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Identification of an IKBKE inhibitor with antitumor activity in cancer cells overexpressing IKBKE

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ABSTRACT

Background: The serine/threonine kinase IKBKE is frequently overexpressed or activated in a variety of human cancers. Ectopic expression of IKBKE induces malignant transformation, cell migration, invasion and chemoresistance. Thus, IKBKE is an attractive target for anti-cancer drug development.

Methods: By screening of NCI Diversity Set and Clinical Collection I and II compound libraries using cell-based assay, we identified several candidates of IKBKE inhibitors, which directly inhibited IKBKE kinase activity in vitro and *in vivo*. One of them, malachite green oxalate (MCCK1), was further characterized. The mechanism was examined by western blot, immunoprecipitation (IP) and Immunofluorescence. We also evaluated in a mouse xenograft model. *In vitro* kinase assay and luciferase reporter assay were also performed in our experiments.

Results: MCCK1 inhibits IKBKE kinase as well as its downstream targets such as IκBα, p65 and IRF3. MCCK1 is a selective inhibitor for IKBKE, with moderate effect on TBK1, but does not inhibit the activation of IKKα/β, STAT3, Erk-1/2, p38 or JNK. The inhibition of IKBKE by MCCK1 resulted in induction of cell growth arrest and apoptosis selectively in human cancer cells that harbor aberrant expression of IKBKE. Furthermore, MCCK1 inhibits tumor growth in nude mice of human cancer cells in which IKBKE is elevated but not of those cancer cells in which it is not.

Conclusion: These data indicate that MCCK1 is an IKBKE inhibitor with anti-tumor activity in vitro and *in vivo* and could be a potential anti-cancer agent for patients with tumors over expressing IKBKE.

1. Introduction

IKBKE (Inhibitor of nuclear factor kappa-B kinase subunit epsilon, also called IKKε) is an essential member of the non-canonical IκB kinase (IKK) family [1]. It shares 27% and 24% identity with IKKα and IKKβ, respectively [2,3]. Accumulating evidences have shown that IKBKE acts as a crucial regulator of innate immunity and involves in inflammation and cancer via multiple signaling pathways, especially modulating NF-κB and interferon signaling [1,4,5]. Inflammatory factors, interferon and viral infection activate IKBKE, its activation subsequently induces NF-κB nuclear accumulation and DNA-binding activity by phosphorylation of IκB-Ser36 which leads to IκB degradation and the transcription of cell growth/survival genes [2]. Several studies support a role for IKBKE-mediated Ser468 andSer536 phosphorylation of the p65 NF-κB subunit in the expression of a specific subset of NF-κB target genes in response to pro-inflammatory signals and viral infection. IKBKE but not IKKα/β phosphorylates CYLD, which is a deubiquitinase of several NF-κB regulators, including TRAF2, TRAF6, and NEMO, to activate the NF-κB pathway [6–8]. Moreover, IKBKE cooperates with another non-canonical IKKs TBK1 (Tank-binding kinase 1) to regulate interferon signaling in response to viral infection. Following activation of toll-like receptors by viral components, IKBKE and TBK1 assemble with TRAF3 and TANK to phosphorylate interferon regulatory factors (IRFs) 3, 5, 7and STAT1 as well as induces phosphorylation of p65/ c-Rel NF-κB subunits [9,10]. Activation of NF-κB and IRFs leads to increased production of pro-inflammatory cytokines/chemokines and typeI IFN genes, which are key players in anti-viral response [11].

Increasing evidence supports that IKBKE functions as anoncogene

Abbreviations: MCCK1, malachite green oxalate; IKBKE, Inhibitor of nuclear factor kappa-B kinase subunit epsilon; IKK, IκB kinase; TBK1, Tank-binding kinase 1; IRFs, interferon regulatory factors; ccRCC, clear cell renal cell carcinoma; NSCLC, non-small cell lung cancer; PDK1, 3-phosphoinositide-dependent protein kinase 1; IACUC, Institutional Animal Care and Use Committee; MBP, Myelin Basic Protein

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which is closely linked to tumorigenesis, progression, poor prognosis and chemoresistance. Michelle et al. performed a comprehensive systematic analysis to observe gene expression for 503 kinases in 93 clear cell renal cell carcinoma (ccRCC) specimens and a replication set of 237 ccRCC tumors [12]. From the analysis results, IKBKE washigher expressed in tumors compared with adjacent normal tissues. Furthermore, it was interesting that only IKBKE was significantly associated with overall survival. Ectopic expression of IKBKE resulted in increased risk of death rate by 5.30-fold. Same alterations of IKBKE are frequently occurring in human ovarian cancer cell lines and primary tumors, especially in high grade and late stage ovarian tumors. Ovarian cancer patients with elevated level of IKBKE have poor clinical outcome than those with lower IKBKE regardless of tumor stages. Abnormal expression of IKBKE renders ovarian cells resistant to cisplatin (CDDP) [13]. Several breast cancer cell lines and approximately 30% of primary human breast carcinomas express high level of IKBKE. Ectopic expression of IKBKE in immortalized mammary epithelial cells at levels found in human cancer cells resulted in malignant phenotype [14]. IKBKE has also been found to be upregulated in a variety of malignant tumors such as non-small cell lung cancer (NSCLC), melanoma and glioma as well as gastric cancer [15–19]. Aberrant IKBKE expression is due to unknown mutation regulating IKBKE transcript levels or to an amplification of the 1q32 region comprising the IKBKE locus [15]. High IKBKE expression is often associated with activation of NF-κB by the accumulation of c-Rel and p65 subunits in the nucleus. Furthermore, IKBKE and TBK1 orchestrate pathways involved in activation of NF-κB and tumorigenesis by direct phosphorylation of p65 or by phosphorylating Akt [1,15,20]. In addition, elevated IKBKE levels were also associated with STAT1 activation in different primary tumors and cell lines, which may also contribute to the oncogenic activities of IKBKE [15].

Together, these observations strongly support the conclusion that IKBKE plays a crucial role in the pathogenesis of a variety of tumors and brings forth it as a promising cancer target for drug development. BX-795 is the first IKBKE inhibitor on the market and simultaneously suppresses TBK1 activity at nanomolar concentrations *in vitro* [21]. It was originally developed as an inhibitor of 3-phosphoinositide-dependent protein kinase 1(PDK1). But now BX-795 shows inhibiting effects towards several other kinases, including Aurora-B, MARK2, JNK and p38 MAP kinases [21,22]. Two relatively specific kinase inhibitors, MRT67307and MRT68921, modified versions ofBX-795, were no longer inhibits JNK or p38 MAP kinases, but still interfered with the activity of PDK1, ULK1 and AMPK-related kinases [22,23]. Therefore, enhancing specificity to target IKBKE only is a challenge for developing IKBKE inhibitors.

To seek high effective inhibitors of IKBKE, we screened NCI Diversity Set and Clinical Collection I and II compound libraries via cell-based assay. MCCK1 (Malachite green oxalate) which was singled out from 76 lead compounds, had the most efficacious inhibition on IKBKE and its downstream signaling. Moreover, MCCK1 exhibited selective anti-tumor activity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell lines, animal care, antibodies and recombinant protein

Cell lines of breast cancer (BT474, MCF7, MDA-231, MDA-435, MDA-468 and T47D), colon cancer (HCT116 and HT29), lung cancer (A549, H292, H1299, H1703, H4006), ovarian cancer (OVCAR3, OVCAR 2008), HEK293 and HeLa were purchased from ATCC. These cells were maintained in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin.

Antibodies against IKBKE, Myc and actin were purchased from Sigma. Anti-p-ERK1/2, -p-p38, -p-Akt, -p-STAT3, -p-JNK, -p-IKKα/β, -p-IKKε/TBK1, -total IKKε, -p-IκBα, -total IκBα, -p-IRF3, -total IRF3, -full length PARP, -cleaved PARP, and -p50/p65 NF-κB antibodies were from Santa Cruz Biotechnology. Recombinant protein MBP was purchased from Active Motif.

2.2. Plasmids

The pCMV-3B-Myc tagged IKBKE, pCMV-IKKα, -IKKβ, - IKKε and -TBK1 were described previously [24]. The reporter plasmids pGL3-NF-κB-Luc and pGL3-IFNβ Luc were purchased from Agilent Technologies.

2.3. Screening for inhibition of IKBKE-transformed cell growth

pCMV-3B-Myc-IKBKE transformed NIH3T3 cells or pCMV-3B vector-transfected NIH3T3 control cells [13,14]were plated into 96-well tissue culture plate. After treatment with 5 μm NCI Diversity Set and Clinic Collection I and II compound, cell growth was detected with CellTier 96 One Solution Cell Proliferation kit (Promega). Compounds that inhibit growth in IKBKE-transformed but not vector-transfected NIH3T3 cells were considered as candidates of IKBKE inhibitor and subjected to additional analysis.

2.4. In vitro kinase assay

In vitro IKBKE kinase assay was performed as previously described [13]. Briefly, immunoprecipitation was performed by incubation of 200 μg protein lysate with anti-IKKε antibody. The immunoprecipitates were subjected to *in vitro* kinase assay. The reaction was performed with kinase buffer (20 mmol/L Hepes, pH 7.6, 10 mmol/L $MgCl_2$, 50 mmol/L NaCl, 0.1 mmol/L sodium vanadate, 20 mmol/L β-glycerolphosphate, and 1 mmol/L dithiothreitol), in the presence of 10 μCi [γ-^{32}P]ATP, 50 μmol/L unlabeled ATP. After incubation at 30 °C for 30 min, the reactions were stopped by adding protein-loading buffer and separated in 12.5% SDS-PAGE gels. The relative amounts of incorporated radioactivity were determined by autoradiography. Each experiment was repeated three times.

2.5. Western blot, immunoprecipitation (IP) and immunofluorescence

Western blot, IP and immunofluorescence were performed as previously described [21]. Briefly, cell lysates were prepared in a lysis buffer and subjected to immune-precipitation and immunoblot analysis. The primary antibodies dilutions were 1:1000–5000 according to the manuals from the Manufactures. For immunofluorescence, HEK293 cells were transfected with myc-IKBKE and pCMV-p50 NF-κB using Lipofectin® 2000 reagent (Invitrogen). After 48 h, cells were treated with 1 μM MCCK1 for 2 h or with DMSO as control, then fixed in 4% paraformaldehyde in PBS and stained with Alexa Fluor® 594 conjugated (Red) anti-Myc-tag antibody and Alexa 488 labeled (Green) anti-p50 antibody and observed under a fluorescence microscope.

2.6. Luciferase reporter assay

The luciferase reporter assay was carried out as previously described [21]. Briefly, HEK293 cells were co-transfected with empty pCMV vector, the luciferase reporter constructs NF-κB-luc or IFNβ-luc and constructs indicated in the figure legend. After incubation for 48 h, cells were treated with increasing dosages of MCCK1 at 0, 0.1, 0.5 and 1 μM for 2 h. Then cells were lysed and luciferase activity was monitor by microplate reader.

2.7. Cell viability and programmed cell death

Cell viability was evaluated using MTT assay. Briefly, cells were diverted into 96-well plate with $1x10^5$cells/well. Following incubation for 24 h, cell were treated with different concentration of MCCK1 for 72 h and then assayed for total cell viability. Apoptosis was performed with the fluorescent Annexin-VApoptosis Detection Kit (Thermo Fisher Scientific) following manufacture's instruction. Cleaved PARP was

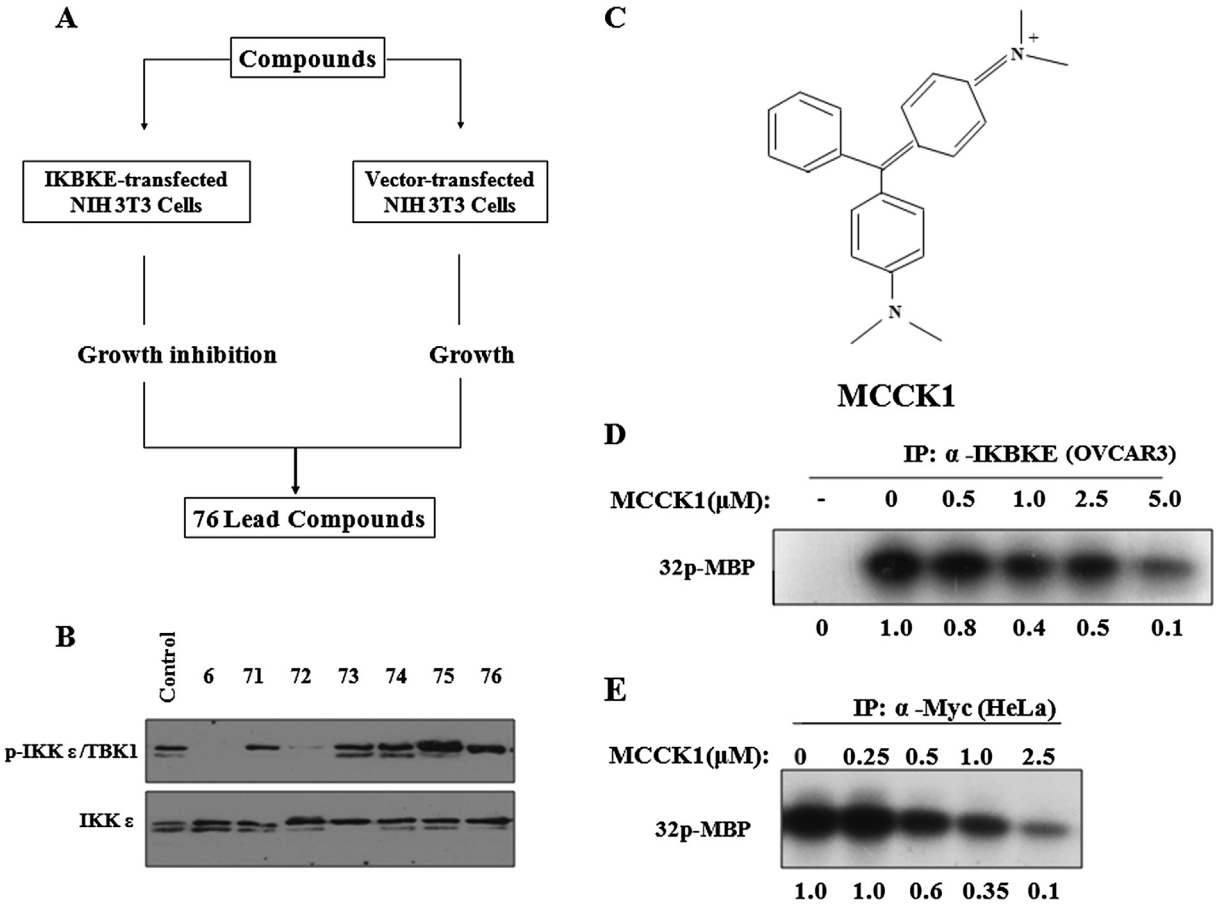

Fig. 1. Identification of MCCK1 as an IKBKE kinase inhibitor. (A) Scheme of the screening of IKBKE inhibitors from NCI Diversity Set and Clinical Collection I and II compound libraries using cell based assay. (B) The effects of 7 lead compounds on phosphorylation levels of IKBKE. Compounds #6 had the best efficacious inhibitory effect. (C) The structure of MCCK1. (D and E) *In vitro* kinase assay. IKBKE was immunoprecipitated from OVCAR-3 and myc-IKBKE transfected HeLa cells following MCCK1 treatment with indicated concentration. The immunoprecipitates were subjected to in vitro kinase assay using MBP as substrate.

detected by western blot.

2.8. EdU staining assay

For EdU staining, cultured tumor cells were plated on slides with a density of $5x10^3$ cells/ml. Cells were exposed to MCCK or DMSO (as control) for 6hr before treatment with 5 μM EdU for 2hr. Cells were then washed with 3% BSA in PBS three times, fixed with 4% paraformaldehyde in PBS for 10 min. After washing with 3% BSA three times, cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min. Cells were then incubated with EdU staining cocktail (Click-iT® EdU Alexa Fluor® 594 Imaging Kit from Invitrogen) kept from lights at room temperature for 30 min. After washing with 3% BSA, samples were then counterstained with 1x DAPI for 10 min at Room temperature. Images were acquired by fluorescence microscope.

2.9. In vivo tumor formation assay

Eight week-old NOD/SCID mice were used. The mice were housed with a regular 12 hr light/12 hr dark cycle and were kept in a pathogen-free environment. Tumor cells were mixed in Matrigeland, then injected into nude mice subcutaneously or intracranially. The mice were randomly divided into two groups. MCCK1 was applied into the mice at 10 mg/kg by intraperitoneal injection every other day for 6 weeks. Tumor growth in these mice was monitored and measured once a week. The tumor volumes were calculated by the equation V (mm^3) = a × b^2/2, where “a” is the largest diameter and “b” is the perpendicular diameter.

2.10. Tunel

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed to detect apoptosis in situ cell death following the manufacturer’s instructions (TUNEL Apoptosis detection kit: UPSTATE, Lake Placid, NY). TUNEL-positive cells displayed brown staining within the nucleus of apoptotic cells. The numbers of TUNEL-positive cells of fields were counted under light microscopy. The extent of apoptosis was calculated and expressed as a number of TUNEL-positive cells from the same field.

2.11. Immunohistochemistry (IHC)

The paraffin-embedded tumor sections were stained for anti-Ki67, anti-Caspase-3 and anti-PCNA (Abcam, Cambridge, UK). Sections (2 μm) were deparaffinized and pretreated with citrate buffer using a heat-induced epitope retrieval protocol. Endogenous peroxidase was blocked with 20% hydrogen peroxide for 15 min at room temperature. The incubation between endogenous peroxidase and anti-Ki67, anti-Caspase-3 or anti-PCNA should achieve 30 min respectively. A biotinylated goat anti-mouse immunglobulin G secondary antibody (Dako, Denmark) was then applied to each slide for 30 min. After washing in Tris-hydrochloric acid buffer (TBS), the slides were incubated with peroxidase-conjugated streptavidin complex reagent (Dako, Denmark) and developed with 3,3′-diaminobenzidine for 5 min. The slides were counterstained and dehydrated.

Animal procedure and care were approved by the IACUC (Institutional Animal Care and Use Committee) at University of South

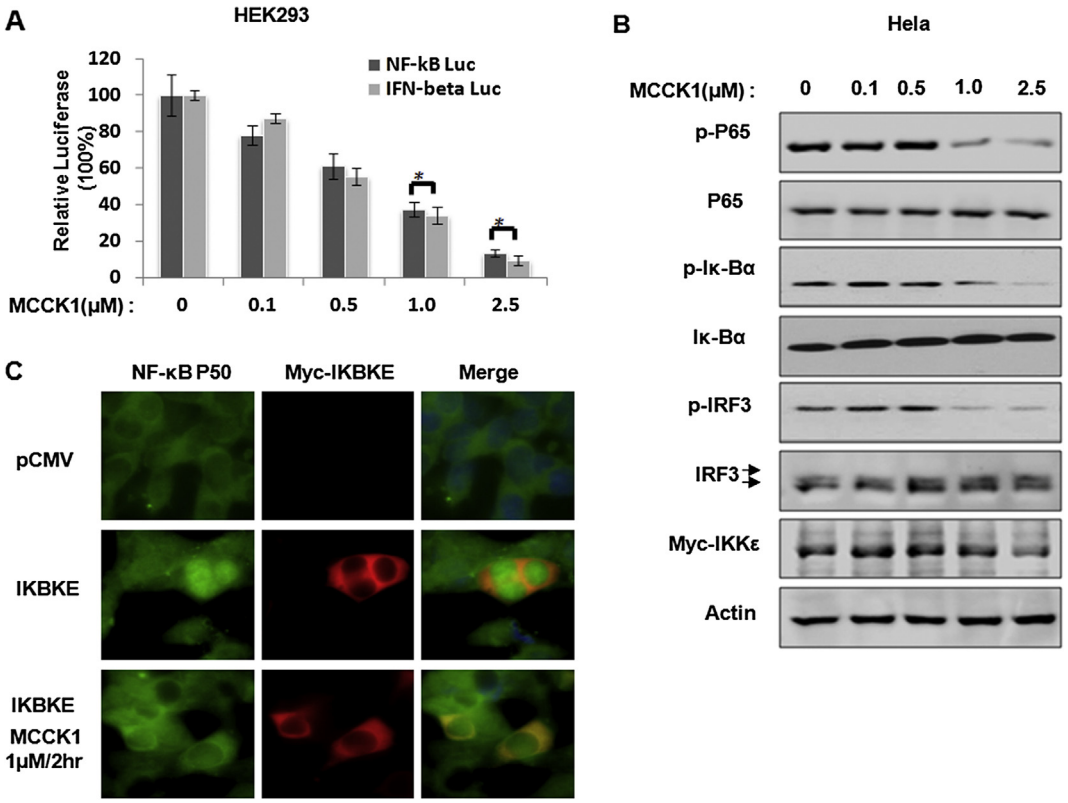

Fig. 2. MCCK1 inhibits the NF-κB and IFN signaling pathway and downstream targets. (A) HEK293 cells were transfected with NF-κB-Luc and IFN-β-Luc reporter plasmids. Following incubation for 48 h and treatment with and without MCCK1 for 2 h, Cells were lysed and the luciferase activity was measured. Each value represents the mean ± SE of triplicate independent samples. The representative column diagrams showing results of relative luciferase. $^{*}p < 0.05$, compared to 0 μM. (B) HeLa cells were treated with MCCK1 for 2 h and then subjected to Western blot analysis with indicated antibodies. (C) HEK293 cells were co-transfected with plasmids encoding Myc-tagged IKBKE and pCMV-p50 NF-κB and then cells were fixed and immunostained with anti-Myc (red) or anti-P50 (green) after incubated with or without MCCK1 (1 μM) for 2 h.

Florida. The tissues were snap frozen and were used.

2.12. Statistical analysis

For luciferase activity and cell survival, the experiments were repeated at least three times in triplicate. The data are represented by means ± SE. Differences between control and testing cells were evaluated by Student's *t* test; all analyses were completed with SPSS software, version 10.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of MCCK1 as an IKBKE kinase inhibitor

We are experienced using cell based assay to screen protein kinase inhibitor and have previously identified specific Akt inhibitor, API-2/triciribine, which inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane [25]. To screen for IKBKE inhibitor, we have established pCMV-3B-Myc-IKBKE stably-transfected NIH3T3, which were fully transformed [13,14], andpCMV-3B-transfected NIH3T3 cell lines. The cells were seeded in 96-well plates. After 24 h of incubation, the chemical compounds from NCI Diversity Set and Clinical Collection I and II libraries were added into the cell culture (3 wells/compound at 5 μM concentration). Following treatment for 12 h, cell growth inhibition was determined using Cell Tier 96 One Solution Cell Proliferation kit. The effective compounds were selected based on that they inhibited the growth of IKBKE transformed NIH3T3 cells, while they had no visible effect on the vector-transfected NIH3T3 cells as showed in Fig. 1A. Out of ~2500 compounds, 76 were identified to selectively inhibitIKBKE-NIH3T3 cell growth. We further tested their effects on the phosphorylation level of IKKε/TBK1 with Western Blot using total IKKε as loading controls (Fig. 1B). Compounds #6 and #72 had a much inhibitory effect on the activation of IKKε/TBK1. Then we checked the libraries and identified the #6 is malachite green oxalate (MCCK1) which contains one benzene and two amino-benzenes (Fig. 1C). To further confirm if MCCK1 directly inhibit the kinase activity of IKBKE, *in vitro* kinase assays of endogenous IKBKE and Myc-tagged IKBKE were carried out with MBP (Myelin Basic Protein) as substrate. MCCK1 at 2–5 μM could efficiently inhibit the phosphorylation of MBP by IKBKE in a dose-dependent manner (Fig. 1D and E). These data suggest that MCCK1 is an efficient inhibitor of IKBKE.

3.2. MCCK1 inhibits downstream targets of IKBKE

To further analyze MCCK1 inhibitory function on IKBKE and its downstream proinflammatory factors at cellular level, we chose two set of reporter systems that function downstream of IKBKE, NF-κB and IFN-β promoter fused with luciferase gene and applied MCCK1 to the HEK293 transfected with these reporter plasmids. The luciferase activities of both NF-κB and IFN-β reporter but not of pGL3 luciferase reporter vector control, were dramatically decreased in the presence of 2.5 μM MCCK1 (Fig. 2A and data not shown). Moreover, we used HeLa cells expressing myc-tagged IKBKE as model system to study which signal pathway was altered by application of MCCK1 via detecting the activation/phosphorylation level of p65 (NF-κB component), IκBα and IRF3. The activation of all the three proteins was dramatically diminished in a dose dependent manner when the cells were exposed to MCCK1 (Fig. 2B). Immunofluorescence studies were performed to detect the subcellular localization of NF-κB p50. When myc-tagged IKBKE

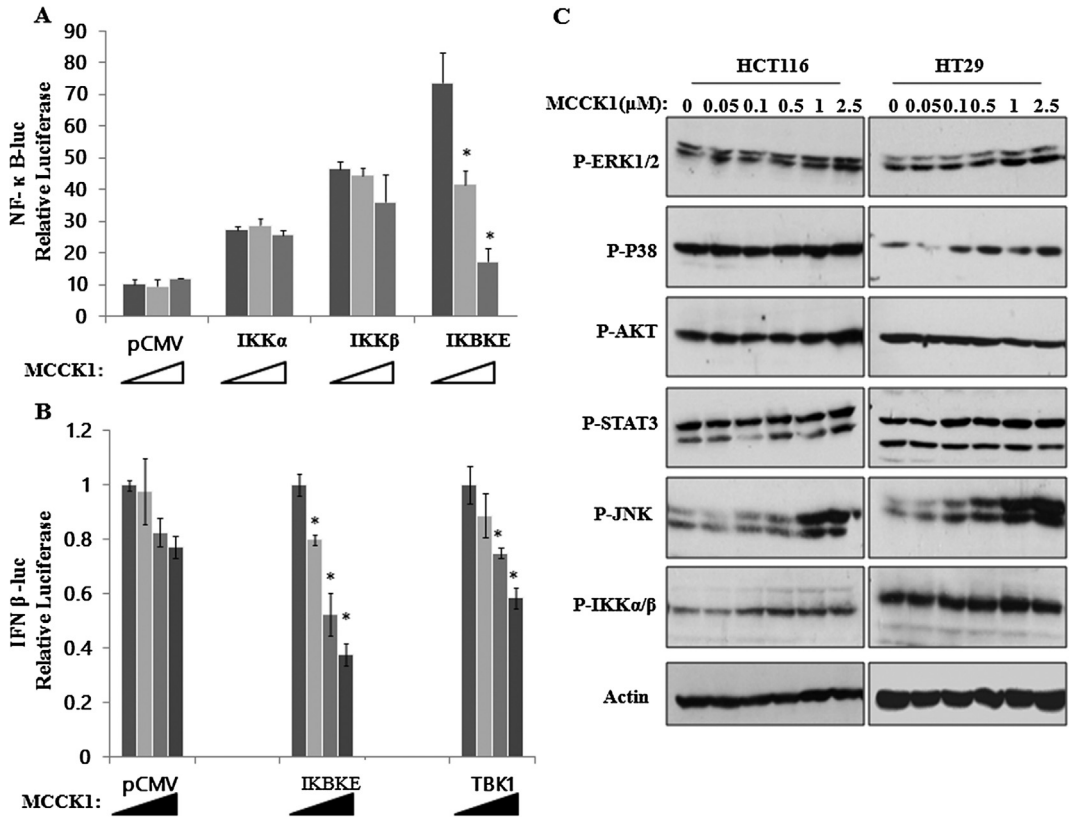

Fig. 3. MCCK1 targeted to IKBKE over other kinases. (A) HEK293 cells were seeded on a 24-well plate, and co-transfected with pMCV-IKKα/β,-IKBKE as well as NF-κB-luc reporter plasmid. After 48 h, cells incubated with MCCK1 for 2 h. The luciferase activity was measured. Each value represents the mean ± SE of triplicate independent samples. (B) HEK293 cells were seeded on a 24-well plate, and co-transfected with pMCV-IKBKE and pMCV-TBK1 as well as together with IFN-β-Luc reporter plasmid. Subsequent procedure was carried out according to NF-κB luciferase reporter assay. (C) HCT116 and HT29 cells were treated with MCCK1 at indicated concentrations and then immunoblotted with the indicated antibodies.

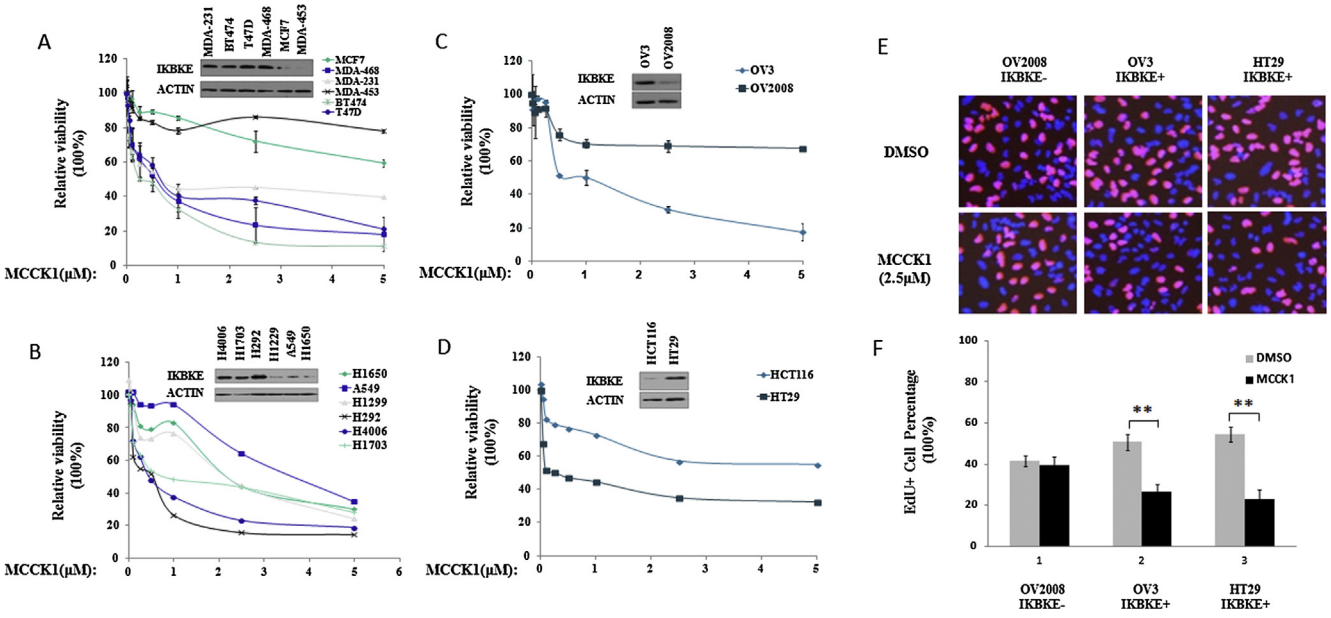

Fig. 4. MCCK1 selectively inhibits the proliferation of tumor cells that express high levels of IKBKE. Cancer cell lines of breast (A), lung (B), Ovary (C) and colon (D) were treated with indicated doses of MCCK1 for 72 h, and cell proliferation was analyzed by MTT assay. (E) EdU incorporation assays were used to detect the proliferation rate of different tumor cells in the presence or absence of MCCK. EdU was labeled in red color and nuclear staining with DAPI was purple. (F) EdU incorporation rate was analyzed using Image-J 2.0 software. The data are shown as means ± SE of three independent experiments. ** *means* $p < 0.01$.

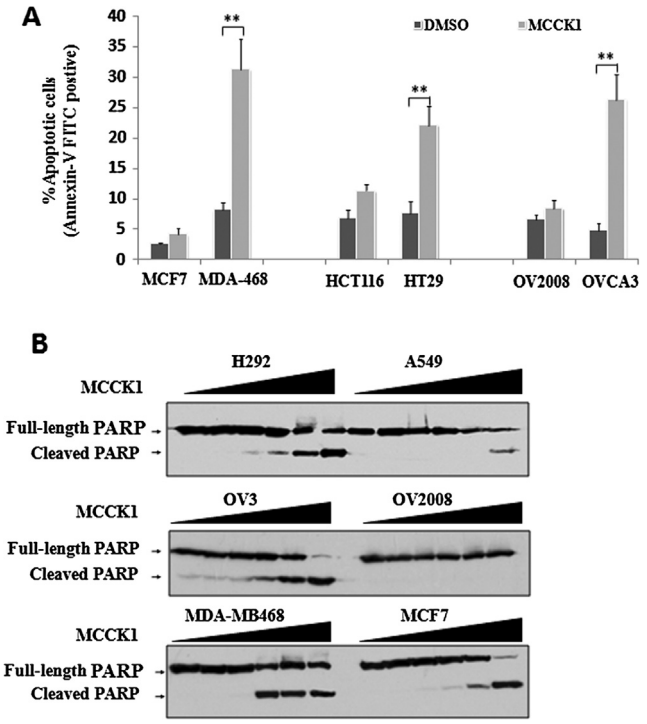

Fig. 5. MCCK1significantly induces apoptosis in IKBKE-high expression tumor cells. The cells were treated with MCCK1 -for 48 h then subjected to Annexin V/PI flow cytometry assay (A) and Western blot for cleaved PARP P(B). The representative column diagrams showing results of apoptosis rate of cells. $^{**}p < 0.01$, compared to control. Data are mean ± SE for the three replicates.

was expressed in the cells, p50 was solely localized in the nuclear in the absence of MCCK1 treatment. Upon MCCK1 treatment for 2 h majority of NF-κB p50 would stay in the cytoplasm where it was degraded via ubiquitin system (Fig. 2C). Taken collectively, these data indicated MCCK1 selectively inhibit IKBKE.

3.3. MCCK1 is highly selective for IKBKE inhibition over other kinases

We next investigated selectivity of MCCK1 toward IKBKE. We took advantage of the high sensitivity of Luciferase assay in HEK293 cells transfected with a variety of signal components pertaining to NF-κB signaling and stress response. As shown in Fig. 3A and B, the activation of IKKα and IKKβ were not affected by exposure to high concentration of MCCK1, just opposite to IKBKE which was significantly demolished in the presence of MCCK1. Indeed, the activity of TBK1 was modest affected by MCCK1 in a dose dependent manner (Fig. 3B). We chose two colon cancer cell lines HCT116 and HT29 as two opposite examples in IKBKE expression pattern (Fig. 4D) and treated them with a series of concentration of MCCK1, then checked the activation level of NF-κB related cell proliferation and survival signaling as shown in Fig. 3C. The activation of IKKα/β, STAT3, Erk-1/2, Aktor p38 was not noticeably changed even exposed to high concentration of MCCK1. In conclusion, MCCK1 is highly specific for IKBKE inhibition.

3.4. MCCK1 selectively suppresses cell growth in the cells expressing elevated IKBKE

Next we examined the different effect of MCCK1 on the cell survival property between cancer cells expressing high and low IKBKE. As shown in Fig. 4A–D, we collected a variety of cancer cell lines, including cancer cells of breast, ovary, lung and colon, and measured their survival curves with MTT assay after exposure to different dose of MCCK1 at 1–5 μM. There were clear separations between IKBKE-high and IKBKE-low cell lines. IKBKE-low cells were much less sensitive to MCCK1 treatment, more than 60% of the cells were viable after exposure to high concentration (5 μM) of MCCK1. However, the cell lines with over expression of IKBKE were much vulnerable to MCCK1, less than 20% of the cells were still alive at 5 μM drug concentration. We also uesd EdU assay to detect the proliferation difference within IKBKE-high and IKBKE-low cell lines, which was shown in Fig. 4E and F. Taken together, MCCK1 is specific toxic to IKBKE overexpressing cancer cells.

3.5. MCCK1 selectively induces programmed cell death

As indicated in Fig. 4, MCCK1 selectively inhibited total cell survival in IKBKE high cancer cell lines, which prompted us to examine the programmed cell death rate of these cancer cell lines after MCCK1 treatment, because IKBKE has been shown to be critical in anti-apoptosis. The apoptosis rate of different cancer cell lines exposed to 5 μM MCCK1 or DMSO (control) was measured by flow cytometry after staining with Annexin-V FITC. As expected, the cell lines with high levels of IKBKE exhibited more apoptosis than cells expressing low levels of IKBKE after MCCK1 treatment (Fig. 5A). Furthermore, we analyzed the cleaved PARP. In consistent with Fig. 5A, all lines expressing high levels of IKBKE regardless their derived organs had much more cleaved PARP than cell lines with low levels of IKBKE after MCCK1 treatment in a dose dependent pattern (Fig. 5B).

3.6. MCCK1 selectively inhibits tumor growth in xenograft nude mouse model

The data above suggest that MCCK1 could be a specific anti-cancer reagent against IKBKE aberrant-expression tumors. To this end, we performed xenograft mouse tumor by injection of 2–10 million either colon cancer cells or ovary carcinoma cells into the NOD/SCID mice. After the tumor developed and grew to average 1.5 cm diameter, the mice were divided into 2 groups (6 mice/ group), one group were received MCCK1 (10 mg/kg, i.p.) and the other were administered with DMSO (control) every other day. The tumor growth was monitored by measuring the tumor volume. Following treatment for 6 weeks, the mice were sacrificed and the tumors were dissected and measured their volumes/weights. The growth curves of either colon tumors (upper two panels in Fig. 6A) or ovary tumors (lower panel of Fig. 6A) clearly showed that the treatment with MCCK1 could dramatically inhibit the tumor growth derived from IKBKE-high cancer cells (left hand Fig. 6A). On the contrary, MCCK1 did not show noticeable inhibition of the tumor growth in IKBKE-low tumors by (right hand Fig. 6A). There was no difference in tumor sizes of IKBKE-low tissues with or without MCCK1 treatment, such as OV2008 and HCT116 (Fig. 6B). While the tumor size differences were much obvious in tumors developed from IKBKE-high cells between the presence and absence of MCCK1, including HT29 (colon tumors) and OVCA3 (ovary carcinoma).

Then after that we performed TUNEL and immunohistochemical experiments. The TUNEL result combined with immunohistochemistry result demonstrated that treatment with MCCK1 significantly promoted apoptosis of IKBKE-high xenograft tumor cells, immunohistochemistry of Caspase-3 and TUNEL confirmed the elevated apoptosis (Fig. 7A). Meanwhile, the low expression of Ki67 and PCNA indicated a suppression of proliferation by treatment with MCCK1 in IKBKE-high cells (Fig. 7B). These data suggest that MCCK1 is a specific anti-tumor drug to treat IKBKE-high positive tumors.

4. Discussion

The NF-κB pathway is a key regulator that activates transcription of genes involved in cell proliferation, survival and invasion. Activation of NF-κB is frequently observed in solid tumors and hematological malignancies and it is a potential driver of cancer growth [26,27]. Various stimulants trigger activation of the IKK (IκBs kinase complex), leading

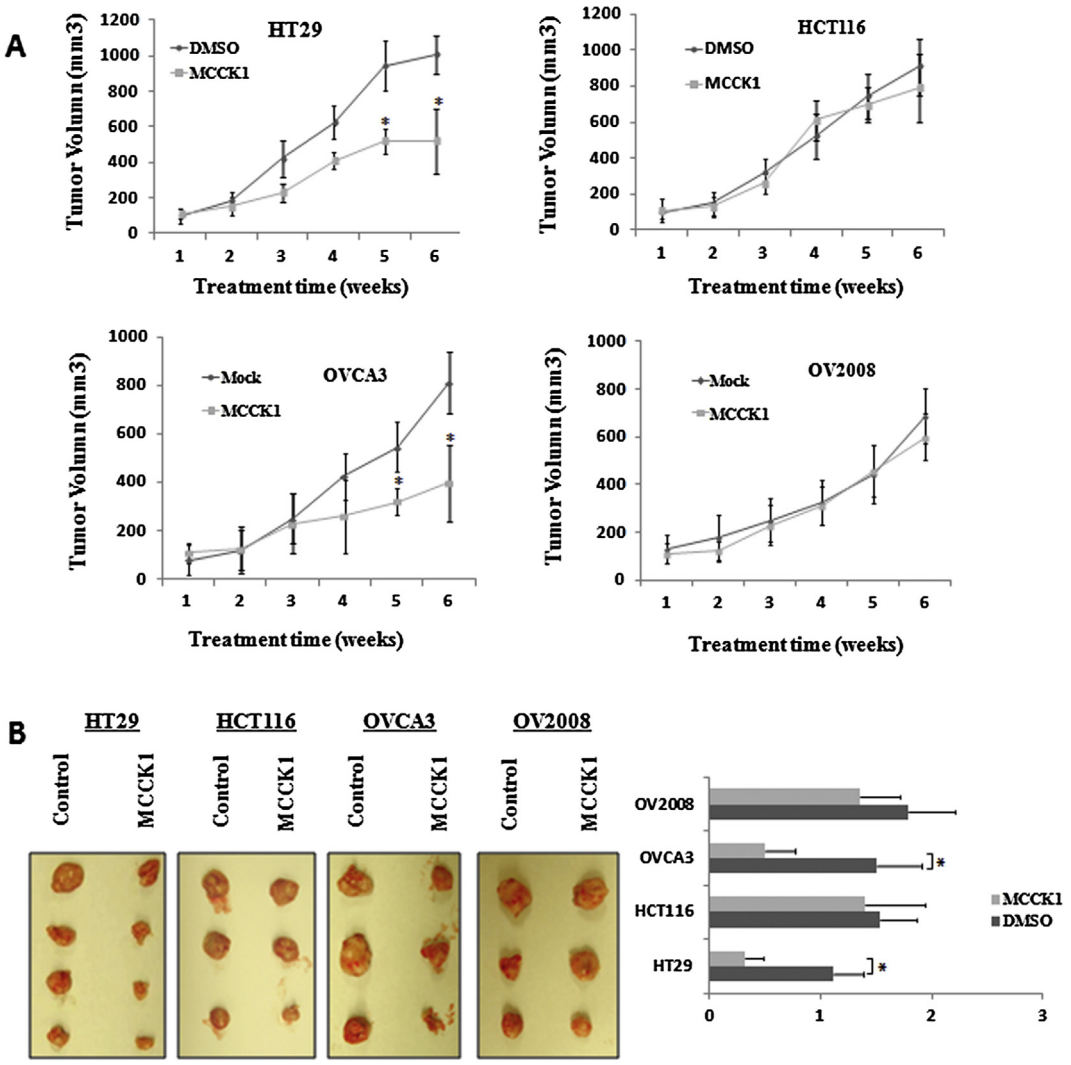

Fig. 6. Selective anti-cancer effects of MCCK1 *in vivo*. Colon cancer (i.e. IKBKE-high HT29 and IKBKE-low HCT116) and ovary cancer (i.e. IKBKE-high OVCAR3 and IKBKE-low OV2008) cells were subcutaneously injected into the flanks of nude mice. When the tumor volumes reached about 100 mm^3, the mice were randomly divided into two groups. One group was administered with MCCK1(10 mg/kg) by intraperitoneal injection every other day for 6 weeks, and the another group was treated with vehicles. (A) Tumor volume was measured at every other day, and plotted for tumor growth curves. (B) At the end of the experiment, mice were sacrificed and tumors were resected, weighed and photographed. The representative graphs showing results of tumor volume. $^{*}p < 0.05$, compared to control. Data are mean ± SE for the three replicates.

to proteasomal degradation of IκBs. Consequently, IKKs are key regulators of NF-κB signaling [28]. The IKK family consists of four kinase members, the canonical kinases IKKα and IKKβ, as well as the non-canonical kinaseTBK1 and IKBKE [29]. NF-κB is generally activated through canonical IKK-dependent phosphorylation. IKBKE and TBK1 are originally characterized as activators of NF-κB, but they are not essential for NF-κB activation [30]. Till now, the pharmaceutical industries have made limited achievements on developing IKKα/IKKβ inhibitors into a new type of anti-inflammatory drugs. Due to NF-κB regulates many physiological functions, non-selective and completely inhibition of NF-κB pathway may lead to severe side effects [31]. IKKα/IKKβ knockout mice always died in embryo or perinatal stages, which illustrates their pivotal roles in mouse development [32]. Whereas the mice with knockout of IKBKE are viable. Therefore pharmaceutical inhibitors, which were selectively targeted on IKBKE, may avoid notorious side effects. The development of specific inhibitors targeting the non-canonical kinases has gradually been a hot area of researching the past few years.

The role of IKBKE in the development of inflammatory and metabolic diseases, as well as cancer, indicate the potential of IKBKE as a promising therapeutic target [33,34]. However, only a few small molecule inhibitors of IKBKE possessing drug-like quality have been described. Moreover, few observations have yet been systematically reported about anti-tumor efficacy of these pharmacological inhibitors *in vivo*. Meanwhile, because existing inhibitors have promiscuously blocked other kinases that would generate unwanted side effects, hence the development of more specific and better IKBKE inhibitors may be enabled by the recent elucidation of the substrate specificity of IKBKE [1,35,36]. And as many following reasons, it is very difficult to develop IKBKE inhibitors that do not target TBK1. First, IKBKE was reported that IKBKE knockout mice have strong viability and fertility. By contrast, TBK1 knockout mice often die at embryonic stage [37]. Second, IKBKE and TBK1 exhibit differential expression patterns, they share the kinase domain and are similar in their ability to activate the NF-kB signaling pathway [1,38]. And they show many overlapping functions in oncogenic signaling pathways including IRF3/7, JAK/STAT and AKT signaling [4,20,33,39]. In a word, the qualities of IKBKE make it possible that it is the appropriate anti-cancer therapeutic target comparing withTBK1.

A couple of new IKBKE inhibitors have recently been reported. The

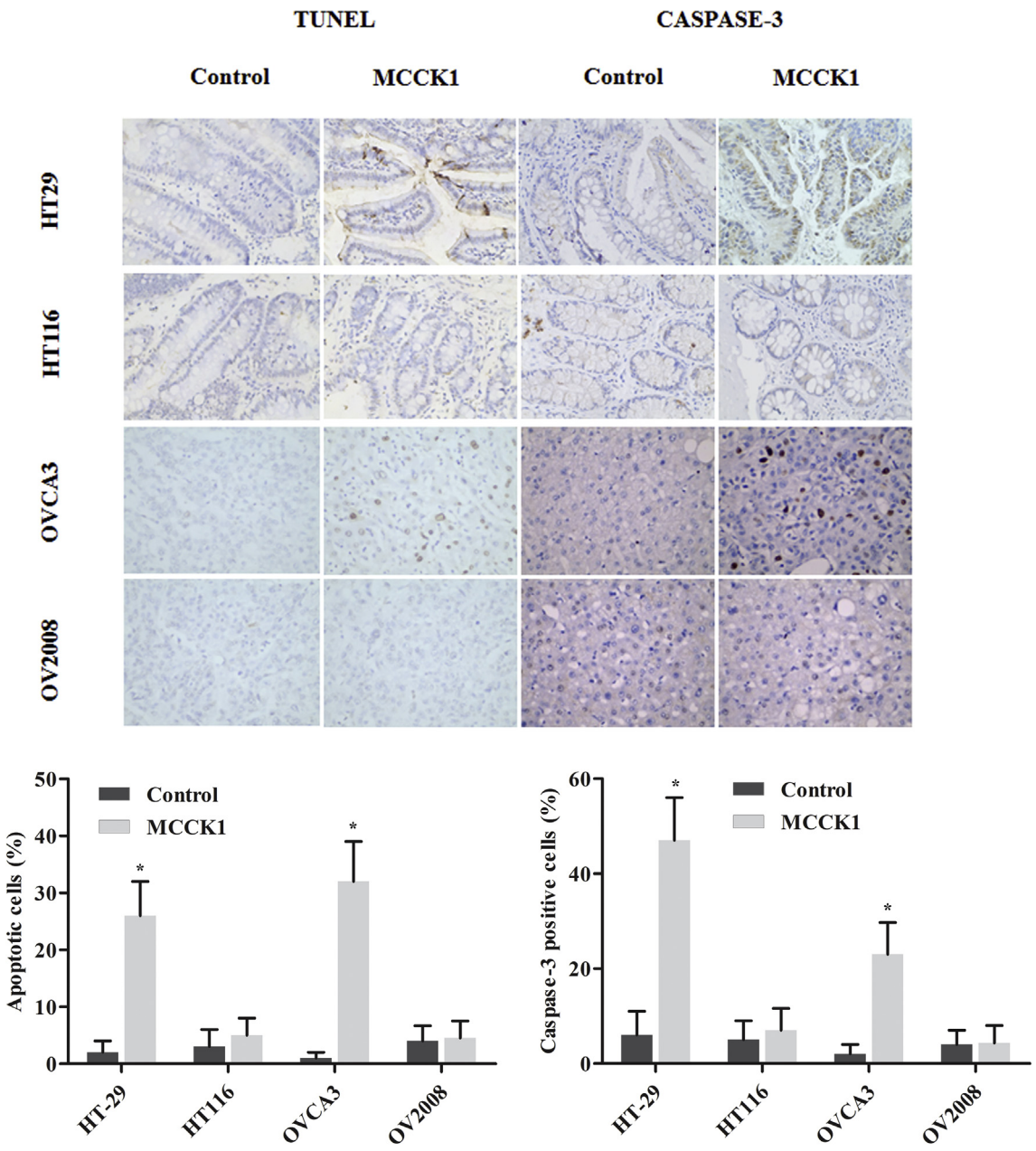

Fig. 7. IHC was used to evaluate the proliferation and apoptosis activity of tumor tissues. TUNEL assay was used to determine the apoptosis in MCCK1 treated IKBKE-high tumors compared with treated IKBKE-low tumors (magnification, ×200). (A) Immunohistochemistry analysis of expression of Ki67, Caspase-3 and PCNA (magnification, ×200). (B). The representative column diagrams showing results of positive rate of cells. $^{*}p < 0.05$, compared to control. The results were presented as the mean of three independent experiments.

derivatives of 6-aryl-azabenzimidole and2,4-diamino-5-cyclopropyl pyrimidines with improved kinase selectivity and drug-like properties were described in 2012[40,41]. Three TBK1/IKBKE dual inhibitors were generated based on a structurally rigid 2-amino-4-(3′-cyano-4′-pyrrolidine)phenyl-pyrimidine scaffold. They potently inhibited cell viability in human breast, prostate and oral cancer cell lines and significantly impaired tumor development in mouse tumor models [42]. The anticancer function of these inhibitors may be partially due to their suppression of TBK1/IKBKE-mediated AKT phosphorylation and VEGF expression. Amlexanox, a clinical medication treated asthma and allergic rhinitis in Japan and aphthous ulcers in America, is thought to be a hopeful dual TBK1 and IKBKE inhibitor with application prospect for cancer and diabetes therapy. It blocked phosphorylation of Akt1 and the MAP-kinases p38 and p42/44 in melanoma cells as well and thus might be involved in the anti-proliferative effects on melanoma cell [17,43]. All these data indicate that targeting IKBKE andTBK1 might be a safe and effective avenue of suppressing cancer development that could avoid notorious side effects associated with complete NF-κB inhibition.

5. Conclusions

In present study, we identified that MCCK1 significant inhibited IKBKE kinase activity and exhibited moderate inhibitory effect on TBK1. Dual inhibition on IKBKE and TBK1 of MCCK1 consequentially reduced activation of NF-κB and interferon signaling. Importantly, MCCK1 has no inhibitory effect on IKKα/β, STAT3 and the MAP kinases. Furthermore, we not only confirmed the inhibitory effect of MCCK1 on the growth rate and apoptosis of a variety of cancer cell lines, but also showed that this inhibitor efficiently suppressed tumor

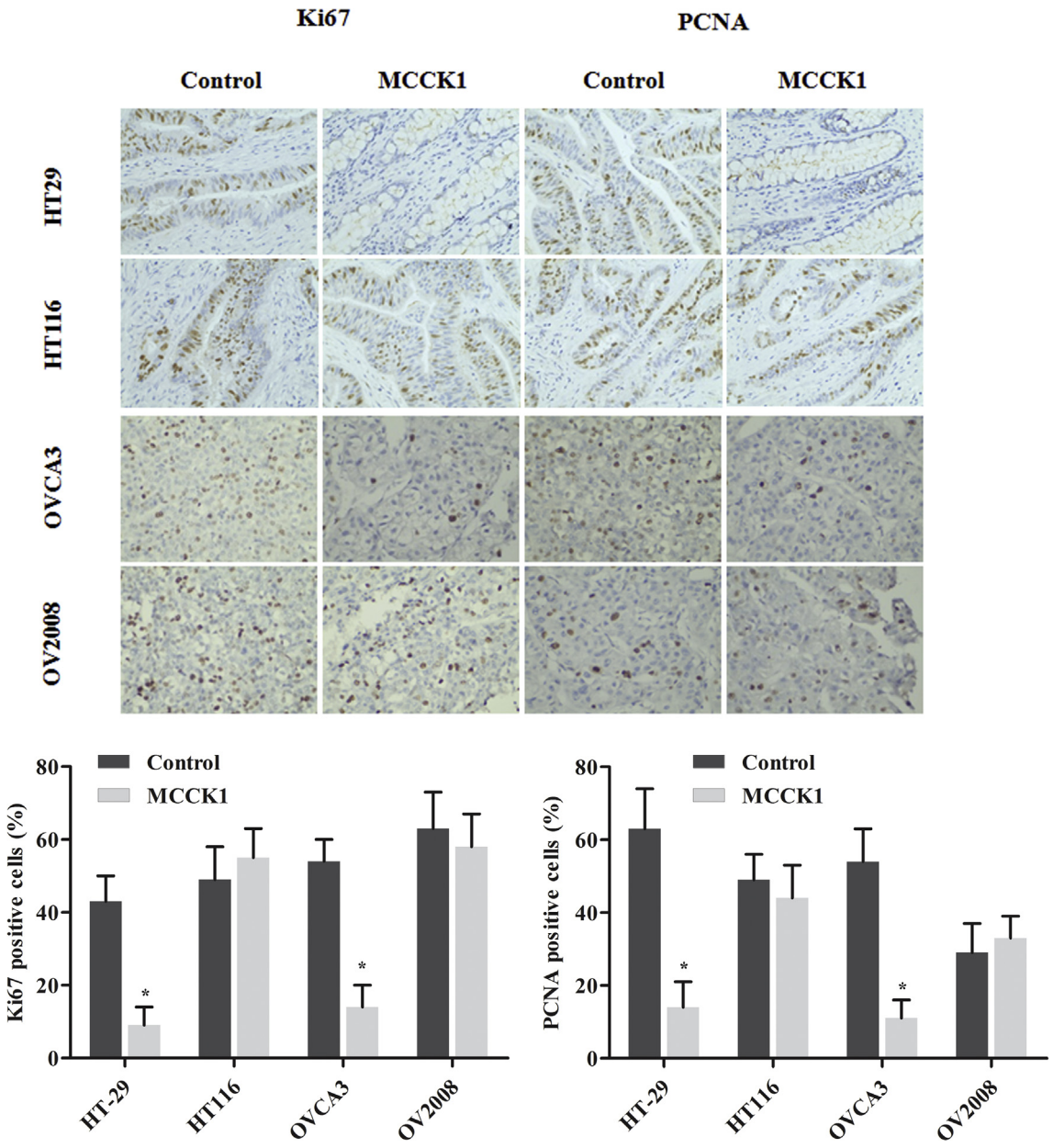

Fig. 7. (*continued*)

growth in nude mice of human cancer cells in which IKBKE is abnormally elevated. Meanwhile, MCCK1 does not suppress AKT phosphorylation in both IKBKE-over expression colon cell line and IKBKE-normal colon cells. It is possible that TBK1/ IKBKE-mediated AKT activation is not involved in the mechanism of MCCK1 of anti-tumor activity in mice. Certainly more evidences should be gathered from other types of tumor cell lines and *in vivo* experiments. And future fundamental investigations are required to determine the effect of MCCK1 on other kinases in IKBKE-overexpression cancer cell lines. In short, our data clearly indicate that MCCK1 is a potential specific IKBKE inhibitor with anti-tumor activity, and could be a novel anti-cancer reagent for patients with tumors over expressing IKBKE.

Ethics approval and consent to participate

Animal procedure and care were approved by the IACUC (Institutional Animal Care and Use Committee) at University of South Florida. The tissues were snap frozen and were used.

Conflict of interest

None.

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References

[1] R.R. Shen, W.C. Hahn, Emerging roles for the non-canonical IKKs in cancer, Oncogene 30 (6) (2011) 631.
[2] T. Shimada, T. Kawai, K. Takeda, M. Matsumoto, J.-I. Inoue, Y. Tatsumi, A. Kanamaru, S. Akira, IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IκB kinases, Int. Immunol. 11 (8) (1999) 1357–1362.
[3] R.T. Peters, S.-M. Liao, T. Maniatis, IKKε is part of a novel PMA-inducible IκB kinase complex, Mol. Cell 5 (3) (2000) 513–522.

[4] J.-F. Clément, S. Meloche, M.J. Servant, The IKK-related kinases: from innate immunity to oncogenesis, Cell Res. 18 (9) (2008) 889.
[5] S.-L. Ng, M.A. Chua, S.M. McWhirter, A. García-Sastre, T. Maniatis, Multiple functions of the IKK-related kinase IKKε in interferon-mediated antiviral immunity, Science 315 (5816) (2007) 1274–1278.
[6] H. Geng, T. Wittwer, O. Dittrich-Breiholz, M. Kracht, M.L. Schmitz, Phosphorylation of NF-κB p65 at Ser468 controls its COMMD1-dependent ubiquitination and target gene-specific proteasomal elimination, EMBO Rep. 10 (4) (2009) 381–386.
[7] M. Adli, A.S. Baldwin, IKK-i/IKKε controls constitutive, cancer cell-associated NF-κB activity via regulation of Ser-536 p65/RelA phosphorylation, J. Biol. Chem. 281 (37) (2006) 26976–26984.
[8] E. Trompouki, E. Hatzivassiliou, T. Tsichritzis, H. Farmer, A. Ashworth, G. Mosialos, CYLD is a deubiquitinating enzyme that negatively regulates NF-κB activation by TNFR family members, Nature 424 (6950) (2003) 793.
[9] K.A. Fitzgerald, S.M. McWhirter, K.L. Faia, D.C. Rowe, E. Latz, D.T. Golenbock, A.J. Coyle, S.-M. Liao, T. Maniatis, IKKε and TBK1 are essential components of the IRF3 signaling pathway, Nat. Immunol. 4 (5) (2003) 491.
[10] A. Koop, I. Lepenies, O. Braum, P. Davarnia, G. Scherer, H. Fickenscher, D. Kabelitz, S. Adam-Klages, Novel splice variants of human IKKε negatively regulate IKKε-induced IRF3 and NF-kB activation, Eur. J. Immunol. 41 (1) (2011) 224–234.
[11] S.-L. Ng, B.A. Friedman, S. Schmid, J. Gertz, R.M. Myers, T. Maniatis, IκB kinase ε (IKKε) regulates the balance between type I and type II interferon responses, Proc. Natl. Acad. Sci. 108 (52) (2011) 21170–21175.
[12] M.A. Hildebrandt, W. Tan, P. Tamboli, M. Huang, Y. Ye, J. Lin, J.-S. Lee, C.G. Wood, X. Wu, Kinome expression profiling identifies IKBKE as a predictor of overall survival in clear cell renal cell carcinoma patients, Carcinogenesis 33 (4) (2012) 799–803.
[13] J.-P. Guo, S.-K. Shu, L. He, Y.-C. Lee, P.A. Kruk, S. Grenman, S.V. Nicosia, G. Mor, M.J. Schell, D. Coppola, Deregulation of IKBKE is associated with tumor progression, poor prognosis, and cisplatin resistance in ovarian cancer, Am. J. Pathol. 175 (1) (2009) 324–333.
[14] J.S. Boehm, J.J. Zhao, J. Yao, S.Y. Kim, R. Firestein, I.F. Dunn, S.K. Sjostrom, L.A. Garraway, S. Weremowicz, A.L. Richardson, Integrative genomic approaches identify IKBKE as a breast cancer oncogene, Cell 129 (6) (2007) 1065–1079.
[15] K. Verhelst, L. Verstrepen, I. Carpentier, R. Beyaert, IκB kinase ε (IKKε): a therapeutic target in inflammation and cancer, Biochem. Pharmacol. 85 (7) (2013) 873–880.
[16] J. Guo, D. Kim, J. Gao, C. Kurtyka, H. Chen, C. Yu, D. Wu, A. Mittal, A. Beg, S. Chellappan, IKBKE is induced by STAT3 and tobacco carcinogen and determines chemosensitivity in non-small cell lung cancer, Oncogene 32 (2) (2013) 151.
[17] C.V. Möser, M. Meissner, K. Laarmann, K. Olbrich, T.S. King-Himmelreich, M.C. Wolters, G. Geisslinger, E. Niederberger, The protein kinase IKKepsilon contributes to tumour growth and tumour pain in a melanoma model, Biochem. Pharmacol. 103 (2016) 64–73.
[18] W. Li, Y. Chen, J. Zhang, L. Hong, N. Yuan, X. Wang, H. Lv, IKBKE upregulation is positively associated with squamous cell carcinoma of the lung in vivo and malignant transformation of human bronchial epithelial cells in vitro, Med. Sci. Monit.: Int. Med. J. Exp. Clin. Res. 21 (2015) 1577.
[19] Y. Fan, M. Ye, L. Wu, S. Lv, M. Wu, B. Xiao, C. Liao, Q. Ji, Y. Chai, X. Zhu, Overexpression of miR-98 inhibits cell invasion in glioma cell lines via down-regulation of IKKε, Age (years) 50 (58.5) (2015).
[20] X. Xie, D. Zhang, B. Zhao, M.-K. Lu, M. You, G. Condorelli, C.-Y. Wang, K.-L. Guan, IκB kinase ε and TANK-binding kinase 1 activate AKT by direct phosphorylation, Proc. Natl. Acad. Sci. 108 (16) (2011) 6474–6479.
[21] K. Clark, L. Plater, M. Peggie, P. Cohen, Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IκB Kinase ϵ a distinct upstream kinase mediates Ser-172 phosphorylation and activation, J. Biol. Chem. 284 (21) (2009) 14136–14146.
[22] K. Clark, M. Peggie, L. Plater, R.J. Sorcek, E.R. Young, J.B. Madwed, J. Hough, E.G. McIver, P. Cohen, Novel cross-talk within the IKK family controls innate immunity, Biochem. J 434 (1) (2011) 93–104.
[23] K.J. Petherick, O.J. Conway, C. Mpamhanga, S.A. Osborne, A. Kamal, B. Saxty, I.G. Ganley, Pharmacological inhibition of ULK1 blocks mTOR-dependent autophagy, J. Biol. Chem. (2015) jbc. C114. 627778.
[24] S. Challa, J.-P. Guo, X. Ding, C.-X. Xu, Y. Li, D. Kim, M.A. Smith, D.W. Cress, D. Coppola, E.B. Haura, IKBKE is a substrate of EGFR and a therapeutic target in non-small cell lung cancer with activating mutations of EGFR, Cancer Res. 76 (15) (2016) 4418–4429.
[25] L. Yang, H.C. Dan, M. Sun, Q. Liu, X.-M. Sun, R.I. Feldman, A.D. Hamilton, M. Polokoff, S.V. Nicosia, M. Herlyn, Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt, Cancer Res. 64 (13) (2004) 4394–4399.
[26] M. Karin, Nuclear factor-κB in cancer development and progression, Nature 441 (7092) (2006) 431.
[27] I. Stein, R. Bramovitch, S. Amit, S. Kasem, E. Gutkovich-Pyest, S. Urieli-Shoval, E. Galun, Y. Ben-Neriah, NF-kappaB functions as a tumour promoter in inflammation-associated cancer, Nature 431 (7007) (2004) 461–466.
[28] H. Häcker, M. Karin, Regulation and function of IKK and IKK-related kinases, Sci. Stke 2006 (357) (2006) re13-re13.
[29] A. Israël, The IKK complex, a central regulator of NF-κB activation, Cold Spring Harbor Perspect. Biol. 2 (3) (2010) a000158.
[30] R.T. Peters, T. Maniatis, A new family of IKK-related kinases may function as IκB kinase kinases, Biochimica et Biophysica Acta (BBA) – Rev. Cancer 1471 (2) (2001) M57–M62.
[31] J. Zhang, M. Tian, Z. Xia, P. Feng, Roles of IκB kinase ε in the innate immune defense and beyond, Virologica Sinica 31 (6) (2016) 457–465.
[32] M.L. Chaisson, D.G. Branstetter, J.M. Derry, A.P. Armstrong, M.E. Tometsko, K. Takeda, S. Akira, W.C. Dougall, Osteoclast differentiation is impaired in the absence of inhibitor of κB kinase α, J. Biol. Chem. 279 (52) (2004) 54841–54848.
[33] T.U. Barbie, G. Alexe, A.R. Aref, S. Li, Z. Zhu, X. Zhang, Y. Imamura, T.C. Thai, Y. Huang, M. Bowden, Targeting an IKBKE cytokine network impairs triple-negative breast cancer growth, J. Clin. Investig. 124 (12) (2014) 5411–5423.
[34] R. Agami, All Roads Lead to IKKε, Cell 129 (6) (2007) 1043–1045.
[35] F. Fujita, Y. Taniguchi, T. Kato, Y. Narita, A. Furuya, T. Ogawa, H. Sakurai, T. Joh, M. Itoh, M. Delhase, Identification of NAP1, a regulatory subunit of IκB kinase-related kinases that potentiates NF-κB signaling, Mol. Cell. Biol. 23 (21) (2003) 7780–7793.
[36] H. Buss, A. Dörrie, M.L. Schmitz, E. Hoffmann, K. Resch, M. Kracht, Constitutive and interleukin-1-inducible phosphorylation of p65 NF-κB at serine 536 is mediated by multiple protein kinases including IκB kinase (IKK)-α, IKKβ, IKKϵ, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription, J. Biol. Chem. 279 (53) (2004) 55633–55643.
[37] H. Hemmi, O. Takeuchi, S. Sato, M. Yamamoto, T. Kaisho, H. Sanjo, T. Kawai, K. Hoshino, K. Takeda, S. Akira, The roles of two IκB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection, J. Exp. Med. 199 (12) (2004) 1641–1650.
[38] A. Chariot, A. Leonardi, J. Müller, M. Bonif, K. Brown, U. Siebenlist, Association of the adaptor TANK with the IκB kinase (IKK) regulator NEMO connects IKK complexes with IKKε and TBK1 kinases, J. Biol. Chem. 277 (40) (2002) 37029–37036.
[39] S. Sharma, N. Grandvaux, G.-P. Zhou, R. Lin, J. Hiscott, Triggering the interferon antiviral response through an IKK-related pathway, Science 300 (5622) (2003) 1148–1151.
[40] T. Wang, M.A. Block, S. Cowen, A.M. Davies, E. Devereaux, L. Gingipalli, J. Johannes, N.A. Larsen, Q. Su, J.A. Tucker, Discovery of azabenzimidazole derivatives as potent, selective inhibitors of TBK1/IKKε kinases, Bioorg. Med. Chem. Lett. 22 (5) (2012) 2063–2069.
[41] E.G. McIver, J. Bryans, K. Birchall, J. Chugh, T. Drake, S.J. Lewis, J. Osborne, E. Smiljanic-Hurley, W. Tsang, A. Kamal, Synthesis and structure–activity relationships of a novel series of pyrimidines as potent inhibitors of TBK1/IKKε kinases, Bioorg. Med. Chem. Lett. 22 (23) (2012) 7169–7173.
[42] J. Li, J. Huang, J.H. Jeong, S.J. Park, R. Wei, J. Peng, Z. Luo, Y.T. Chen, Y. Feng, J.L. Luo, Selective TBK1/IKKi dual inhibitors with anticancer potency, Int. J. Cancer 134 (8) (2014) 1972–1980.
[43] S.M. Reilly, S.-H. Chiang, S.J. Decker, L. Chang, M. Uhm, M.J. Larsen, J.R. Rubin, J. Mowers, N.M. White, I. Hochberg, An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice, Nat. Med. 19 (3) (2013) 313.