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Inhibition of PI3K by ZSTK474 suppressed tumor growth not via apoptosis but G_0/G_1 arrest

Shingo Dan [a], Hisashi Yoshimi [a,b], Mutsumi Okamura [a], Yumiko Mukai [a], Takao Yamori [a,*]

[a] Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan
[b] Research Laboratory, Zenyaku-Kogyo Co., Ltd., 2-23-7 Ohizumi-machi, Nerima-ku, Tokyo 178-0062, Japan

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ABSTRACT

Phosphoinositide 3-kinase (PI3K) is a potential target in cancer therapy. Inhibition of PI3K is believed to induce apoptosis. We recently developed a novel PI3K inhibitor ZSTK474 with antitumor efficacy. In this study, we have examined the underlying mode of action by which ZSTK474 exerts its antitumor efficacy. In vivo, ZSTK474 effectively inhibited the growth of human cancer xenografts. In parallel, ZSTK474 treatment suppressed the expression of phospho-Akt, suggesting effective PI3K inhibition, and also suppressed the expression of nuclear cyclin D1 and Ki67, both of which are hallmarks of proliferation. However, ZSTK474 treatment did not increase TUNEL-positive apoptotic cells. In vitro, ZSTK474 induced marked G_0/G_1 arrest, but did not increase the subdiploid cells or activate caspase, both of which are hallmarks of apoptosis. These results clearly indicated that inhibition of PI3K by ZSTK474 did not induce apoptosis but rather induced strong G_0/G_1 arrest, which might cause its efficacy in tumor cells.

Aberration of PI3K pathway is often found in cancer [1]. Among the genes coding for PI3Ks, PIK3CA has been reported to be frequently activated by gene amplification [2,3] and by somatic hotspot mutations [4]. Akt, one of the most important downstream effectors of PI3K, activates cell proliferation via activation of mTOR/p70S6kinase [1]. Akt also cancels out the action of the cellular apoptotic machinery by inactivating pro-apoptotic proteins BAD and caspase-9 via phosphorylation [5,6]. These results, taken together, suggest that the PI3K pathway plays a significant role in the proliferation and survival of cancer cells. Therefore, targeting the PI3K pathway to inhibit proliferation and induce apoptosis in cancer cells has been thought to be a promising approach for developing novel anticancer therapies [7].

Among all the compounds identified to inhibit the PI3K activity, wortmannin and LY294002 are the first generation of PI3K inhibitors. Wortmannin was originally isolated from a fungus [8], and was shown to inhibit the kinase activity of PI3K irreversibly by covalently attaching to it [9]. On the other hand, LY294002, a derivative of multiple kinase inhibitor quercetin, reversibly inhibited PI3K [10]. These two compounds induced apoptosis in colorectal cancer [11], pancreatic cancer [12], and androgen-sensitive prostate cancer cells [13], raising the possibility of becoming anticancer drugs; however, the idea was soon dropped because of their liver- and skin-related toxicities [14,15]. Moreover, these compounds inhibit not only PI3K but also other protein kinases such as mTOR, DNA-PK and casein kinase 2 [16,17].

In our previous study, we reported a novel PI3K inhibitor ZSTK474 that showed potent antitumor activity against human cancer xenograft without severe toxicity [18]. ZSTK474 is an ATP-competitive inhibitor that inhibited PI3K activity >10-fold more strongly than LY294002, and unlike other PI3K inhibitors, did not inhibit mTOR, DNA-PK and other 139 protein kinases [18–21]. We also presented preliminary results showing that inhibition of PI3K by ZSKT474 induced rather weaker apoptosis in vitro than expected, and instead, it induced strong G_0/G_1 arrest of cell cycle [18]. Here, we have examined in detail the underlying mode of action by which a pure PI3K inhibitor ZSTK474 exerted its antitumor activity, especially, involvement of apoptosis, against human cancer xenografts derived from different origin in vivo and in vitro.

Materials and methods

Cell lines and cell culture: Human cancer cell lines used in this study are: prostate cancer, PC-3; lung cancer, A549 and NCI-H226; glioblastoma, SNB-75; and colorectal cancer, KM-12 and HCT-116. All cell lines were grown in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd.) supplemented with 1 μg/mL kanamycin and 5% v/v heat-inactivated fetal bovine serum (Moregate Exports, Bulimba, Queensland, Australia). Cells were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

Drugs: ZSTK474 was provided by Zenyaku-Kogyo Co., Ltd. (Tokyo, Japan). LY294002 and doxorubicin were purchased from

* Corresponding author. Fax: +81 3 3570 0484.
E-mail address: yamori@jfcr.or.jp (T. Yamori).

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CalbioChem (San Diego, CA) and Kyowa Hakko Co., Ltd. (Tokyo, Japan), respectively. For in vitro studies, these compounds were dissolved in dimethyl sulfoxide (DMSO). ZSTK474 was suspended in 5% hydroxypropylmethylcellulose (HPMC) in water as a solid dispersion form for animal experiments.

Cell cycle analysis: Cells were harvested, washed with PBS and fixed in 70% ethanol. The fixed cells were washed with PBS and resuspended in 1 mg/ml ribonuclease A in PBS, followed by incubation at 37 °C for 30 min. Cells were stained using PI solution (50 mg/ml propidium iodide, 0.1% sodium citrate, 0.1% Nonidet P-40) and were analyzed by using a Becton–Dickinson FACSCalibur flow-cytometer provided with the CellQuest software (Braintree, MA).

Animal experiments: Animal care and treatment was performed in accordance with the guidelines of the Animal Use and Care Committee of the Japanese Foundation for Cancer Research, and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice with BALB/c genetic backgrounds were purchased from Charles River Japan (Yokohama, Japan). Mice were maintained under specific pathogen-free conditions and provided with sterile food and water ad libitum. Human tumor xenografts were generated by subcutaneously inoculating 3 mm × 3 mm × 3 mm tumor fragment of PC-3 or other six cell lines (A549, DMS273, KM-12, HCT-15, MKN1 and SK-OV3) to nude mice. On day 23 (early stage) and day 49 (advanced stage) from the date of tumor inoculation, ZSTK474 was orally administered at 400 mg/kg of body weight following the schedule indicated below. For other six cell lines, daily administration of ZSTK474 (from day 0 to day 14, except days 3 and 10) was started when tumor size reached 100–300 mm^3. Length (L) and width (W) of the subcutaneous tumor mass were measured by calipers in live mice, and the tumor volume (TV) was calculated as: $TV = (L \times W^2)/2$. Antitumor activity was calculated as: $T/C\ (\%) = TV_D/TV_C \times 100$, where TV_D is the tumor volume after administration of ZSTK474 and TV_C is the tumor volume of respective control. To assess toxicity, we measured the body weight of the tumor-bearing mice. Mice were finally sacrificed and tumors were excised and frozen in liquid N_2 for further examination.

Immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL): For immunohistochemistry and TUNEL analysis, tumor tissues were fixed in 10% neutral formalin and embedded in paraffin. Tissue sections (4 μm) were deparaffinized in xylene and then in a series of (100% to 50%) ethanol solutions. Immunohistochemistry-specific antibodies against human Ki67 antigen (DAKO cytomation A/S, Glostrup, Denmark), phospho-Akt (Ser-473) (Cell Signalling Technologies, Danvers, MA), cyclin D1 (Abcam, Cambridge, MA) were used for hybridization and the bound antibodies were visualized by using DAKO EnVision kit containing a secondary horseradish peroxidase (HRP) conjugated anti-mouse polymer antibody complex. Sections were counterstained with Mayer's hematoxylin. Analysis of apoptotic cell death by TUNEL was performed using the Apoptosis in situ Detection Kit (Wako chemicals, Osaka, Japan) according to the manufacturer's instruction.

Preparation of nuclear extract and cytosolic extract: Nuclear and cytosolic fractions were prepared using an NE-PER kit (Pierce, IL) according to the manufacturer's instructions. Concentrations of proteins in the nuclear and cytosolic extracts were determined by using a Protein Assay Kit (Pierce, IL).

Immunoblot analysis: Equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the separated proteins were transferred onto Immobilon P PVDF membrane (Millipore, Billerica, MA). The membrane was incubated with a primary antibody as indicated in each experiment. The antibodies for Akt, phospho-Akt (Ser-473), phospho-pRB (Ser-780 and Ser-807/Ser811) were purchased from Cell Signaling Technologies. The antibodies for pRB, cyclin D1 and p27kip1 were purchased from BD Biosciences Pharmingen (San Jose, CA), Biosource (Camarillo, CA) and BD Transduction Laboratories (San Jose, CA), respectively. The bound antibody was detected using an appropriate anti-mouse or rabbit immunoglobulin secondary antibody and the ECL Plus™ Western Blotting Detection Systems (GE Healthcare Biosciences KK, Tokyo, Japan).

Measurement of caspase activation: Ten micrograms of cytosolic fraction from each sample was mixed in 50 μl of ICE buffer containing 20 mM HEPES–NaOH (pH 7.5), 2 mM dithiothreitol, 10% v/v glycerol and 1 mM DEVD–MCA (Peptide Institute, Osaka, Japan) as a specific substrate for caspase-3. The reaction mixture was incubated at 37 °C for 30 min and increase in the AMC fluorescence was measured (λ_{ex} = 380 nm, λ_{em} = 460 nm). Data are the mean of the results performed in triplicate.

Results

Inhibition of in vivo tumor proliferation by ZSTK474 without inducing apoptosis

To examine the effect of ZSTK474 on in vivo tumor growth, ZSTK474 was orally administered to nude mice bearing xenografted tumor derived from subcutaneously implanted human prostate cancer PC-3 cells. As we reported previously [18], ZSTK474 effectively suppressed the in vivo tumor growth, without inducing extensive body weight loss (Fig. 1A–C). Surprisingly, the inhibitory effect of ZSTK474 was observed even when the tumors were in the advanced stages. Immunohistochemical analysis revealed that ZSTK474 down-regulated the expression of phosphorylated Akt (Ser-473), suggesting that PI3K was certainly inhibited in vivo. Moreover, administration of ZSTK474 decreased the expression of nuclear proteins cyclin D1 and Ki67, both of which are hallmarks of proliferation, but did not cause any increase in the TUNEL-positive apoptotic cells in the tumor tissue (Fig. 1D). Suppression of tumor growth without inducing apoptosis was also observed in xenografts derived from lung cancer (A549 and DMS273), colorectal cancer (KM-12 and HCT-15), gastric cancer (MKN1), and ovarian cancer (SK-OV3) (Table 1 and Supplemental Fig. 1). The present results clearly suggested that ZSTK474 exerted its potent antitumor activity in vivo by inhibiting proliferation, and not by apoptosis.

Induction of G_0/G_1 phase cell cycle arrest, but not apoptosis, by ZSTK474 in vitro

Next, we examined the effect of ZSTK474 on cell cycle distribution and induction of apoptosis in vitro. As shown in Fig. 2A, ZSTK474 induced accumulation of diploid cells (cells in G_0/G_1 phase) but extensive induction of subdiploid cells was not observed in prostate cancer (PC-3), lung cancer (A549) and colorectal cancer (KM-12) cells (Fig. 2A). Similar results were obtained in other cell lines derived from lung cancer (NCI-H226), glioblastoma (SNB-75) and colorectal cancer (HCT-116) (Supplemental Fig. 2). We further examined whether ZSTK474 induces apoptotic caspase activation in cancer cells (Fig. 2B). As a result, activation of caspase was not observed when the cells were treated for 48 h with 10 μM of ZSTK474, a concentration that is ~100 times higher than its GI_{50} for the PC-3 cells (0.1 μM). Another PI3K inhibitor LY294002 did not induce caspase activation. Similar results were obtained in five other cancer cell lines examined (Fig. 2B and Supplemental Fig. 2B). These results indicated that ZSTK474 is a potent inducer of G_0/G_1 arrest, but not of apoptosis, in cancer cells both in vitro and in vivo.

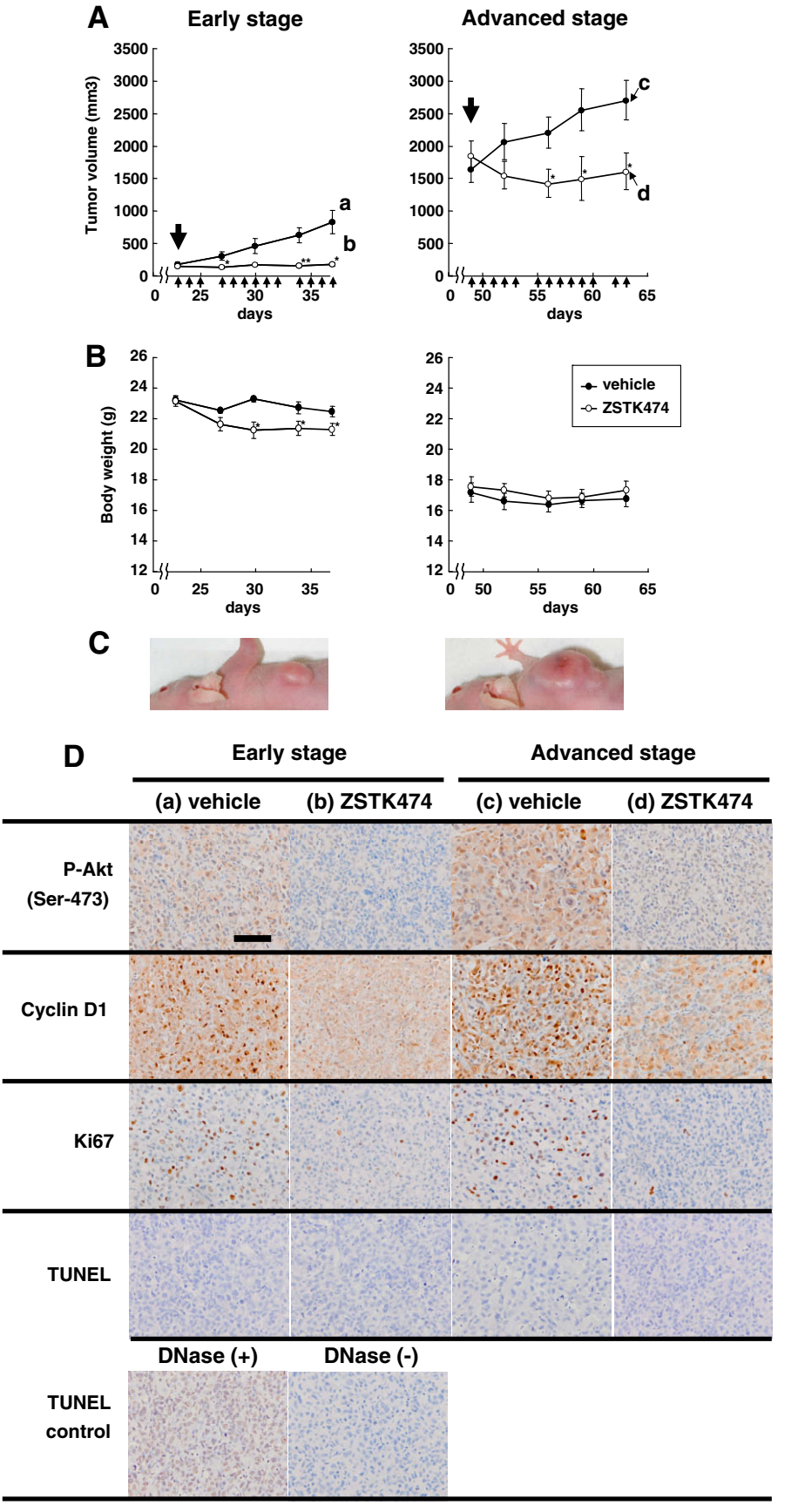
A
Early stage
Advanced stage
Tumor volume (mm3)
3500
3000
2500
2000
1500
1000
500
0
a
b
c
d
0 25 30 35
0 50 55 60 65
days
B
Body weight (g)
26
24
22
20
18
16
14
12
vehicle
ZSTK474
C
D
Early stage
Advanced stage
(a) vehicle
(b) ZSTK474
(c) vehicle
(d) ZSTK474
P-Akt (Ser-473)
Cyclin D1
Ki67
TUNEL
DNase (+)
DNase (-)
TUNEL control

Table 1
Antitumor activity and apoptosis induction in human cancer xenografts after the administration of ZSTK474.

Cells	Induction of TUNEL-positive cells	*T/C* (%)
A549	–	25.5
DMS273	–	19.6
KM-12	–	48.2
HCT-15	–	47.5
MKN1	–	44.8
SK-OV3	–	41.3
PC-3	–	24.9

Effects of ZSTK474 on cell cycle-related proteins

We next examined the effect of ZSTK474 on cell cycle-related proteins by immunoblot analysis (Fig. 3). In accord with our previous study using A549 cells [18], the expression of phospho-Akt (Ser-473) in PC-3 cells was completely suppressed within 1 h after exposure to 0.3 μM ZSTK474, and it remained suppressed to at least 48 h. Similar to the results of in vivo immunohistochemistry (Fig. 1D), ZSTK474 treatment reduced the relative amount of cyclin D1 in the nucleus, but not in the cytoplasm. Moreover, a CDK inhibitor, p27kip1, was significantly induced by ZSTK474. In parallel, relative amounts of the phosphorylated pRB also declined. Taken together, these results suggested that inactivation of cyclin D1 and induction of p27kip1 were involved in dephosphorylation of pRB and the G_0/G_1 arrest of cell cycle.

Discussion

Inhibition of PI3K is believed to induce apoptosis in cancer cells, and induction of apoptosis in cancer cells has been believed to be an important property for a good anticancer drug [22,23]. However, in this study, we found that ZSTK474 exerted strong antitumor activity via G_0/G_1 arrest of cell cycle, but not by apoptosis. The in vivo and in vitro results described here suggested that ZSTK474 is a promising antitumor agent which can control tumor progression especially in patients with advanced stage tumors.

It was thought that inhibition of PI3K would not only inhibit tumor growth, but would also cause apoptosis, since anti-apoptotic function of PI3K is believed to play one of the most important role in tumorigenesis [1,24]. Indeed, the PI3K inhibitor LY294002 triggered apoptosis in colorectal cancer [11], pancreatic cancer [12], and androgen-sensitive prostate cancer cells [13]. In this study, we however clearly demonstrated that ZSTK474 effectively suppressed the growth without inducing apoptosis both in vivo and

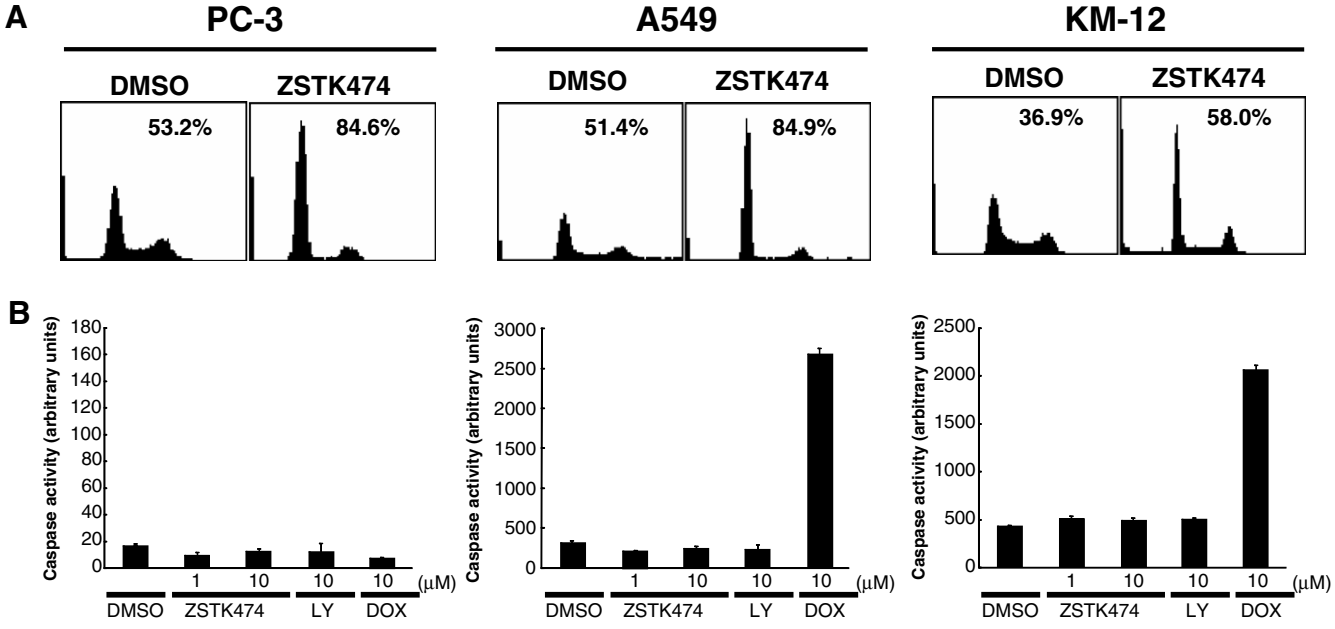

Fig. 2. Induction of G_0/G_1 cell cycle arrest, but not apoptosis, in human cancer cells after exposure to ZSTK474 in vitro. (A) Human cancer cell lines were exposed to ZSTK474 at 1 μM (PC-3), 2 μM (A549) or 3 μM (KM-12) for 24 h. Cells were harvested, fixed in 80% EtOH, and genomic DNA was stained with propidium iodide. Histograms of DNA content were obtained by using a Becton–Dickinson FACSCalibur. The number in each histogram is the percentage of diploid cells, i.e. cells in G_0/G_1 phase. (B) Estimation of apoptotic caspase activation by ZSTK474, LY294002 and doxorubicin. Cells were exposed to ZSTK474 (1 and 10 μM), LY294002 (LY, 10 μM) and doxorubicin (DOX, 10 μM) for 48 h and induction of apoptosis was examined by specific cleavage of DEVD tetrapeptide by caspase-3.

Fig. 1. Antitumor activity, suppression of phospho-Akt and cyclin D1 expression, and estimation of apoptosis induced after the administration of ZSTK474 to mice xenografted with tumor derived from the human prostate cancer PC-3 cells. (A) Antitumor activity of ZSTK474 on xenografted tumors derived from PC-3 cells. Twenty-four nude mice were subcutaneously inoculated with 3 mm × 3 mm × 3 mm tumor fragment of PC-3 cells on day 0. From day 23 (early stage) and day 49 (advanced stage) ZSTK474 was orally administered daily (six mice for each stage) at 400 mg/kg of body weight, except for days 26 and 33 (for early stage) and days 54 and 61 (for advanced stage), as indicated by arrows. Length and the width of each tumor were measured and the tumor volume was calculated as described in Materials and methods. Tumor growth was effectively suppressed by administration of ZSTK474, even when the tumor volumes were ~200 mm^3 (day 23), ~1000 mm^3 (day 40), and ~1700 (day 49) mm^3 in size. (B) Changes in the body weights of mice used in (A) above before and after the administration of ZSTK474. (C) Representative images of tumors on day 15 (early stage) and day 40 (advanced stage) prior to the administration of ZSTK474. (D) Immunohistochemistry and TUNEL assay after administration of ZSTK474. Mice were sacrificed 4 h after ZSTK474 administration on day 37 (early stage) and day 63 (advanced stage), and excised sections of tumors were analyzed by immunohistochemistry to assess the expression of Ser-473 phosphorylated Akt (P-Akt), cyclin D1 and Ki67. Relative expression levels of these proteins decreased after the administration of ZSKT474 in both stages. In addition, TUNEL assay was performed in order to determine the progression of apoptosis after the administration of ZSTK474. ZSTK474 did not induce apoptosis. Scale bar = 100 μm. Error bars = standard deviations. $^{*}P < 0.05$ and $^{**}P < 0.01$, two-sided t-test, compared with respective control.

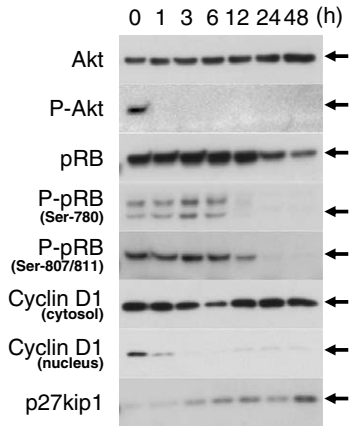

Fig. 3. Immunoblot analysis of PI3K-downstream factors and cell cycle-related proteins. PC-3 cells were treated with 0.3 μM ZSTK474 for the indicated periods. Expression of Akt and phospho-Akt (Ser-473) were determined in cytoplasmic fractions of each sample. Expression of cyclin D1 was determined in the cytoplasmic and nuclear fractions. Expressions of pRB, phospho-pRB (Ser-780 and Ser-807/811) and p27kip1 were determined in the nuclear extract. Decrease in expression of phospho-Akt was observed within 1 h of exposure to ZSTK474. Cyclin D1 levels in the nucleus also decreased within 1 h of exposure to the drug. Expression levels of phospho-pRB (Ser-780) and phospho-pRB (Ser-780 and Ser-807/811), both of which are specifically phosphorylated by cyclin D/CDK4 holoenzyme, decreased within 12 h of exposure to the drug, and finally disappeared. p27kip1, a CDK inhibitor, was significantly induced after exposure to the drug.

in vitro. In vivo, ZSTK474 inhibited the growth of xenografted tumors derived from the PC-3 cells, and the growth inhibition was accompanied by a reduction in the amount of nuclear cyclin D1 and Ki67, but no appearance of apoptosis. Suppression of tumor growth without inducing apoptosis was also observed in xenografts derived from additional six different human cancer cell lines. In vitro, ZSTK474 evenly induced G_0/G_1 arrest, and not apoptosis, in all of six human cancer cell lines examined. Moreover, we confirmed this in additional five cancer cell lines (data not shown). Among the six cell lines examined in this study, PC-3 is a PTEN-negative cell line [25] and HCT-116 has the H1047R gain-of-function hotspot mutation on the kinase domain of the PIK3CA gene [26]. Thus, these cancer cell lines underwent G_0/G_1 arrest but not apoptosis after exposure to ZSTK474 whether or not they had the functional PTEN gene or gain-of-function PI3K mutations. Moreover, induction of G_0/G_1 arrest, and not apoptosis, was observed even when the cells were treated with excess amount of ZSTK474 (Fig. 2B). Similar results were observed when the cells were treated with 10 μM of another PI3K inhibitor, LY294002, a concentration that was sufficient to dephosphorylate Akt [18]. Taken together, we concluded based on these results that the inhibition of PI3K activity by a specific PI3K inhibitor generally causes G_0/G_1 arrest of cell cycle, but not apoptosis, in cancer cells.

It was surprising that ZSTK474 exerted a strong antitumor activity in human cancer xenograft without inducing apoptosis (Figs. 1 and 2) because it is widely believed that induction of apoptosis is a pre-requisite for an agent to exert antitumor effect [22,23]. That ZSTK474 induced cell cycle arrest but not apoptosis, might contribute to its low toxicity in vivo. Indeed, ZSTK474 induced reversible dephosphorylation of Akt and did not induce apoptosis or critical defects in normal tissues such as kidney, lung, small intestine and skin (data not shown). These characteristics of ZSTK474 might be due to its high selectivity for PI3K and its ability to inhibit PI3K in ATP-competitive and reversible manner [18–21]. Another important point to note is that ZSTK474 effectively suppressed tumor growth even in advanced stage tumors (Fig. 1A), suggesting that ZSTK474 could be used clinically for controlling tumors in advanced stages.

In summary, we demonstrated in this study that ZSTK474 is a potent inducer of G_0/G_1 arrest, but not an inducer of apoptosis. The G_0/G_1 arrest was accompanied by a reduction of the nuclear cyclin D1 and Ki67, induction of p27kip1 and hypophosphorylation of pRB. Although the precise mechanism underlying the antitumor effect of ZSTK474 is not yet fully understood, these properties may contribute to its favorable efficacy with low toxicity, which makes a strong case in favor of ZSTK474 to become a promising anticancer drug.

Acknowledgments

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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.bbrc.2008.12.015.

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