, free eIF4E increases mRNA translation and the nuclear export of mRNAs involved in cell proliferation, survival, angiogenesis and metastasis [13, 14].

Upregulation of the mTOR signaling pathway occurs in approximately 70 % of all types of cancers [12, 15–17]. The mTOR pathway is a major tumor-initiating pathway in hepatocellular carcinoma [12]. In lung adenocarcinoma, 90 % reveals mTOR activation regardless of EGFR status [18]. Aberrant activation of mTOR signaling pathway is involved in chemotherapy resistance and malignant transformation as well [19–21]. Increasing evidence demonstrates that mTOR signaling pathway has become an attractive target for cancer therapy and targeting mTOR signaling has been exploited as a promising tumor-selective therapeutic strategy [16]. Rapamycin, a macrocyclic lactone produced by *Streptomyces hygroscopicus*, is the first defined mTOR inhibitor [22, 23]. Several rapamycin analogs with more favorable pharmaceutical characteristics have been developed, including CCI-779, RAD001, AP23573, 32-deoxorapamycin (SAR943) and zotarolimus (ABT-578) [24]. Preclinical studies have shown their anti-proliferative activity against a diverse range of cancer types, and clinical trials have demonstrated promising anticancer efficacy in certain types of cancers [25–28].

In recent years, there has been significant interest in the search for mTOR signaling antagonists from natural products. Several naturally occurring compounds have been found to downregulate mTOR signaling and exhibit potent anticancer activity. Cryptotanshinone, a natural compound isolated from plant *Salvia miltiorrhiza bunge*, has the capability to inhibit mTOR signaling pathway [29]. Curcumin, a polyphenol natural product of the plant *Curcuma longa*, inhibits mTOR signaling pathway through disrupting the mTOR–raptor complex and has been undergoing early clinical trials as a novel anticancer agent [30]. EGCG, an important natural product from green tea, is a dual PI3K/mTOR inhibitor and could inhibit the cell proliferation of MDA-MB-231 and A549 cells [31].

To discover novel mTOR signaling pathway inhibitors, we screened a natural compound library with a high content screen assay based on the subcellular localization of eIF4E, which is tightly regulated by the mTOR-dependent phosphorylation of 4E-BPs [32, 33]. Two novel inhibitors of mTOR signaling, 1,4-O-diferuloylsecoisolariciresinol (IM-1) and Pierreione B (IM-2), were discovered. Both of the compounds markedly induced nuclear translocation of eIF4E in cancer cells and reduced the phosphorylation levels of the mTORC1 downstream targets, S6K and 4E-BP1. IM-1 and IM-2 exhibited obvious cell cytotoxicity and significantly induced apoptosis in a variety of cancer cell lines.

Materials and methods

Reagents

Cell culture media and fetal bovine serum (FBS) were provided by HyClone (Logan, UT). Antibodies of S6K, phospho-S6K (Thr389), phospho-S6 (Ser235/236), 4E-BP1, phospho-4E-BP1 (Thr37/46) and phopho-Akt (Ser473) were from Cell Signaling Technology; anti-eIF4E (BD Transduction Laboratories); anti-S6 and Akt1 (Santa Cruz); anti-actin (Sigma-Aldrich). Goat anti-mouse IgG Alexa Fluor® 546 was purchased from Invitrogen, and all the other secondary antibodies were from Sigma-Aldrich. The library of compounds was supplied by the State Key Laboratory of Phytochemistry and Plant Resources in West China (Kunming Institute of Botany, Chinese Academy of Sciences, China). PI-103 was purchased from Selleckchem; 4,6-diamidino-2-phenylindole (DAPI) and rapamycin were from Sigma-Aldrich. Recombinant human IGF-1 was from Peprotech. CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was from Promega.

Cell lines and cell culture

Human hepatocellular carcinoma cell lines SMMC-7721 and Hep G2, human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231, human lung adenocarcinoma epithelial cell line A549 and human colon adenocarcinoma cell line SW480 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China); mouse embryonic fibroblast (MEF) cells were obtained from Kunming Institute of Zoology (Chinese Academy of Sciences, China). MEF, Hep G2, MCF-7, MDA-MB-231 and SW480 cells were cultured in Dulbecco's modified Eagle medium, and A549 and SMMC-7721 cells were sustained in RPMI-1640 medium, supplemented with 10 % FBS, 100 µg/mL streptomycin and 100 U/mL penicillin in a humidified atmosphere with 5 % CO_2 according to supplier's instructions.

Immunofluorescence assay

Cells were seeded and cultured in 96-well plates (5,000 cells/well) and treated with tested compounds dissolved in DMSO. After 5 h incubation, cells were washed twice with phosphate-buffered saline (PBS) prior to fixation with 4 % paraformaldehyde (10 min, 37 °C) and permeabilization with 0.1 % triton X-100 (5 min) followed by blocking in 2 % bovine serum albumin (BSA) in PBS. Cells were prepared for immunofluorescence by incubation with the mouse monoclonal anti-eIF4E antibody (1:400 in

2 % BSA) overnight and then washed three times in PBS followed by incubation with anti-mouse IgG Alexa Fluor® 546 antibody (1:1,000 in 2 % BSA) and DAPI staining for 5 min at room temperature.

High content screening

To monitor the subcellular location of eIF4E, Array-Scan® VTI HCS Reader (Thermo Scientific, USA) was used to image 100 fields per well for each 96-well plate; 20× objective lens and BGRFR filters as well as autofocus mode were performed to capture the images. For the cellular imaging, 100 images per well were taken, visualizing approximately 3,000 individual cells. For all screening assays, eIF4E location was monitored and analyzed by determining the eIF4E nuclear–cytoplasmic intensity of each field per well using ArrayScan® VTI HCS Reader according to manufacturer's instruction.

Western blotting

The cell lysates were prepared in lysis buffer (50 mM Tris pH 7.6, 0.2 mM EDTA, 10 mM $MgCl_2$, 0.2 % Triton X-100, protease inhibitor cocktail) and homogenized on ice. The protein extracts were boiled for 10 min and were stored at −20 °C. The protein samples were separated on 12 % SDS-PAGE mini gels and transferred to PVDF membranes. The membranes were blocked in 5 % nonfat milk and then incubated with antibody according to manufacturer's protocol followed by incubation with secondary antibody. Specific proteins were incubated with enhanced chemiluminescence reagent and detected using Luminescent Image Analyzer LAS-4000 mini System (GE Healthcare, USA).

Cell cytotoxicity assay

Cell viability was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay in 96-well plates according to the protocol of CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA). Briefly, 100 µL of cell suspensions were seeded into each well of 96-well plate and allowed to adhere for 24 h. Cancer cells were exposed to the tested compounds in triplicates for 48 h; 20 µL of CellTiter 96® AQ$_{ueous}$ One Solution Reagent was added in each well, and the cells were further incubated at 37 °C for 1–2 h. Cell viability was detected by measuring the absorbance at a wavelength of 490 nm. Concentrations of 50 % inhibition of cell viability (IC_{50}) were determined on the basis of the relative survival curve.

Apoptosis assay

Cells were treated with various concentrations of IM-1 and IM-2, respectively, for 48 h. Cells were then collected and stained using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen™) according to manufacturer's instruction. In brief, cells were washed with cold PBS twice and with binding buffer once, about 10^5 cells were resuspended in 100 µL Annexin V-binding buffer, followed by incubation with FITC-conjugated Annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. For each sample, the cells were resuspended in 500 µL Annexin V-binding buffer and 10^4 cells were analyzed using BD FACSCalibur flow cytometry (BD FACSCalibur™).

Results

Screening for mTOR signaling pathway inhibitors

Firstly, we set up the assay based on the observation that the nuclear accumulation of eIF4E occurs in a 4E-BP-dependent manner specifically upon mTOR signaling inhibition [34–36]. In this assay, we used high content technology to visualize the ratio of nuclear and cytoplasmic distribution of eIF4E. To evaluate the sensitivity of the assay in our study, we investigated the effects of two well-known mTOR inhibitors, rapamycin and PI-103, on the subcellular localization of eIF4E in MEF cells. As expected, eIF4E in untreated MEF cells was dominantly localized in cell cytoplasm and the eIF4E nuclear-to-cytoplasmic (eIF4E [nuc:cyto]) ratio was 0.85. Treatments of 100 nM rapamycin and 1 µM PI-103 induced the nuclear translocation of eIF4E and increased the eIF4E [nuc:cyto] ratios to 1.30 and 2.0, respectively (Fig. 1a, b).

We screened 1,100 compounds from the natural compound library. MEF cells were treated with tested compounds for 5 h at the initial concentration (40 µM). Compounds causing 1.5-fold increase in eIF4E [nuc:cyto] ratios are considered "positive hits." To avoid "false-positive," compounds, which dramatically altered cell number or cell morphology, were subjected to secondary screen with lower concentrations. As shown in Fig. 1c, 9 compounds with more potent ability to increase eIF4E [nuc:cyto] ratios than rapamycin were identified.

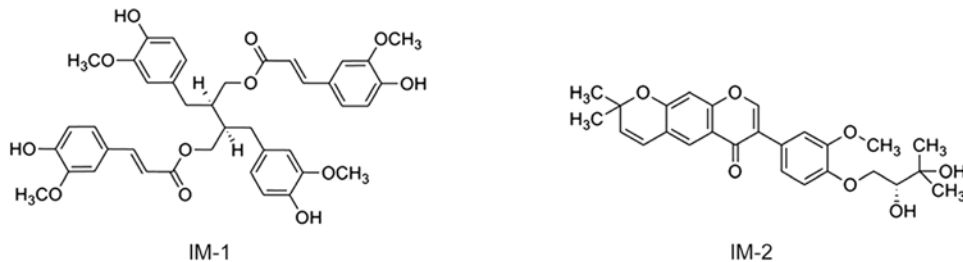## IM-1 and IM-2 inhibited mTOR signaling pathway in cancer cells

Dose-dependent assay was further performed with the positive hits obtained from the primary screening to indentify particularly potent compounds with minimal cellular cytotoxicity (data not shown). Two active compounds, IM-1
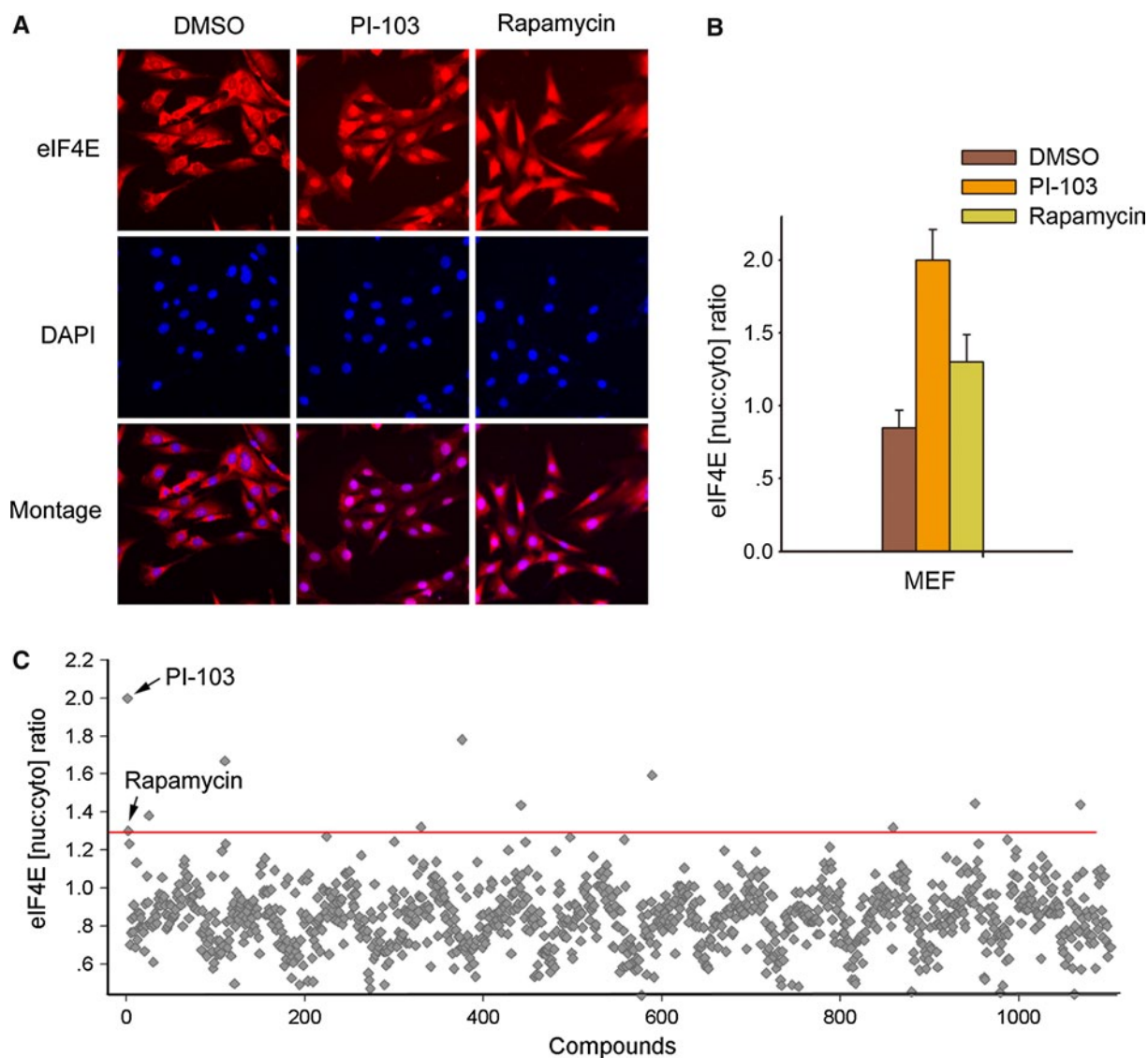
Fig. 1 Nuclear translocation of eIF4E and high content screening for mTOR signaling inhibitors. **a** MEF cells were treated with 1 μM PI-103 and 100 nM rapamycin for 5 h, respectively, and 0.1 % DMSO as a vehicle control. Immunofluorescence (*red*) intensity reflects eIF4E subcellular distribution, and cell nuclei are defined by DAPI staining of DNA (*blue*). Cells were visualized with high content reader. **b** Nuclear-to-cytoplasmic ratio of eIF4E in MEF cells was determined via high content reader. **c** Screening for mTOR signaling inhibitors. MEF cells were treated with tested compounds for 5 h, with PI-103 (1 μM) and rapamycin (100 nM) as positive controls. eIF4E nuclear-to-cytoplasmic ratio were determined via high content reader
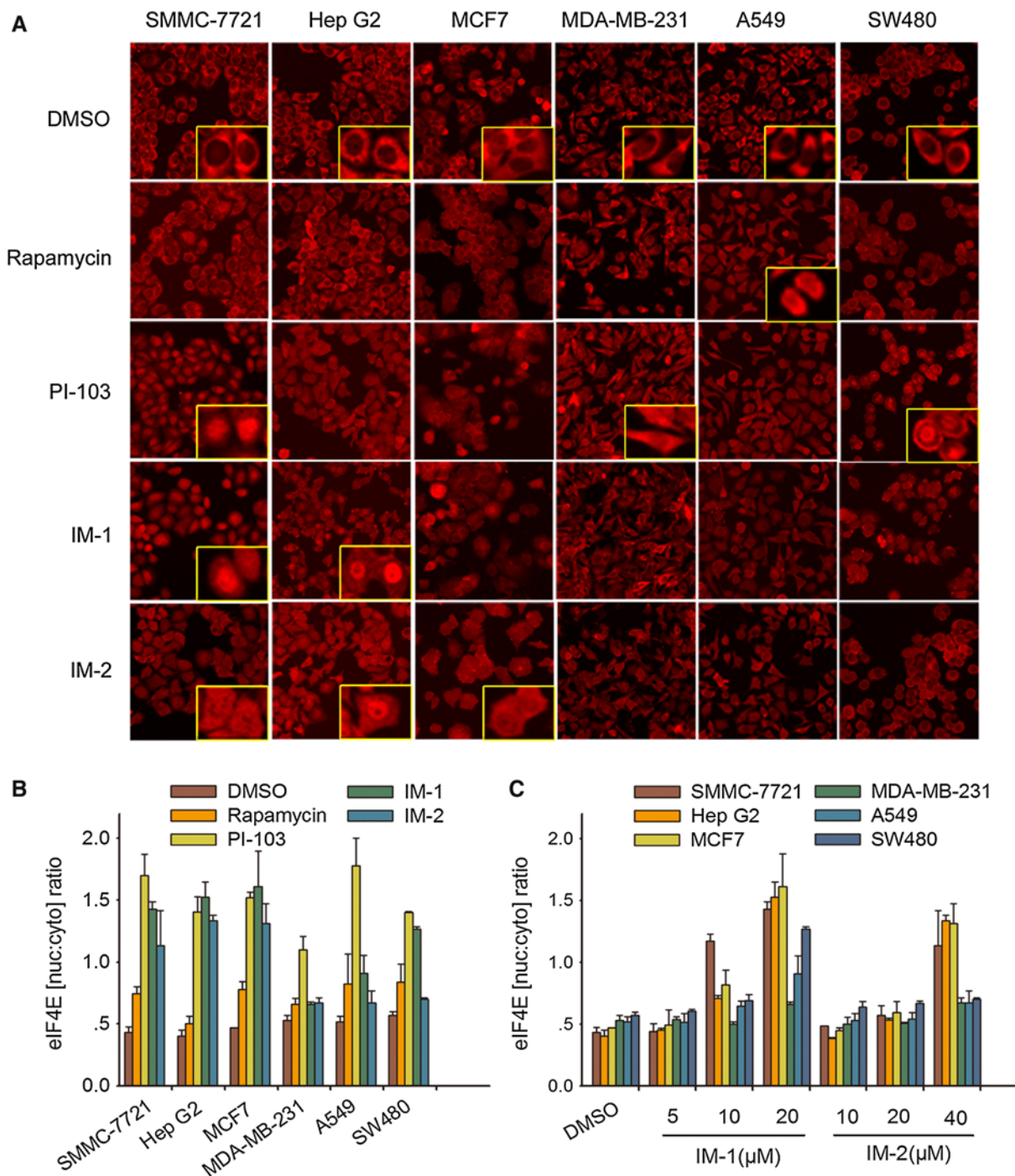
Fig. 2 Chemical structures of IM-1 and IM-2

and IM-2, exhibited good properties and were selected for further study. The chemical structures of IM-1 and IM-2 are displayed in Fig. 2.

Aberrant activation of mTOR signaling pathway occurs in multiple types of cancer cells. As expected, in all the six cancer cell lines (SMMC-7721, Hep G2, MCF7,

Fig. 3 Effects of IM-1 and IM-2 on eIF4E nuclear translocation in cancer cells. **a** Six cancer cell lines (SMMC-7721, Hep G2, MCF7, MDA-MB-231, A549 and SW480) were treated with 20 μM IM-1 and 40 μM IM-2 for 5 h, respectively, with 1 μM PI-103, and 100 nM rapamycin used as positive controls and 0.1 % DMSO as a vehicle control. eIF4E distribution was visualized by immunofluorescence, and the insets are the magnification of part of cells shown in the corresponding panels. **b** Quantification of the subcellular distribution of eIF4E shown in **a**. **c** eIF4E [nuc:cyto] ratios in cancer cells treated with different concentrations of IM-1 and IM-2 for 5 h

MDA-MB-231, A549 and SW480), eIF4E was localized dominantly in cytoplasm, with even lower eIF4E [nuc:cyto] ratio than in MEF cells (Figs. 1a, b, 3a, b). The effects of IM-1 and IM-2 on eIF4E nuclear translocation

in cancer cells were evaluated. Both of the compounds exhibited strong activities to induce eIF4E nuclear accumulation (Fig. 3a), with 4.1-fold (IM-1) and 3.6-fold (IM-2) increases in eIF4E [nuc:cyto] ratios comparing with

the control (DMSO) in hepatocellular carcinoma Hep G2 cells (Fig. 3b). Further, the cells were treated with different concentrations of IM-1 and IM-2, respectively, for 5 h and eIF4E [nuc:cyto] ratios were determined. The results showed that IM-1 and IM-2 induced the eIF4E nuclear translocation in most of the cancer cells in a dose-dependent manner (Fig. 3c). As a positive control, PI-103 treatment induced more significant eIF4E nuclear translocation in all the six cancer cell lines than in MEF cells (Figs. 1b, 3b).

The effects of IM-1 and IM-2 on downstream targets of mTOR complexes

S6K and 4E-BP1 are the two best-characterized effectors of mTORC1 signaling pathway. In order to determine the effects of IM-1 and IM-2 on downstream target proteins of mTORC1, SMMC-7721 and Hep G2 cells were treated with IM-1 and IM-2 for 5 h, respectively, and the phosphorylation levels of S6K and 4E-BP1 were determined by Western blot analysis. As shown in Fig. 4a, both compounds remarkably decreased the phosphorylation of S6K, S6 and 4E-BP1 and induced band shift of 4E-BP1. These results indicate that IM-1 and IM-2 are able to inhibit mTORC1 signaling pathway and subsequently induce eIF4E nuclear translocation in cancer cells.

In order to investigate whether or not IM-1 and IM-2 affect mTORC2 activity, we tested the effects of IM-1 and IM-2 on the phosphorylations of Akt (Ser473). As shown in Fig. 4b, treatment with IM-1 for 5 h, followed by IGF-1

stimulation, inhibited the phosphorylation of Akt (Ser473) in Hep G2 cells. However, the phosphorylation of Akt (Ser473) was upregulated by IM-1 in SMMC-7721 cells. IM-2 increased the phosphorylation of Akt (Ser473) in both SMMC-7721 and Hep G2 cells. The results suggest that IM-1 could inhibit mTORC2 activation induced by IGF-1 in Hep G2 cells but not in SMMC-7721 cells, whereas IM-2 has no effect on mTORC2 inhibition in the two tested hepatocellular carcinoma cells.

### IM-1 and IM-2 exhibited cytotoxicity and induced apoptosis in cancer cells

mTOR signaling pathway played a pivotal role in regulation of cell growth, proliferation and survival. Hence, we investigated the cytotoxicity of IM-1 and IM-2 against in six cancer cell lines with MTS assay. As shown in Fig. 5a, IM-1 exhibited obvious cytotoxicity against all the six cancer cell lines. Except SW480 and MDA-MB-231, IM-2 showed concentration-dependent cytotoxicity against other four cancer cell lines. The IC$_{50}$ values of IM-1 and IM-2 against each cell line are shown in Fig. 5b.

Further, we evaluated the apoptosis in IM-1- and IM-2-treated cancer cells. SMMC-7721 cells were exposed to IM-1 and IM-2, respectively, for 48 h, and cell apoptosis assay was performed with AnnexinV/PI staining and flow cytometry. As shown in Fig. 6a, both IM-1 and IM-2 induced obvious apoptosis of SMMC-7721 cells in a concentration-dependent manner. IM-1 treatment significantly promoted cell apoptosis (40 %) at 20 µM and IM-2

Fig. 4 Effects of IM-1 and IM-2 on downstream targets of mTOR complexes. **a** SMMC-7721 and Hep G2 cells were treated with IM-1 and IM-2, respectively, for 5 h at indicated concentrations. Phosphorylated S6K, S6 and 4E-BP1 and the corresponding total proteins were analyzed by Western blot assay. **b** SMMC-7721 and Hep G2 cells were pretreated with 100 nM rapamycin (R), IM-1 and IM-2, respectively, for 5 h at indicated concentrations and then stimulated with 50 ng/ml IGF-1 for 30 min. Phosphorylation of Akt (Ser473) and total proteins were analyzed by Western blot assay
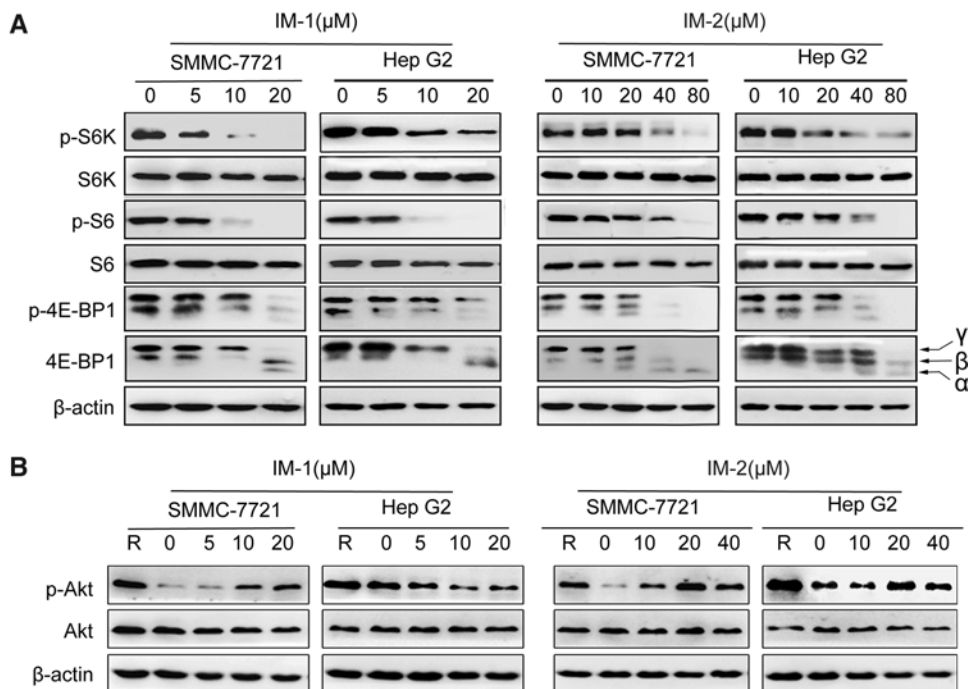
Fig. 5 Effects of IM-1 and IM-2 on cell viability. **a** cancer cells were incubated with IM-1 and IM-2 at indicated concentrations for 48 h. Cell viability was detected by MTS assay and represented with relative viability versus control. **b** IC$_{50}$ values of IM-1 and IM-2 against cancer cells were shown with mean ± SD
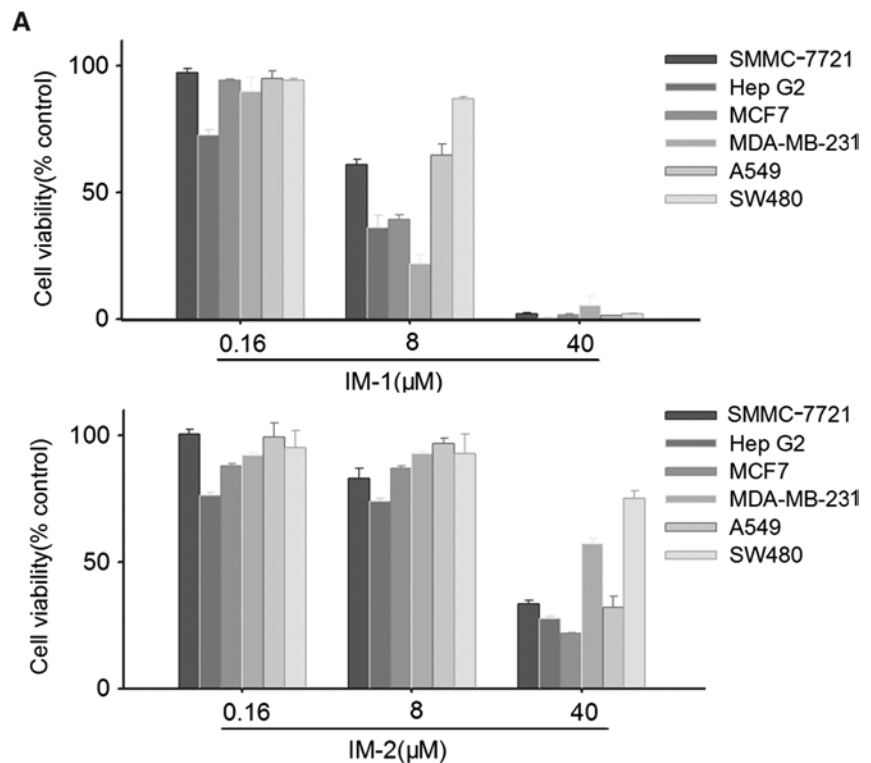
| Cell lines	IC$_{50}$(mean ± SD, μM)	
	IM-1	IM-2
SMMC-7721	10.79 ± 0.45	23.29 ± 1.10
Hep G2	5.71 ± 0.64	21.29 ± 0.75
MCF7	5.85 ± 0.25	20.18 ± 0.19
MDA-MB-231	11.10 ± 0.74	>40
A549	11.57 ± 0.97	25.69 ± 1.25
SW480	16.08 ± 0.15	>40

significantly induced apoptosis (64 %) at 40 μM, compared with control cells (Fig. 6b).

Discussion

Dysregulation of mTOR signaling pathway often occurs in a variety of human malignant diseases. Inhibition of mTOR signaling pathway has great potential to become a tumor-selective therapeutic strategy [24]. eIF4E is a critical node in an RNA regulon that impacts nearly every stage of cell cycle progression [37, 38]. The eIF4E availability and activity are tightly regulated by the mTOR-dependent phosphorylation of 4E-BPs [36]. In cancer cells, hyperactive mTOR signaling leads to eIF4E cytoplasmic translocation and elevated eIF4F activity. This consequently enables a disproportionate increase in the translation of pro-growth/survival mRNAs, such as cyclin D1, survivin, Bcl-2 and Mcl-1, or

some genes regulating angiogenesis (VEGF, FGF-2) [39]. Overexpression of eIF4E promotes tumorigenesis and has been involved in cancer development and progression [40]. Blocking eIF4E function suppresses cellular transformation, tumor growth, invasiveness and metastasis [33, 41].

In the present study, we employed a high content screen assay based on eIF4E nuclear accumulation to identify novel inhibitors targeting mTOR signaling pathway [36], 1,100 compounds from natural products and their derivatives were primarily screened in MEF cells, and 9 compounds showed comparable eIF4E [nuc:cyto] ratios to rapamycin. Among the positive compounds, IM-1 and IM-2 showed the most potent effects on eIF4E nuclear accumulation in hepatocellular carcinoma SMMC-7721 and Hep G2 cells. S6K and 4E-BP1 are the two best-characterized downstream targets of mTORC1 signaling pathway. It was reported that 4E-BP1 (Thr37/46) phosphorylation is sufficient to block eIF4E binding with 4E-BPs [11]. Our data
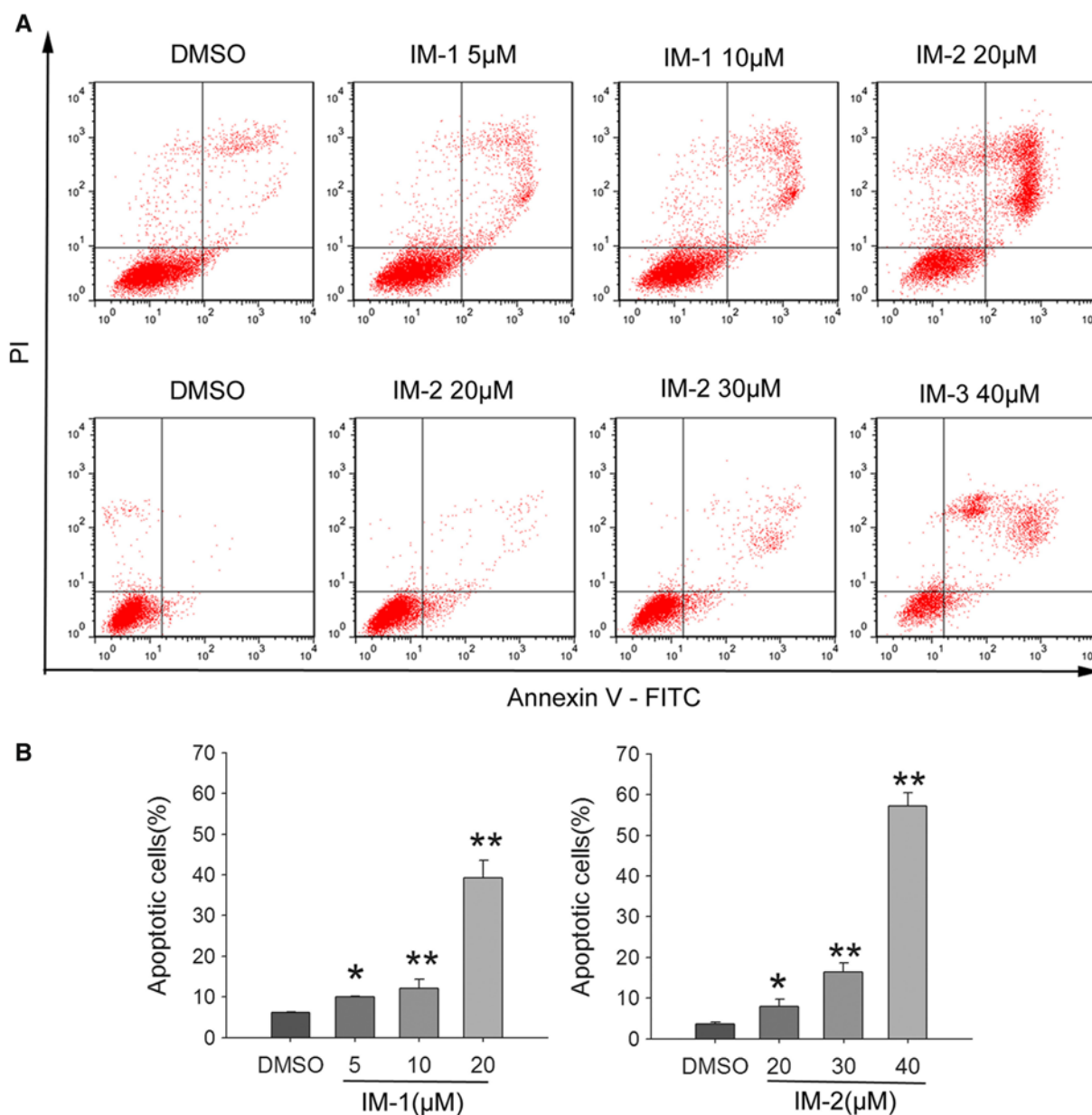
Fig. 6 Effects of IM-1 and IM-2 on apoptosis of SMMC-7721 cells. **a** One representative flow cytometry analysis of apoptosis. SMMC-7721 cells were treated with IM-1 and IM-2 at indicated concentrations for 48 h, respectively. Cell population FITC⁻/PI⁻, FITC⁺/PI⁻, FITC⁺/PI⁺, FITC⁻/PI⁺ were regarded as living, early apoptotic, late apoptotic and necrotic cells, respectively. **b** Quantification of flow cytometry analysis of apoptosis. Results are presented as mean ± SD of three separate experiments. *$p < 0.05$, **$p < 0.01$ versus control

demonstrated that IM-1 and IM-2 significantly decreased the phosphorylation levels of 4E-BP1, S6K and its target S6 in SMMC-7721 and Hep G2 cells and further confirmed that IM-1 and IM-2 are two mTORC1 signaling pathway inhibitors.

We found that IM-1 inhibited mTORC2-mediated phosphorylation of Akt (Ser473) in Hep G2 cells, but increased in SMMC-7721 cells, which might be due to the different contexts of the two cell lines. IM-2 increased mTORC2-mediated phosphorylation of Akt (Ser473) in both Hep G2

and SMMC-7721 cells. mTORC1 activation is postulated to cause a negative feedback loop through S6K, which phosphorylates insulin receptor substrate 1 (IRS1), inducing its degradation, whereas inhibition of S6K activity results in accumulation of IRS1 and activation of its downstream kinases, such as AKT [42]. The mechanisms under AKT activation by IM-1 and IM-2 remain to be determined.

IM-1, isolated from a folk medicine known as *Trichosanthes*, has been shown to exhibit strong cytotoxicity against several cancer cells [43]. IM-2, a cytotoxic pyranoisoflavone,

was uncovered from bioassay-guided fractionation of extract of the leaves and twigs of *Antheroporum pierrei* [44]. In our study, we found that IM-1 and IM-2 exerted cytotoxicity in a panel of cancer cells with high basal of mTOR signaling and significantly induced apoptosis of SMMC-7721 cancer cells. These data suggest the cancer inhibitory activities of the two compounds might be due to cell apoptosis induced through targeting the mTOR signaling. Since the two compounds have distinct structures, different action mechanisms and targets toward mTOR signaling of them are expected and worthy of further investigated.

In the present study, we found there were more eIF4E located in cytoplasm in all cancer cells than in MEF cells (Figs. 1b, 3b). Treatment with PI-103 induced more significant eIF4E nuclear translocation in cancer cells, particularly in hepatocellular carcinoma cells, than in MEF cells. These results indicate that the cancer cells with hyperactive mTOR signaling are more sensitive to mTOR inhibitors than MEF cells in this screen assay. Therefore, to avoid potential mTOR signaling inhibitors with anticancer activity being missed, it could be more reasonable to screen compounds against specific cancer cells than MEF cells.

In conclusion, with a powerful and useful approach of high content screening, IM-1 and IM-2, two novel inhibitors of mTOR signaling pathway, were discovered in our study. Both of the compounds exhibited potent cytotoxic activity and induced apoptosis in cancer cells. These compounds could be promising candidates for anticancer drug development by targeting mTOR signaling pathway and as such warrants further exploration.

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