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A unique P-glycoprotein interacting agent displays anticancer activity against hepatocellular carcinoma through inhibition of GRP78 and mTOR pathways

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ABSTRACT

P-glycoprotein (P-gp) overexpression has been demonstrated in many malignancies being a predominant mechanism by which cancer cells develop multidrug resistance. Several categories of P-gp inhibitors have been demonstrated to potentiate anticancer effect induced by cancer chemotherapeutic drugs through competitive inhibition of P-gp pumping activity. Few studies show the agent that selectively acts on P-gp and, by itself, causes cell apoptosis while remain P-gp-deficient cells unaffected. KNG-I-322, a desmosdumotin B derivative, displayed a direct interaction with P-gp and demonstrated selective anti-proliferative and apoptotic activities in P-gp overexpressed Hep3B/VIN other than P-gp-deficient Hep3B cells. KNG-I-322 induced an inhibitory effect on the phosphorylation of $mTOR^{Ser2448}$, $p70S6K^{Thr389}$ and $4E\text{-}BP^{Thr37/46}$ in Hep3B/VIN but not Hep3B cells. The inhibition was fully blocked by the knockdown of P-gp using siRNA techniques. Notably, the P-gp inhibitor, verapamil, also directly interacted with P-gp but significantly diminished KNG-I-322-induced anti-proliferative activity. After the mechanism study, the data showed that KNG-I-322 induced a dramatic down-regulation of GRP78 expression, which was significantly inhibited by verapamil and completely diminished by the knockdown of P-gp. The protein profile analysis of detergent resistant membranes showed that upon the stimulation by KNG-I-322, the level of P-gp expression in non-raft fractions was dramatically increased and, concomitantly, the GRP78 expression was significantly decreased. Taken together, the data suggest that KNG-I-322 induces anticancer activity in Hep3B/VIN cells through a direct interaction with P-gp, leading to the inhibition of mTOR pathways and the induction of GRP78 down-regulation. The data support that KNG-I-322 is a selective anticancer agent against P-gp-overexpressed other than P-gp-deficient cancer cells.

1. Introduction

P-glycoprotein (P-gp), a drug transporter, belongs to a member of the ATP-binding cassette (ABC) transporter superfamily that confers efflux of its substrates from intracellular into extracellular fluid, leading to a decrease of intracellular substrate concentrations [1]. P-gp, which expresses in numerous tissues such as liver, kidney and intestine, is responsible for the distribution and elimination of its substrates. However, P-gp overexpression has been demonstrated in a lot of malignancies being a predominant mechanism by which cancer cells develop multidrug resistance [2]. A lot of cancer chemotherapeutic drugs, such as paclitaxel, vincristine and mitoxantrone, are P-gp substrates and are capable of inducing overproduction of P-gp that is responsible for maintaining drug concentration below cytotoxic levels in cancer cells [3,4]. Furthermore, ionizing radiation can also stimulate P-gp overproduction in cancer cells, leading to chemoresistance to a variety of anticancer drugs [5,6]. Because of the resulted obstacle to the efficacy of anticancer treatment, P-gp has become a therapeutic target for evading multidrug resistance (MDR). A feasible approach to overcoming MDR is the blockade of the transporter pumping activity and, accordingly, several inhibitors/modulators have been developed [7].

Flavonoids are a family of polyphenolic phytochemicals including flavones and isoflavones. A large body of evidence shows that diet high in flavonoids is associated with reduced incidence of cancers [8,9]. Flavonoids are extensively studied to display anticancer activity through numerous signaling pathways [8–10]. Besides, a lot of studies have reported that flavonoids form a category of P-gp inhibitors and enhance the bioavailability and activity of several anticancer drugs, such as paclitaxel and

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doxorubicin [11,12]. These studies highlight the potential of flavonoids for the development of cancer chemotherapeutic drugs based on the inhibition of P-gp activity. Recently, our colleagues have discovered that flavone desmosdumotin B showed little activity against human oral epidermoid carcinoma KB cells but, interestingly, exhibited an effective anticancer activity against a subline of parental KB cells after selection with vincristine (KB-VIN, P-gp overexpression) [13]. The mechanism has yet to be studied. KNG-I-322 is a desmosdumotin B derivative, displaying higher anticancer activity and selectivity against KB-VIN versus KB cells [13]. In this study, we selected and cultured a subline of hepatocellular carcinoma Hep3B cells, designating Hep3B-VIN which over-expressed P-gp. KNG-I-322 also showed effective anticancer activity against Hep3B-VIN other than parental Hep3B cells. Several pharmacological and biochemical assessments have been carried out to characterize the anticancer mechanism of KNG-I-322. After the study, we found that KNG-I-322 was a unique agent that acted on P-gp and down-regulated anti-apoptotic protein, such as glucose-regulated protein 78 (GRP78), and inhibited the mammalian target of rapamycin (mTOR) signaling pathways in P-gp-overexpressing hepatocellular carcinoma cells. To our knowledge, this is the first study demonstrating that a flavone targets P-gp and regulates GRP78 and mTOR-mediated function.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and all other cell culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antibodies to P-gp, GRP78, Ki-67, GAPDH, heat shock protein-27 (HSP27), HSP70, HSP90 and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to mTOR, phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389}, phospho-4E-BP1$^{Thr37/Thr46}$, caspase-3 and α-tubulin were from Cell Signaling Technologies (Boston, MA). Paclitaxel, vincristine, camptothecin, sulforhodamine B (SRB), propidium iodide (PI), phenylmethylsulfonylfluoride (PMSF), leupeptin, dithiothreitol and rhodamine 123 were obtained from Sigma–Aldrich (St. Louis, MO). The synthetic procedure and identification of KNG-I-322 was published elsewhere [13].

2.2. Cell lines and cell culture

The cancer cell line Hep3B was purchased from American Type Culture Collection (Rockville, MD). Hep3B/VIN was obtained from Hep3B, where overexpression of P-gp was induced by growing cells in the presence of vincristine. The cells were cultured in RPMI 1640 medium with 10% FBS (v/v) and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. SRB assays

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of compound addition (T_0). After additional incubation of dimethyl sulfoxide (DMSO) or the compound for 48 h, cells were fixed with 10% TCA and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of the compound (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $100 - [(T_x - T_0)/(C - T_0)] \times 100$. Growth inhibition of 50% (IC_{50}) is determined at the compound concentration which results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. Rhodamine efflux

The uptake was measured after a 30-min incubation with 5 μM rhodamine 123 at 37 °C. Efflux was determined after a 90-min incubation at 37 °C in the absence or presence of the indicated compound. Flow cytometric analysis (Becton Dickinson FACS Calibur, NJ, USA) was performed after two further washes.

2.5. Measurement of P-gp ATPase activity

The luminescent P-gp ATPase assay kit (Promega Corporation, WI, USA) was used to detect the effects of compounds on recombinant human P-gp in a cell membrane fraction. The assay was based on ATP-dependent light-generating reaction of firefly luciferase. The assay was performed according to the supplier. ATP was incubated with P-gp in the absence or presence of the compound. The P-gp ATPase reaction was stopped, and the remaining unmetabolized ATP was detected as a luciferase-generated luminescent signal. The decreased luminescence of reactions treated with 100 μM verapamil relative to 100 μM Na_3VO_4-treated reactions represents 100% of the stimulated P-gp ATPase activity.

2.6. Western blotting

After the treatment, cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml of lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF and 100 μM sodium orthovanadate. In some experiments, mitochondrial/cytosol fractionation kit (Biovision, Mountain View, CA) was used to separate mitochondrial and cytosolic fraction. Total protein was quantified, mixed with sample buffer and boiled at 90 °C for 5 min. Equal amount of protein (30 μg) was separated by electrophoresis in 8 or 12% SDS–PAGE, transferred to PVDF membranes and detected with specific antibodies. The immunoreactive proteins after incubation with appropriately labeled secondary antibody were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.7. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted (20 μg). The PCR primers pairs used for genes amplification were demonstrated in Table 1. After denatur-

Table 1
Primers used for RT-PCR experiments.

Gene	Primers	Base pair
MDR1	Fw 5′-CTCATCGTTTGTCTACAGTTC-3′ Rev 5′-GCTTTCTGTCTTGGGCTTGAGATCCACG-3′	288 bp
MRP1	Fw 5′-CATGAAGGCCATCGGACTCT-3′ Rev 5′-CAGGTCCACGTGCAGACA-3′	259 bp
MRP2	Fw 5′-GACTATGGGCTGATATCCAGTGT-3′ Rev 5′-AGGCACTCCAGAAATGTGCT-3′	490 bp
GRP78	Fw 5′-GCCTGTATTTCTAGACCTGCC-3′ Rev 5′-TTCATCTTGCCAGCCAGTTG-3′	150 bp
GAPDH	Fw 5′-TCCTTGGAGGCCATGTGGGCCAT-3′ Rev 5′-TGATGACATCAAGAAGGTGGTGAAG-3′	240 bp

ation at 94 °C for 2 min, PCR was performed in a Robocycler Gradient 96 (Stratagene, La Jolla, CA) for 30 cycles. Each reaction cycle includes denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were analyzed on 1.5% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA), and visualized in the presence of 1 μg/ml ethidium bromide staining using BioDoc-It Imaging System (UVP, Upland, CA, USA).

2.8. Small interfering RNA (siRNA) transfection

All of the MDR1 siRNA duplexes were designed and synthesized according to the published study [14]. For transfection, PC-3 cells were seeded into 60-mm tissue culture dishes with 30% confluence and grown for 24 h to 50–60% confluence. Each dish was washed with serum-free Opti-MEM (Life Technologies, Ground Island, NY), and 2 ml of the same medium was added. Aliquots containing siRNA in serum-free Opti-MEM were transfected into cells using Lipofectamine 2000 following the manufacturer's instructions. After incubation for 6 h at 37 °C, cells were washed with medium and incubated in 10% FBS-containing RPMI-1640 medium for 48 h. Then, the cells were treated with or without KNG-I-322 for the indicated time and the subsequent experiments were performed.

2.9. Sucrose density gradients

The detergent-resistant membranes were isolated using lysis conditions and centrifugation on discontinuous sucrose gradients [15]. Briefly, the cells were washed with ice-cold PBS and lysed for 30 min with 1% Triton X-100 in TNE buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1 mM PMSF. Cells were homogenized and centrifuged (200 × g, 8 min). The nuclei and cellular debris were pelleted and the supernatant (850 μl) was mixed with 850 μl 10% (w/v) sucrose in TNE buffer. Sucrose (1700 μl, 40%) in TNE buffer was loaded to the bottom of a Beckman 13 mm × 51 mm centrifuge tube and 1700 μl 35% (w/v) sucrose in TNE buffer was overlaid. The diluted lysate was then transferred on the top of the 35% (w/v) sucrose in TNE buffer. After centrifugation (257,000 × g, 18 h at 4 °C) the fractions (each of 400 μl) were collected from the top gradient. The volume of 20 μl of each fraction was subjected to Western blot analysis.

2.10. Data analysis

The compound was dissolved in DMSO. The final concentration of DMSO was 0.1% in cell culture media. Data are presented as the

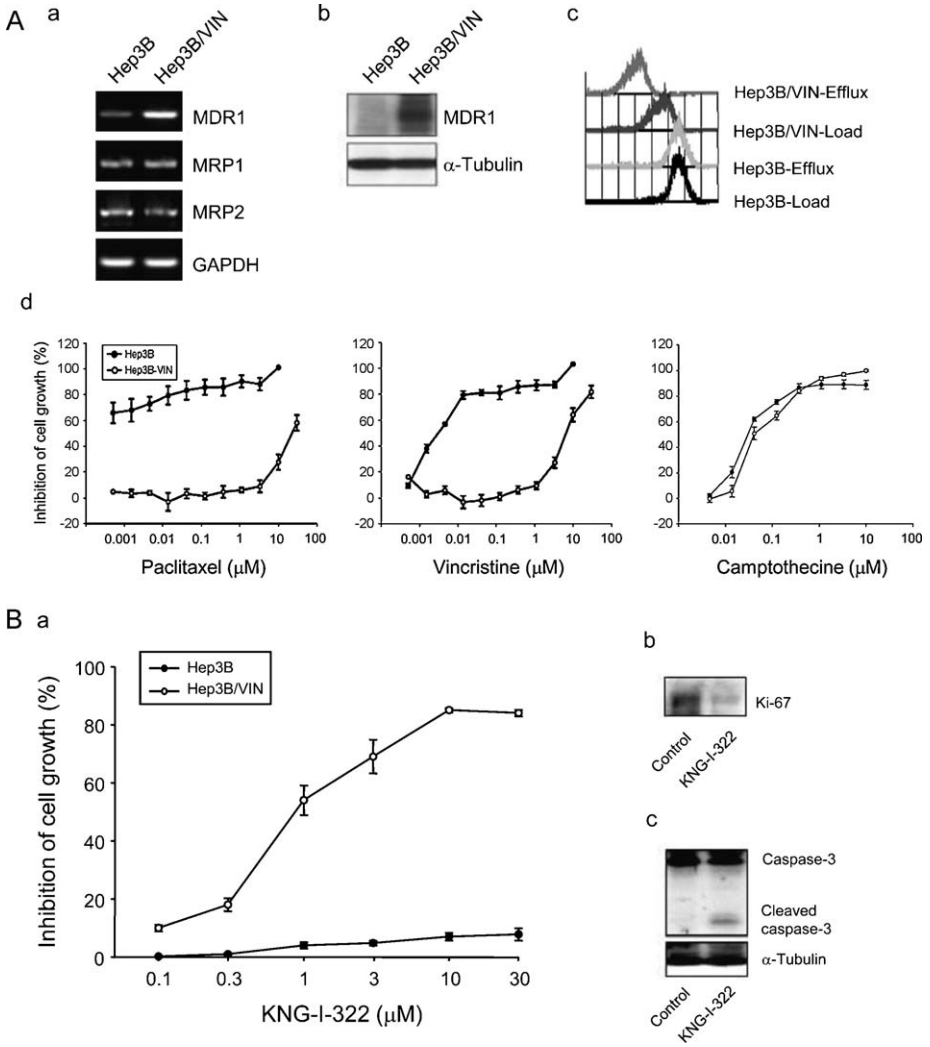

Fig. 1. Identification of drug-resistant cells and examination of anticancer activity. (A) The MDR1 expression was examined by RT-PCR (a) and Western blot analysis (b). The pumping activity was determined by rhodamine 123 efflux assays (c). The anti-proliferative activity of several drugs in a 48-h treatment was tested by SRB assays (d). (B) KNG-I-322 was exposed to Hep3B (a) or Hep3B/VIN cells (a, b and c) for 48 h. The anti-proliferative activity was examined by SRB assays (a) or the cells were harvested for the identification of indicated proteins by Western blot analysis (b and c). Data are expressed as mean ± SEM of four individual experiments.

mean ± SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance followed by a *t*-test and *p*-values less than 0.05 were considered significant.

3. Results

3.1. Identification of resistant cell line and anticancer activity

The human MDR-1 gene encoding for MDR transporter (P-gp) is often associated with reduced intracellular drug concentration in cancer cells caused by increased drug efflux. Both RT-PCR and Western blot analysis showed that vincristine-resistant Hep3B/VIN cells over-expressed P-gp but not the other multidrug resistance proteins MRP1 and MRP2 (Fig. 1A). The P-gp efflux activity was confirmed by rhodamine 123 efflux assay. Besides, the P-gp-mediated drug resistance was validated by the dramatic decrease of anti-proliferative activity of paclitaxel and vincristine (two P-gp substrates) but not camptothecin (Fig. 1A). Interestingly, KNG-I-322 did not show any activity in Hep3B cells but displayed concentration-dependent inhibition of cell proliferation with an IC_{50} of 0.8 μM in Hep3B/VIN cells. The inhibition of Ki-67 expression, a cellular marker of proliferation, and induction of caspase-3 activity in Hep3B/VIN cells confirmed the anti-proliferative and apoptotic activity of KNG-I-322 (Fig. 1B).

3.2. Effect of KNG-I-322 on the expression and activity of P-gp

Since KNG-I-322 selectively displayed anti-proliferative activity in Hep3B/VIN cells, the effect on the expression and efflux pump activity of P-gp was examined accordingly. The data demonstrated that KNG-I-322 did not change the expression level and activity of P-gp (Fig. 2A and B). In contrast, verapamil (a P-gp inhibitor) inhibited efflux pump activity of P-gp in a concentration-dependent fashion (Fig. 2B). Verapamil has been well identified to directly bind to P-gp on specific sites and is a potent stimulator of P-gp ATPase activity [16]. The data in Fig. 2C demonstrated that verapamil induced ATPase activity of P-gp from membrane preparation. Of note, KNG-I-322 showed similar activity to verapamil. However, genistein (a flavone as a negative control) at similar concentration level did not stimulate any P-gp ATPase activity (Fig. 2C). It revealed that KNG-I-322 displayed a direct interaction with P-gp but did not block efflux pump activity of P-gp.

3.3. Effect of KNG-I-322 on mTOR-mediated translational pathways

The mTOR controls cell growth in response to amino acids and growth factors. Recent studies reveal that the arrest of cell-cycle caused by numerous cellular stresses or ATP depletion is associated with the inhibition of mTOR-signaling pathways [17,18]. To test if the blockade of mTOR pathways involved in KNG-I-322-mediated anti-proliferative mechanism, the phosphorylation state of several target proteins was examined. The data showed that KNG-I-322 exhibited little effect in Hep3B cells. In contrast, it induced an inhibitory effect on the phosphorylation of $mTOR^{Ser2448}$, $p70S6K^{Thr389}$ and $4E\text{-}BP^{Thr37/46}$ in a time-dependent manner (Fig. 3). It was consistent with the data that KNG-I-322 specifically induced anticancer activity against Hep3B/VIN other than Hep3B cells.

3.4. Identification of the critical role of P-gp

To substantiate the critical role of P-gp in KNG-I-322-mediated signaling pathways, the knockdown of P-gp was carried out by the transfection of Hep3B/VIN cells with P-gp siRNA. The efficiency of the transfection was more than 90%. As a result, KNG-I-322-mediated inhibition of cell proliferation was significantly blocked (Fig. 4A). Furthermore, the level of KNG-I-322-induced inhibition of phosphorylation of $p70S6K^{Thr389}$ and $4E\text{-}BP^{Thr37/46}$ was dramatically rescued (Fig. 4B). The data suggest that P-gp plays a central role on KNG-I-322-mediated signaling pathways in Hep3B/VIN cells.

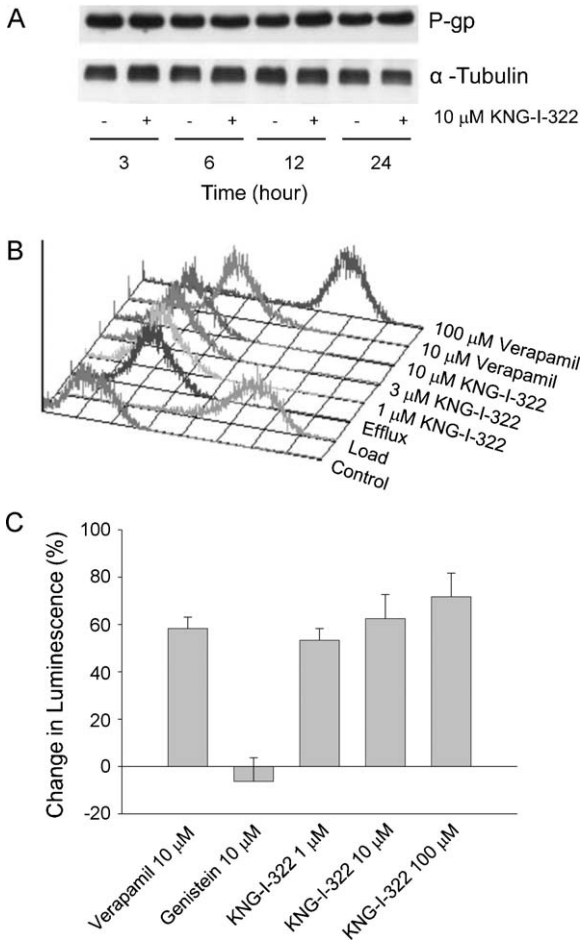

Fig. 2. Effect of KNG-I-322 on the expression and activity of P-gp. Hep3B/VIN cells were incubated with KNG-I-322 for various times (A) or 90 min (B). P-gp expression and pumping activity were examined by Western blot analysis (A) and rhodamine efflux assays (B), respectively. Data are representative of three independent experiments. (C) P-gp ATPase was incubated with the compound for 10 min. The P-gp ATPase activity was determined as described in Section 2. Data are expressed as mean ± SEM of four individual experiments.

3.5. Effect of KNG-I-322 on GRP78 expression

GRP78, a predominant endoplasmic reticulum (ER) chaperone, promotes cell proliferation, survival and metastasis. It also induces resistance of cancer cells to a variety of cancer chemotherapies [19]. There are several lines of evidence suggesting that GRP78 plays a crucial role in the development of HBV-related hepatocarcinogenesis [20]. In this study, KNG-I-322 did not modify the protein expression of GRP78 in Hep3B cells but significantly decrease its protein level in Hep3B/VIN cells (Fig. 5A). A concentration-dependent study showed an IC_{50} value of 6.7 μM (Fig. 5B). However, KNG-I-322 had little effect on mRNA expression of GRP78 in Hep3B/VIN cells (Fig. 5C), indicating the inhibition of translational other than transcriptional pathways to KNG-I-322

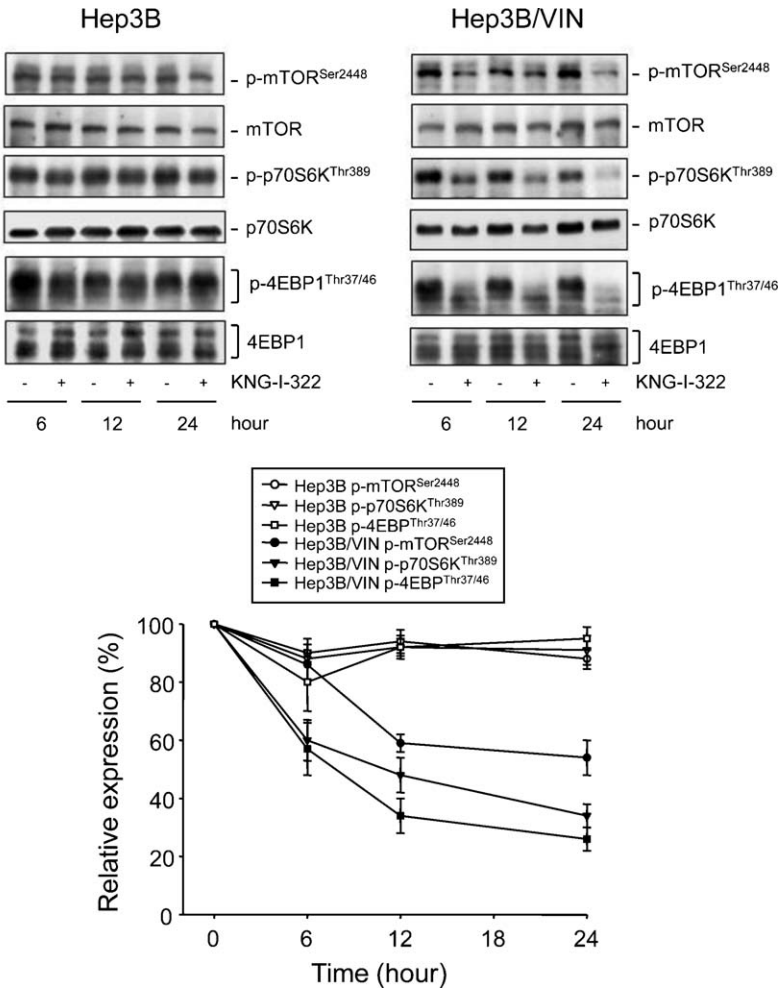

Fig. 3. Effect of KNG-I-322 on several protein expressions. Hsp3B/VIN cells were treated with KNG-I-322 (10 μM) for the indicated times. The proteins were detected by Western blot analysis and quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). Data are representative of three independent experiments.

action. Besides, the knockdown of P-gp almost completely rescued KNG-I-322-induced down-regulation of GRP78 expression (Fig. 5D), suggesting that GRP78 functioned as a downstream effector of P-gp in Hep3B/VIN cells.

3.6. Different regulation of verapamil and KNG-I-322 on GRP78 expression

In this study, verapamil and KNG-I-322 have been demonstrated to act directly on P-gp (Fig. 2C). Interestingly, verapamil significantly diminished KNG-I-322-induced anti-proliferative activity (Fig. 6A). The IC_{50} was shifted from 0.58 to 10.85 μM. Accordingly, the expressions of several heat shock proteins including HSP27, HSP70 and HSP90 as well as GRP78 were examined since these proteins have been extensively identified to be associated with cell proliferation and resistance to cancer chemotherapies [21]. As a consequence, the expressions of these molecular chaperones were not affected by verapamil and KNG-I-322 except for GRP78, which was down-regulated by KNG-I-322 while significantly rescued by verapamil in Hep3B/VIN cells (Fig. 6B).

3.7. Effect of KNG-I-322 on membrane distribution of P-gp and GRP78

The protein profile analysis of detergent resistant membranes was used to determine the effect of KNG-I-322 on the localization of P-gp and GRP78. Hep3B/VIN cells were fractionated. The subcellular fractions along sucrose gradients showed that P-gp localized to fractions containing caveolin-1, a resident protein of lipid rafts. In contrast, GRP78 was distributed in both lipid raft and non-raft fractions (Fig. 7). Upon the stimulation of KNG-I-322, the level of P-gp expression in non-raft fractions was dramatically increased and, concomitantly, the GRP78 expression was significantly decreased (Fig. 7). However, the immunoprecipitation assay revealed that there was no direct association between P-gp and GRP78 (data not shown).

4. Discussion

Various cellular stresses, including glucose deprivation, the exposure to carcinogenic chemicals and fractionated X irradiation, and the treatment with anticancer drugs, have been suggested to induce the overproduction of P-gp in a wide variety of cancer types [1,2,22–25]. In this study, a long-term treatment of sub-lethal concentrations of vincristine resulted in the overproduction of P-gp other than MRP1 and MRP2 in Hep3B cells. The data support that P-gp is one of the most susceptible MDR proteins being induced under cellular stress in hepatoma cells.

The activity of P-gp can be determined by its membrane ATPase assay. Both stimulation and inhibition of P-gp ATPase activity are suggested to show an interaction with P-gp.

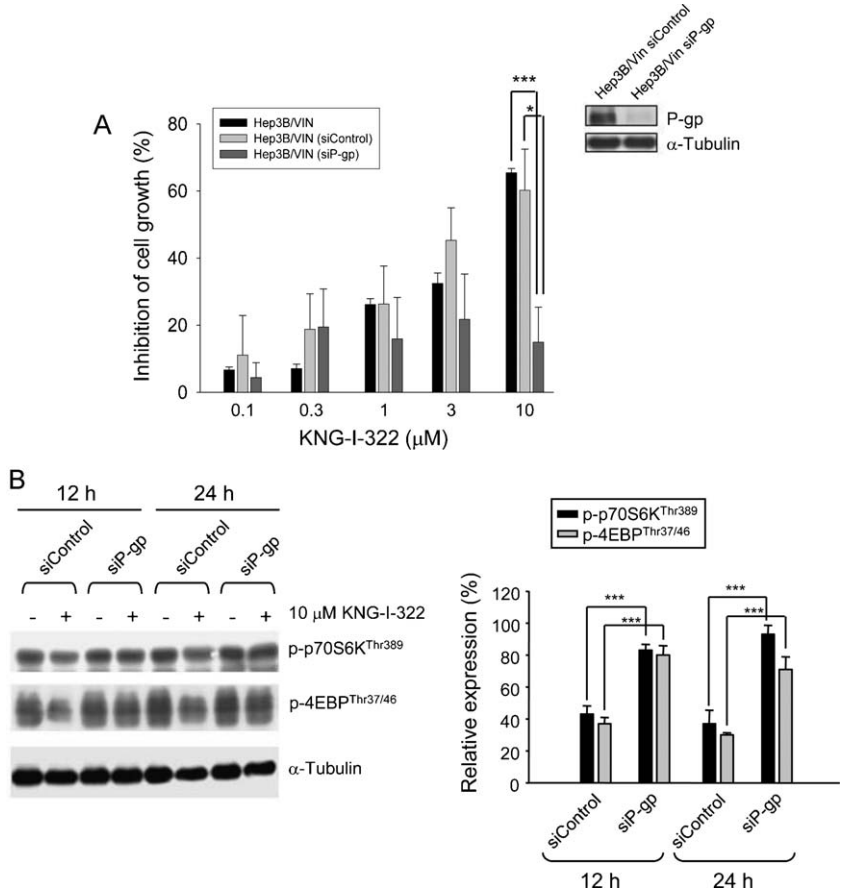

Fig. 4. Identification of the function role of P-gp. Hep3B/VIN cells were transfected with or without P-gp siRNA. The cells were treated with KNG-I-322 for 48 h (A) or the indicated times (B). The anti-proliferative activity was examined by SRB assays (A) or the cells were harvested for the identification of indicated proteins by Western blot analysis (B). Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the respective control.

Accumulating evidence shows that several drugs of P-gp substrates induced different degrees of stimulation of P-gp ATPase activity. These substrates will also competitively hinder the efflux of other P-gp substrates. In contrast, blockers of ATPase activity may not be P-gp substrates but may inhibit transport of other compounds [26,27]. The P-gp ATPase activity assay showed that KNG-I-322, similar to verapamil, effectively stimulated ATPase activity. However, the data from rhodamine 123 efflux assays revealed that KNG-I-322 did not compete with rhodamine 123 efflux. The data suggest that KNG-I-322 interacts with P-gp in an allosteric site that turns on the ATPase activity but the transporter cannot extrude this compound. The critical role of P-gp on KNG-I-322-mediated anti-proliferative activity was substantiated with the knockdown of P-gp expression by siRNA technique.

Collateral sensitivity to anticancer drugs in cell lines could be related to the altered level of critical protein expression. It has been suggested that cisplatin-resistant human ovarian adenocarcinoma IGROV-1 cells showed collateral sensitivity to paclitaxel conferred by p53 mutation in spite of varied expression of glutathione-dependent system [28,29]. Kotoh and the colleagues reported that the cisplatin-resistant bladder carcinoma T24 cells showed an increased level of topoisomerase I and developed a collateral sensitivity to camptothecin derivatives, which target topoisomerase I [30]. Moreover, numerous compounds have been identified to selectively kill MDR cancer cells indicating the central role of MDR proteins in producing collateral sensitivity [31]. These studies reveal that the collateral sensitivity may be attributed to a variety of factors. In this study, the direct interaction of KNG-I-322 with P-gp and the blockade of KNG-I-322-mediated effect in P-gp knockdown cells provided evidence supporting that P-gp plays a critical role in vincristine-resistant Hep3B cells showing collateral sensitivity.

Several hypotheses have been made for P-gp-involved collateral sensitivity. Karwatsky and the colleagues reported that apoptosis was preferentially induced in verapamil-treated P-gp expressing cells, an effect that was mediated by the production of reactive oxygen species (ROS) in response the high ATP demand by P-gp [32]. It has been speculated that metal ion interaction explains the anticancer activity of the P-gp-selective compounds [31]. It has been suggested that iron-chelating drugs may kill cancer cells through the deprivation of essential iron and blockade of the function of highly specific metal-enzyme targets. P-gp may increase the susceptibility of P-gp-expressing cells to this mechanism [31]. We speculated that KNG-I-322 was not an iron-chelating agent because of the lack of functional groups capable of binding metal ions. Besides, KNG-I-322 did not induce the production of ROS (data not shown) although displayed a direct interaction with P-gp. The mechanism of P-gp-involved collateral sensitivity to KNG-I-322 action has yet to be elucidated.

mTOR is a serine/threonine protein kinase that regulates cell growth by integrating nutrient- and growth factor-derived signals. Accumulating evidence shows that rapamycin (a specific mTOR inhibitor) effectively blocks the proliferation of hepato-

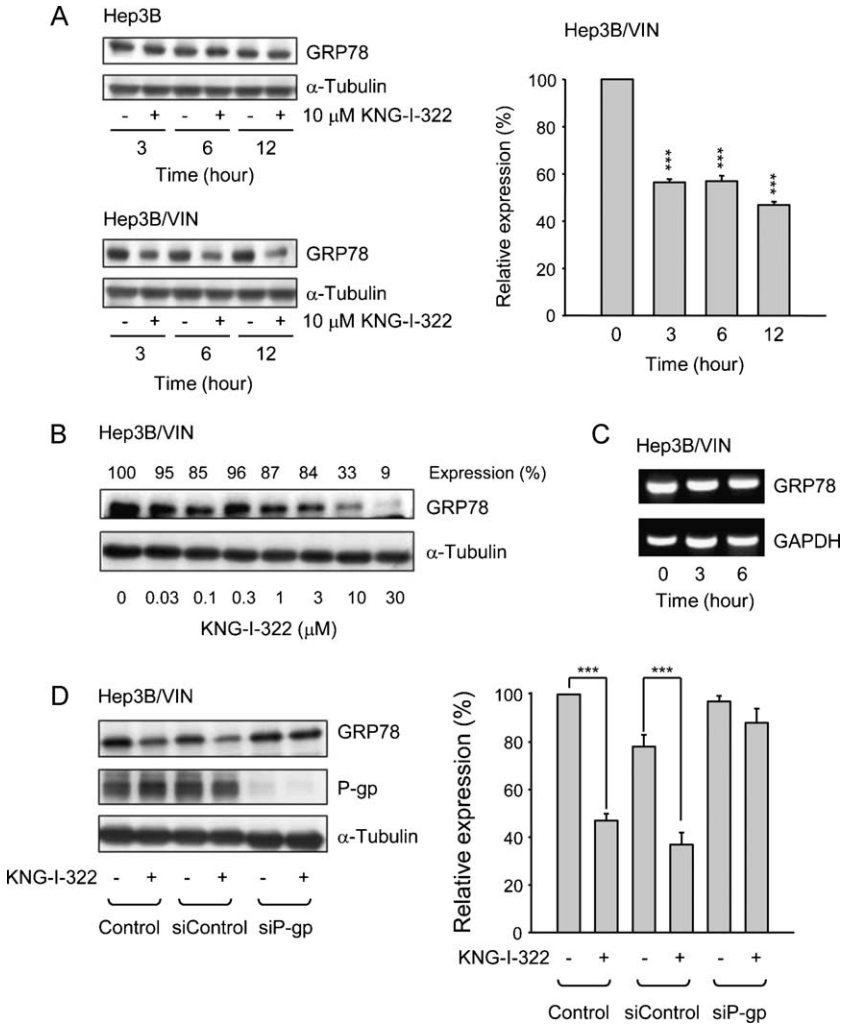

Fig. 5. Effect of KNG-I-322 on GRP78 expression and its regulation by P-gp. Cells were treated with KNG-I-322 for various times (A and C) or 6 h (B and D). The cells were harvested for the identification GRP78 expression in mRNA (C) or protein levels by RT-PCR and Western blot analysis, respectively. Data are quantified using the computerized image analysis system ImageQuant (Amersham Biosciences) and are expressed as mean ± SE of three independent experiments. ***$p < 0.001$ compared with the respective control.

cellular carcinoma (HCC) in both *in vitro* and *in vivo* models [33]. Besides, the stimulation of mTOR activity occurs in about 50% of patients with HCC [34]. Accordingly, mTOR is one of the major strategic targets for the development of anticancer agents, in particular the treatment against HCC. The data in this study provided evidence that the inhibition of mTOR pathways involved in KNG-I-322-mediated effect. mTOR served as a downstream effector of P-gp since the knockdown of P-gp expression significantly rescued mTOR-mediated signals. KNG-I-322 is also indicative of potential candidate in the mTOR strategy against HCC.

GRP78, belonging to HSP70 protein family, is a major ER chaperone that binds Ca^{2+} and serves as a central regulator under ER stress [19]. Accumulating evidence reveals that GRP78, a pro-survival component, is induced in a wide variety of cancer cells and malignant tissues [35]. Not only pro-survival property, GRP78 also promotes the invasion of cancer cells including HCC and induces resistance to a wide variety of therapies [19,36]. Accordingly, it has been suggested that combination therapy repressing GRP78 expression may be a potential approach in cancer chemotherapy. Recent discovery shows that GRP78 may locate at the cell surface of cancer cells other than in normal tissues [19]. Our data demonstrated that GRP78 was present in both lipid raft and non-raft portions of Hep3B/VIN cell surface. Because the knockdown of P-gp fully rescued the GRP78 expression, it has been suggested that P-gp may control the fate of GRP78, but in an indirect way because there is a lack of direct interaction between these two proteins. The distinct regulation of GRP78 expression between verapamil and KNG-I-322, at least in part, explained that verapamil reduced KNG-I-322-induced anti-proliferative activity in Hep3B/VIN cells. Notably, verapamil by itself induced a moderate increase of GRP78 expression. It might be explained by the induction of ER stress [37]. Numerous flavonoids have been studied to be capable of inducing ER stress and promoting cell survival [38]. For the anticancer strategy, the induction of GRP78 expression may blunt the anticancer efficacy. KNG-I-322 is in the right direction to suppress GRP78 protein level and to display anticancer activity.

In conclusion, the data suggest that KNG-I-322 selectively displays anticancer activity against Hep3B/VIN cells through P-gp-dependent inhibition of mTOR translational pathways and down-regulation of GRP78 expression. Besides, KNG-I-322 did not modify the function of endogenous P-gp in cells derived from normal canine kidney tissue. The data support that KNG-I-322 is a potential compound for further development.

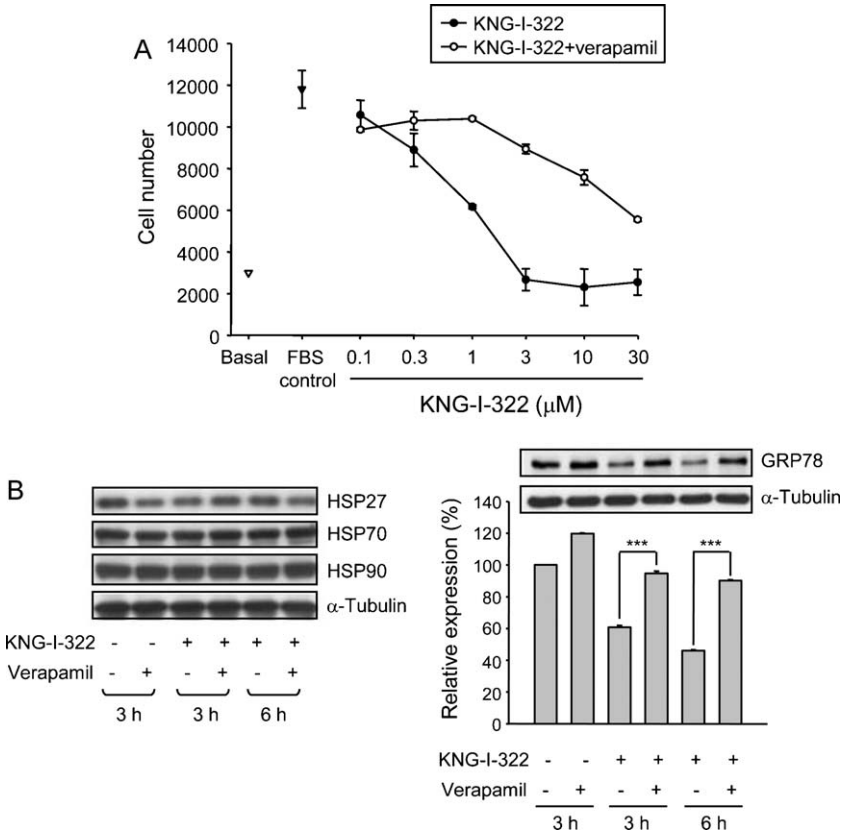

Fig. 6. Effect of verapamil on KNG-I-322-mediated activity. Hep3B/VIN cells were exposed to the indicated compound for 24 h (A) or the indicated times (B). After treatment, the cell proliferation was examined by SRB assays (A) or the cells were harvested for the identification of indicated proteins by Western blot analysis (B). Data are quantified using the computerized image analysis system ImageQuant (Amersham Biosciences) and are expressed as mean ± SE of three independent experiments. $^{***}p < 0.001$ compared with the respective control.

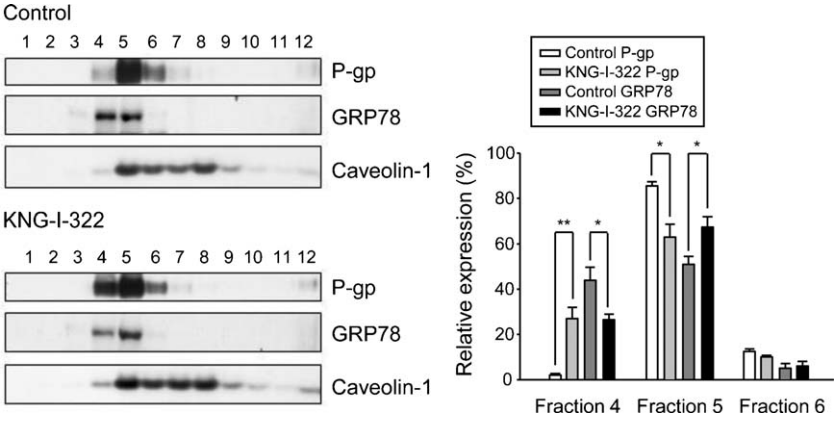

Fig. 7. Effect of KNG-I-322 on the distribution of several proteins. Hep3B/VIN cells were treated with KNG-I-322 for 6 h. After treatment, cells were lysed and fractionated by centrifugation. The proteins were detected by Western blot analysis of discontinuous sucrose gradients. Data are quantified using the computerized image analysis system ImageQuant (Amersham Biosciences) and are expressed as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.02.013.

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