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ZSTK474, a specific phosphatidylinositol 3-kinase inhibitor, induces G1 arrest of the cell cycle *in vivo*

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ABSTRACT

Phosphatidylinositol 3-kinase (PI3K) is regarded as a promising therapeutic target because it is often activated in cancer. We previously reported that ZSTK474, a specific PI3K inhibitor, inhibits tumour cell proliferation *via* G1 arrest of the cell cycle without inducing apoptosis *in vitro*. However, it remained unclear whether ZSTK474 induces G1 arrest to exert antitumour efficacy *in vivo*. We recently developed a live imaging system, named Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci), to visualise cell cycle distribution. Here, by using this system, we tested whether ZSTK474 induces G1 arrest in tumour cells *in vivo*, as well as *in vitro*. Fucci-introduced human breast cancer MCF-7 cells and cervical cancer HeLa cells were subcutaneously xenografted in nude mice. ZSTK474 was administered to the tumour-bearing mice for 5 days, and the cell cycle distribution in the xenografted tumours were analysed by monitoring fluorescence in live mice. We demonstrate that ZSTK474 induces G1arrest along with tumour suppression *in vivo*. Moreover, we show that ZSTK474 suppresses the tumour growth without inducing apoptosis. Interestingly, such increase in G1 cells and tumour suppression was maintained during long-term (3-month) administration of ZSTK474. These results suggest that ZSTK474 exerts its *in vivo* antitumour efficacy *via* G1 arrest but not *via* apoptosis as long as it is administered, and could be used for months as maintenance therapy for patients with advanced cancers.

1. Introduction

The PI3K pathway is frequently activated in cancer by a gain-of-function hotspot mutation of the *PIK3CA* gene[1,2] and loss of Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) expression,[3,4] thereby activating tumourigenesis and tumour growth. Thus, PI3K is thought to be a promising target for cancer therapy. We previously reported a selective PI3K

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inhibitor ZSTK474 with potent antitumour activity and low toxicity *in vivo*.[5–7] Subsequently, a number of PI3K inhibitors have been developed, and some, including ZSTK474 and NVP-BEZ235, have already entered clinical trials.[8–12]

Inhibition of the PI3K pathway was expected to induce apoptosis, cell cycle arrest and cell growth inhibition. However, in our previous study, we demonstrate that inhibition of PI3K by ZSTK474 induces strong G1 arrest of the cell cycle but scarcely any apoptosis *in vitro*.[6] Consistent with these findings, NVP-BEZ235, a dual PI3K/mammalian Target Of Rapamycin (mTOR) inhibitor, induced G1 arrest but not apoptosis *in vitro*.[13,14] Moreover, we demonstrated that tumour cells did not undergo apoptosis upon administration of ZSTK474 in tumour xenografts derived from various types of human cancers implanted in nude mice.[6] Therefore, the antitumour efficacy of PI3K inhibitors appears to be exerted by a cytostatic mechanism *via* G1 arrest. However, the studies with regard to G1 arrest by PI3K inhibitors were performed *in vitro*. Thus, it remains unclear whether PI3K inhibitors also induce G1 arrest *in vivo*.

We previously developed an imaging system, named Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci), to visualise cell cycle distribution in living cells by introducing fluorescent cell cycle-specific marker proteins using a lentiviral expression vector.[15] Here, we used the Fucci system to investigate cell cycle distribution after administration of ZSTK474 both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell lines and cell culture

Human breast cancer MCF-7 cells expressing mCherry-hCdt1 (30/120) and mAG-hGeminin (1/110) and cervical cancer HeLa cells expressing mKO-hCdt1 and mAG-hGeminin were described previously.[15] These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 5% v/v heat-inactivated foetal bovine serum (FBS) (Moregate Exports, Bulimba, Queensland, Australia) and 1 μg/mL kanamycin. Cells were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2. Authentication of cell lines was performed by short tandem repeat analysis using PowerPlex16 Systems (Promega, Madison, WI) (data not shown).

2.2. Drugs

ZSTK474 was kindly provided by Zenyaku Kogyo Co., Ltd. (Tokyo, Japan). NVP-BEZ235 and GDC-0941 were purchased from Selleck (Houston, TX) and Symansis (Timaru, New Zealand), respectively. Paclitaxel and cisplatin were purchased from Sigma–Aldrich (St. Louis, MO) and Nippon Kayaku (Tokyo, Japan), respectively.

2.3. Real-time visualisation of cell cycle progression using a fluorescence microscope

Fucci-introduced cells were seeded at 3.5×10^4 cells/well in a 12-well glass-bottomed plate, and incubated overnight in a CO_2 incubator. Cells were administered with the indicated drugs and cell cycle progression was successively monitored (every 15 min) using a IX81 fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Animal experiments and in vivo visualisation of the cell cycle

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 National Institutes of Health (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*. Tumour xenografts were generated by subcutaneously inoculating 1.5×10^7 cells suspended in 100 μL of Hanks' Balanced Salt Solution (Gibco BRL) at each of two sites per mouse. Daily oral administration of ZSTK474 (400 mg/kg) was started when tumour size reached 100–300 mm^3. Length (*L*) and width (*W*) of the subcutaneous tumour mass were measured every other day using a pair of calipers. The tumour volume (*TV*) was calculated as: $TV = (L \times W^2)/2$. Cell cycle distribution in tumours implanted in nude mice was monitored each day 4 h after drug administration by using an OV100 *in vivo* fluorescent imaging system (Olympus). Fluorescence intensity was quantified by using Image J software (National Institute of Health, MD). For microscopic analysis of cell cycle distribution, frozen tumours were fixed in 4% v/v paraformaldehyde and washed with H_2O. Tissue sections (14-μm) were examined using a IX81 fluorescence microscope (Olympus).

2.5. Immunohistochemistry

Tumour tissues fixed in 10% neutral formalin were embedded in paraffin. Tissue sections (4-μm) were deparaffinised in xylene and then in a series of (100–50%) ethanol solutions. Specific antibodies against phospho-Akt (Ser-473), phospho-S6, phospho-4E-BP1, phospho-RB (S807/S811) and cleaved PARP (Cell Signalling Technologies, Danvers, MA) were used for hybridization and the bound antibodies were visualised by using DAKO EnVision kit containing a secondary horseradish peroxidase (HRP) conjugated anti mouse polymer antibody complex. Sections were counterstained with Mayer's haematoxylin.

3. Results

3.1. Real-time visualisation of the induction of G1 arrest in cancer cells after treatment with ZSTK474

First we examined cell cycle progression *in vitro* after exposure to PI3K inhibitors in human breast cancer MCF-7 cells using the Fucci system (Fig. 1, Supplementary Movies 1 and 2). Control cells exhibited a combination of red and green nuclei and proliferated in parallel to cell cycle progression over a 48 h observation period. Upon exposure to a specific PI3K inhibitor ZSTK474, the number of cells with red nuclei gradually increased and those with green nuclei disappeared within

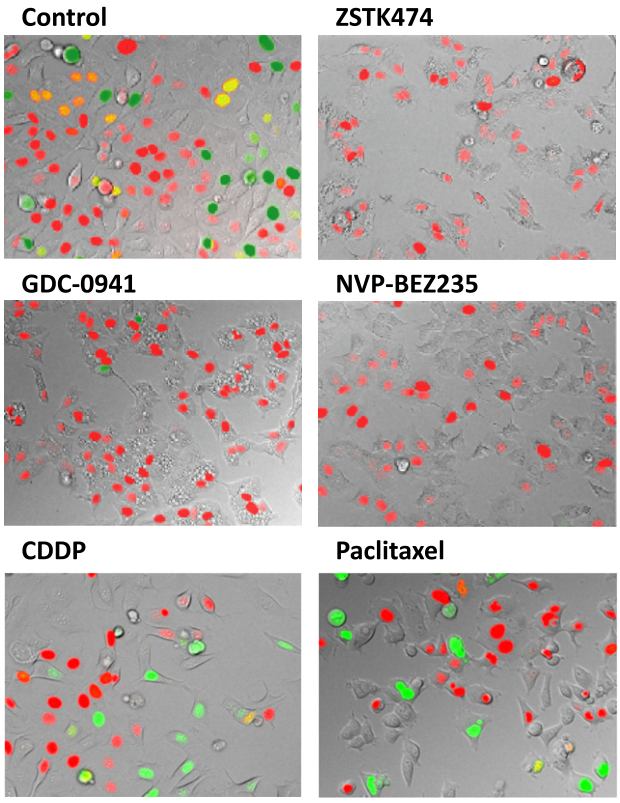

Fig. 1 – *In vitro* visualisation of cell cycle distribution in Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci)-introduced human breast cancer MCF-7 cells. Fucci-MCF-7 cells were treated with ZSTK474 (5 μM), GDC-0941 (5 μM), NVP-BEZ235 (150 nM), cisplatin (30 μM) or paclitaxel (10 nM) for 24 h. Cell cycle distribution was monitored by using a fluorescence microscope at 24 h after exposure. Red cells in G1 phase increased and green cells in S/G2/M phase decreased upon treatment with phosphatidylinositol 3-kinase (PI3K) inhibitors, but not with cisplatin and paclitaxel.

24 h of exposure to ZSTK474, along with inhibition of cell proliferation. Similar results were obtained when cells were exposed to other PI3K inhibitors GDC-0941 and NVP-BEZ235. Induction of G1 arrest was confirmed by conventional flow cytometric analysis (Fig. S1). However, when cells were treated with cisplatin and paclitaxel, growth inhibition did not coincide with an increase of red nuclei. We next examined the phosphorylation status of PI3K-downstream pathway members including Akt, ribosomal S6 protein (S6) and eukaryotic initiation factor 4E binding protein (4E-BP1). As shown in Fig. S2, these proteins were dephosphorylated after treatment with PI3K inhibitors. Similarly, retinoblastoma protein (Rb) was also dephosphorylated. Interestingly, cleaved poly (ADP ribose) polymerase (PARP) was not detected after treatment with these PI3K inhibitors. Our results show that these PI3K inhibitors inhibit the PI3K pathway, and that hyperphosphorylated Rb protein is reduced in parallel to the induction of G1 arrest of the cell cycle.

3.2. *In vivo imaging of G1 arrest after administration of ZSTK474 in human tumours xenografted in nude mice*

We next carried out *in vivo* analysis of cell cycle after administration of ZSTK474 in xenografted tumours derived from Fucci-MCF7 cells subcutaneously implanted in nude mice. As shown in Fig. 2A, tumour growth was effectively suppressed upon daily administration of ZSTK474 for five days. Macroscopic analyses using the OV100 fluorescence intravital imaging system revealed that the tumours turned from yellow to red as a result of induction of red fluorescence (G1 cells) and the reduction of green fluorescence ($S/G_2/M$ cells) after the second administration of ZSTK474 (Day 2, Fig. 2C). In agreement with this observation, R/G ratio (relative red/green fluorescence intensity ratio) was significantly elevated (Fig. 2B). Microscopic analysis confirmed that the number of green cells dramatically reduced and almost all cells became red in the tumour section resected on Day 5 (Fig. 2D), but no such increase in G1 cells was observed after administration of paclitaxel and cisplatin (data not shown). Similar results were obtained by using xenografted tumours derived from human cervical cancer HeLa cells. These results indicate that the PI3K inhibitor ZSTK474 induces simultaneous G1 cell cycle arrest and suppression of tumour growth *in vivo*.

3.3. *Effect of ZSTK474 on phosphorylation status of PI3K pathway proteins and pRB*

We next examined whether ZSTK474 inhibits the PI3K pathway *in vivo*. As shown in Fig. 3, ZSTK474 reduced the expression levels of phosphorylated Akt, ribosomal S6 protein and 4E-BP1, indicating that the PI3K-Akt-mTOR signalling pathway was indeed inhibited. As expected, ZSTK474 reduced the level of phosphorylated Rb protein in parallel to induction of G1 arrest. Similar results were obtained in xenografted tumours derived from human ovarian cancer SK-OV3 and prostate cancer PC-3 cells after daily administration of ZSTK474 over a two week period (Fig. S3). From these results, we conclude that ZSTK474 inhibits the PI3K pathway during G1 arrest *in vivo*.

3.4. *Antitumour effect of ZSKT474 was not accompanied by apoptosis*

A previous study reported that NVP-BEZ235 selectively induced apoptosis in breast cancer cells carrying a hotspot mutation in the *PIK3CA* gene.[16] Therefore, we attempted to examine the involvement of apoptosis during the tumour regression process induced by ZSTK474 in MCF-7-derived tumours carrying a *PIK3CA* hotspot mutation (E545K). No significant enhancement of apoptotic cells was observed after administration of ZSTK474 as determined by emergence of cleaved PARP (Fig. 3). Similar results were obtained in tumours derived from *PIK3CA*-mutated SK-OV3 cells (H1047R) and those from PTEN-null PC-3 cells (Fig. S3). In agreement with these observations, no enhancement in cleaved PARP was observed when Fucci-MCF7 cells were treated with ZSTK474 *in vitro* (Fig. S2). These results clearly indicate that ZSTK474 exerts a strong *in vivo* antitumour activity against

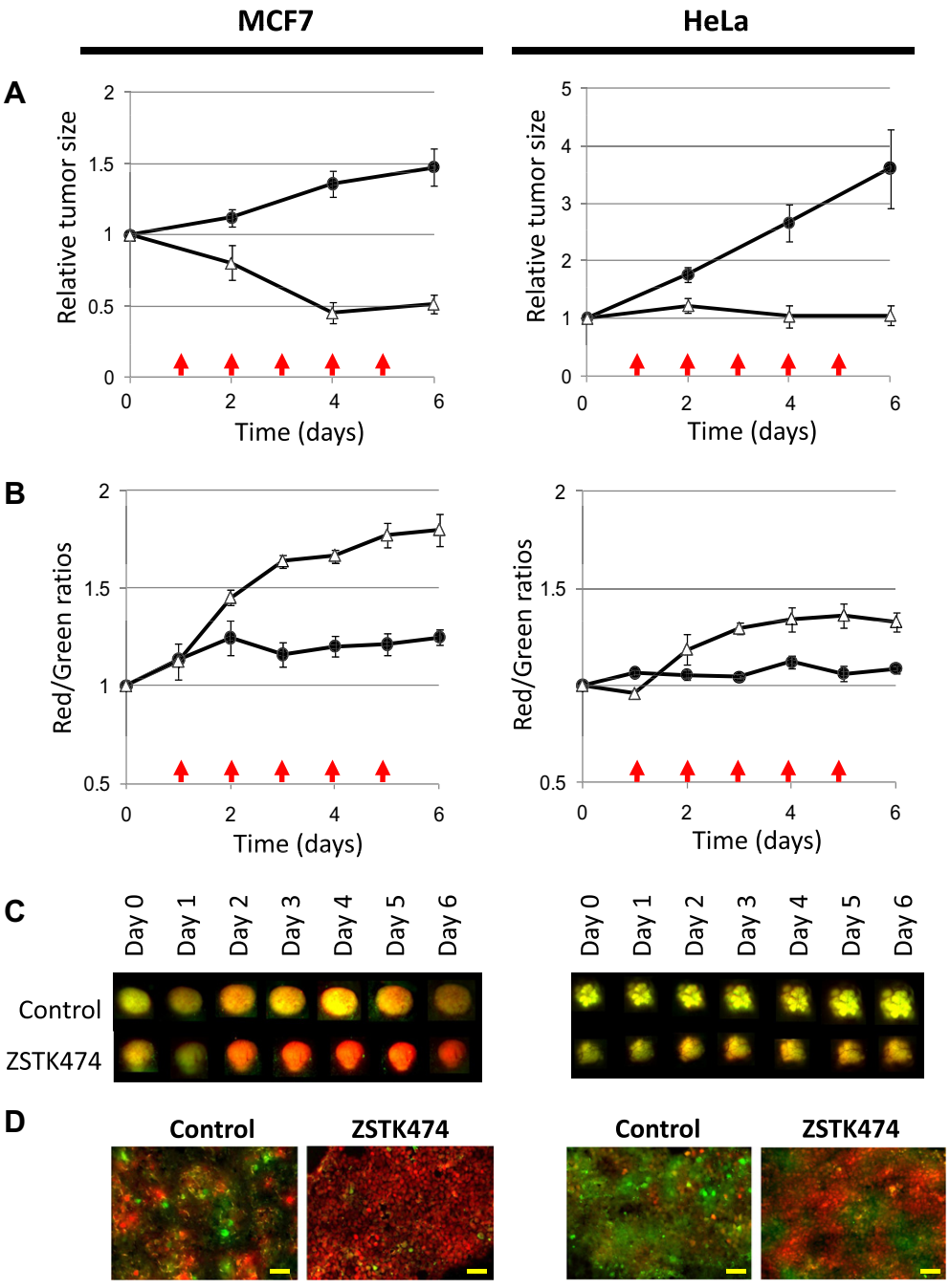

Fig. 2 – *In vivo* visualisation of cell cycle distribution in Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci)-introduced cancer cells. Fucci-MCF-7 cells and Fucci-HeLa cells were subcutaneously injected in nude mice to generate tumour xenografts. Then, ZSTK474 (400 mg/kg) was orally administered to the tumour-bearing mice (n = 6) from Day 1 to Day 5 (as indicated by the red arrows). (A) Tumour sizes in ZSTK474-administered mice (n = 6) and control mice (n = 6) were measured every other day (4 h after drug administration). Tumour sizes were then normalised to the respective size at day 0. ZSTK474 significantly suppressed tumour growth. (B) Macroscopic analyses using the OV100 fluorescence intravital imaging system were performed to investigate cell cycle distribution in live mice. Red and green fluorescent intensities were measured from each image. Relative fluorescence intensities (R/G ratio) were then calculated. The R/G ratios in each group (n = 12) were averaged and the averaged ratio at Day 0 was normalised to 1. (C) Typical images overlaying red and green fluorescence on each day are shown. (D) Microscopic images of frozen tumour sections resected on Day 5 from mice administered with ZSTK474. ZSTK474 drastically reduced the number of green cells with almost all cells turning red. This observation indicates G1 arrest of the cell cycle. Scale bars = 100 μm.

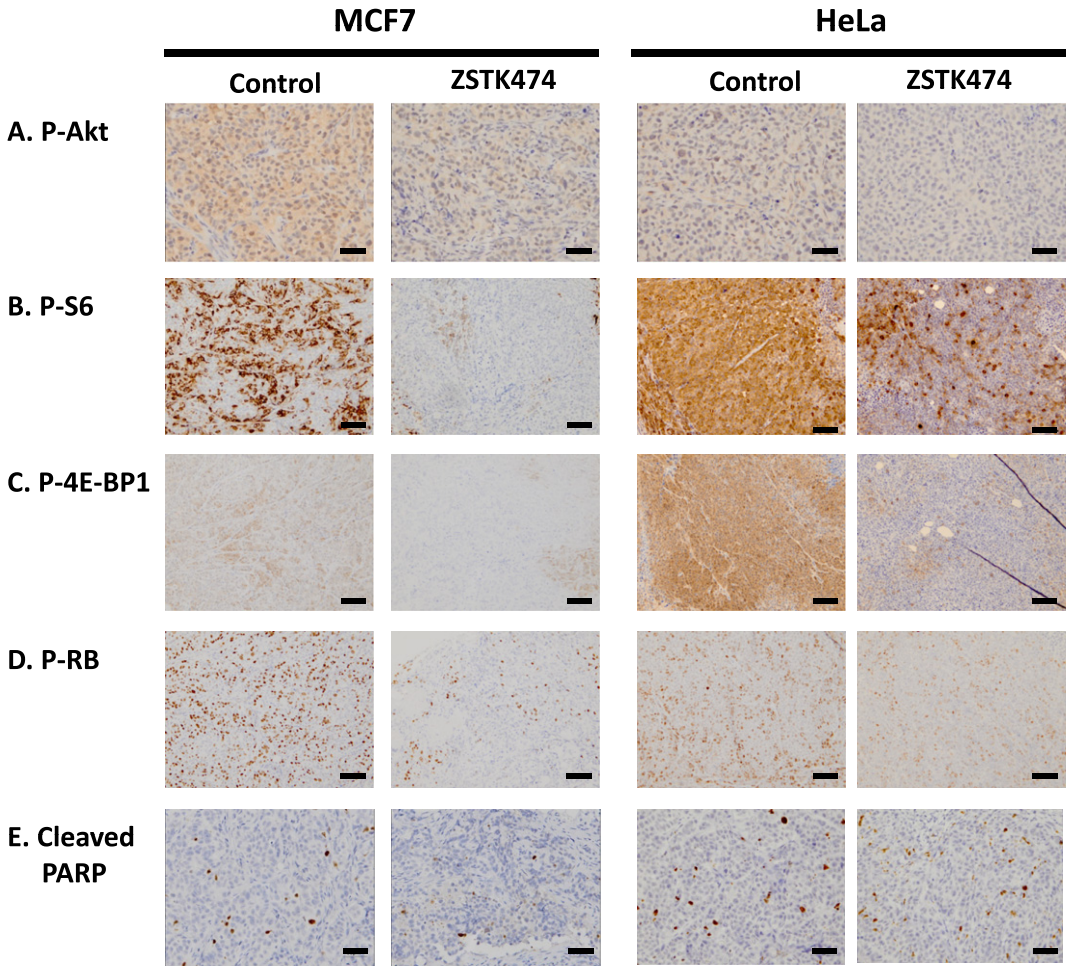

Fig. 3 – *In vivo* effects of ZSTK474 on the phosphorylation levels of phosphatidylinositol 3-kinase (PI3K) pathway members and Rb protein. Tumours derived from Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci)-MCF-7 cells and Fucci-HeLa cells were resected on Day 5. The sections were then subjected to immunohistochemistry to examine expression levels of phospho-Akt (A), phospho-S6 (B), phospho-4E-BP1 (C) phospho-Rb (D) and cleaved PARP (E) by using specific antibodies for respective proteins. Scale bars = 100 μm.

these *PIK3CA*-mutated and PTEN-deficient tumours without inducing apoptosis.

3.5. *Reduction of cell size after administration of ZSTK474*

Thus far, we have demonstrated that ZSTK474 exerts a strong antitumour activity, and this antitumour effect is accompanied by G1 arrest, but not by apoptosis, despite the observed tumour regression of MCF-7-derived tumours after administration of ZSTK474. A careful investigation of MCF-7-derived tumour sections revealed that tumour cells appeared to shrink after administration of ZSTK474 for five days (Fig. 2D). Therefore, we tested whether ZSTK474 reduced tumour cell size (Fig. 4A and B). Our results clearly show that ZSTK474 significantly increases the tumour cell density. Previous studies have reported that the PI3K pathway regulates cell size *via* mTOR and its downstream targets S6K1 and 4E-BP1.[17] In fact, ZSTK474 reduces cell size in the G1 phase of the cell cycle *in vitro* (Fig. S4) as well as lowering the degree of phosphorylation of S6 and 4E-BP1 both *in vivo* (Fig. 3B and C) and *in vitro* (Fig. S2). Similar results were obtained for SK-OV3- and PC-3-derived tumours (tumour cell density increased by 90% and 32%, respectively. Fig. S3). From these results we concluded that ZSTK474 reduces cell size *in vivo* and *in vitro via* inhibition of S6K1 and 4E-BP1.

3.6. *Accumulation of G1 cells and inhibition of tumour growth during long-term administration of ZSTK474*

In Figs. 2 and S3, we demonstrated that ZSTK474 strongly suppressed tumour growth in parallel to accumulation of G1 cells and/or hypophosphorylation of pRB after daily administration for five days or two weeks. We next attempted to investigate the effect of long-term administration of ZSTK474 on tumour growth and cell cycle distribution. As shown in Fig. 5B, ZSTK474 increased G1 cell population in Fucci-HeLa-derived tumour xenografts within a week, and such increase was maintained during long-term daily administration for up to 100 days. In agreement with this observation, ZSTK474 efficiently blocked tumour growth (Fig. 5A). The result suggested

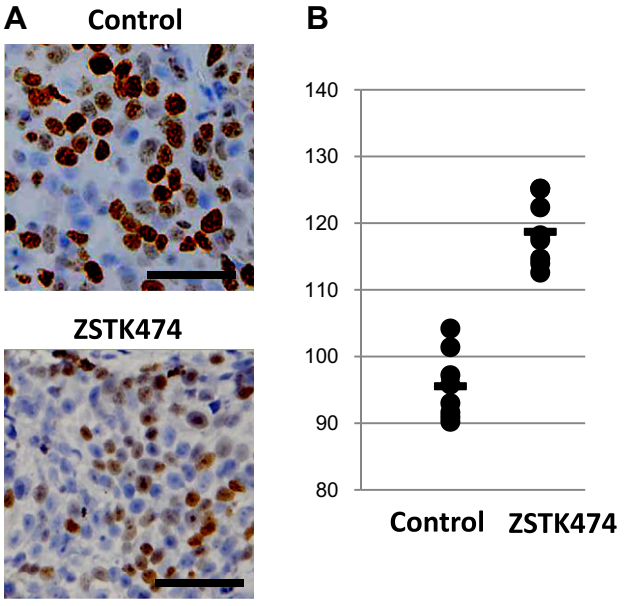

Fig. 4 – Morphological changes of tumour cells derived from Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci)-MCF7 cells after administration of ZSTK474. (A) Tumours resected on Day 5 were subjected to immunohistochemistry to detect Ki-67. Tumour cells appeared to shrink. Scale bars = 50 μm. (B) Difference in tumour cell densities (i.e. number of tumour cells per unit area) in tumours resected from control mice and those from ZSTK474-administered mice. Data are the averages of eight different fields (2.0×10^4 μm^3) from each sample.

that ZSTK474 exerts its antitumour effect by inducing G1 arrest and this effect lasts at least three months.

4. Discussion

In the current study, we have used the Fucci system to investigate cell cycle distribution in tumours derived from MCF-7 cells and HeLa cells subcutaneously implanted in nude mice after administration of ZSTK474. We have demonstrated for the first time that a PI3K inhibitor ZSTK474 induces G1 arrest in tumour cells *in vivo* in parallel to suppression of tumour growth. We also confirmed that ZSTK474 did not induce apoptosis to exert its *in vivo* antitumour effect, even in *PIK3CA*-mutated tumours. These results suggest that ZSTK474 exerts its antitumour efficacy mainly *via* G1 arrest, but not apoptosis. Moreover, we demonstrated that simultaneous G1 arrest and suppression of tumour growth lasted at least three months as long as daily administration of ZSTK474 continued. These observations indicate that ZSTK474 could be used clinically for controlling tumours in advanced stages. In fact, we previously demonstrated that ZSTK474 strongly inhibited growth of tumours derived from human prostate cancer PC-3 cells xenografted in nude mice, even when the tumour volumes were ~1700 mm^3 in size.[6]

Inhibition of PI3K is believed to induce apoptosis in cancer cells. Indeed, the PI3K inhibitor LY294002 triggered apoptosis in colorectal and pancreatic cancer cells.[18,19] However, previous reports, including our own, have demonstrated that some

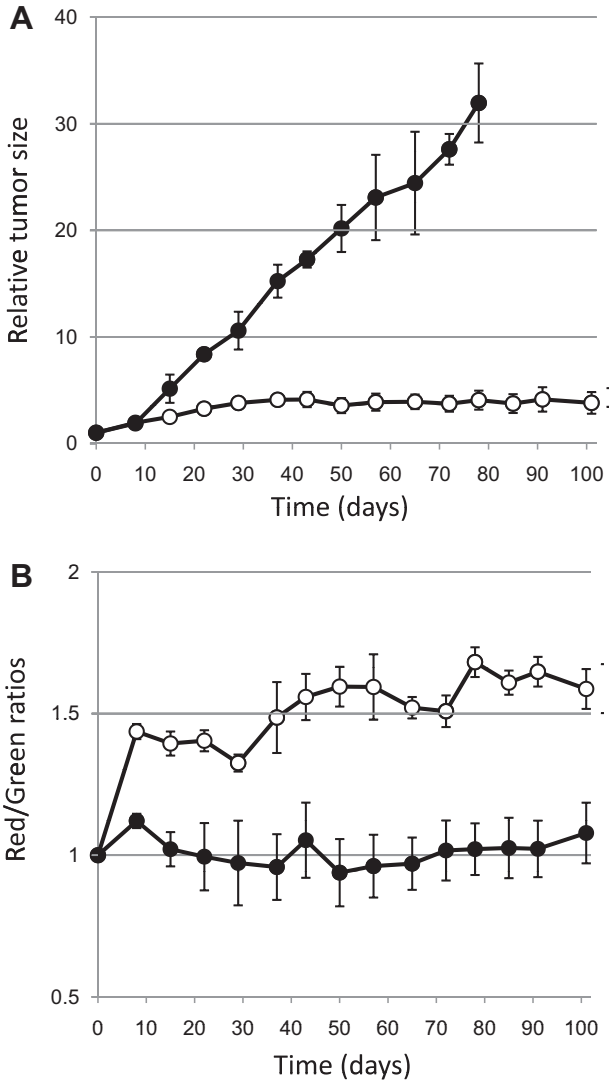

Fig. 5 – Accumulation of G1 cells and inhibition of tumour growth during long-term administration of ZSTK474. Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci)-HeLa cells were subcutaneously inoculated at each of two sites per mouse (n = 3) to generate tumour xenografts. Then, ZSTK474 was orally administered to the tumour-bearing mice from Monday to Friday at a dose of 400 mg/kg (800 mg/kg on Friday). (A) Tumour sizes in ZSTK474-administered mice (n = 6) and control mice (n = 6) were measured once a week. Measurement of tumour size in control mice was discontinued on day 77 because of the tumour rupture. Tumour sizes were then normalised to the respective size at day 0. (B) Macroscopic analyses using the OV100 fluorescence intravital imaging system were performed to investigate cell cycle distribution in live mice. Red and green fluorescent intensities were measured from each image, and relative fluorescence intensities (R/G ratio) were then calculated. The R/G ratios in each group (n = 6) were averaged and the averaged ratio at Day 0 was normalised to 1.

PI3K inhibitors under active development, such as ZSTK474 and NVP-BEZ235, exert their antitumour efficacy *in vitro* by

G1 arrest rather than inducing apoptosis.[5,6,13,14] Recently, NVP-BEZ235 and GDC-0941 were shown to selectively induce apoptosis in breast cancer cells with PI3K mutation and/or Human Epidermal growth factor Receptor 2 (HER2) amplification.[16,20] However, we previously demonstrated that the mutation status of *PIK3CA* did not correlate with the *in vitro* efficacy of ZSTK474 across a panel of 39 human cancer cell lines (JFCR39), nor with the *in vivo* drug efficacies of ZSTK474 across 24 human cancer cell lines implanted in nude mice.[21] In this study, we clearly demonstrate that ZSTK474 induces G1 arrest along with tumour suppression *in vivo* by using Fucci-introduced cancer cells. Moreover, ZSTK474 did not induce apoptosis along with tumour suppression in MCF-7- and SK-OV3-derived tumours despite the fact that these cell lines carry an activating mutation on the *PIK3CA* gene. Of note, SK-OV3 cells overexpress the HER2 protein.[21]

We observed that ZSTK474 reduced tumour cell size during tumour suppression. Previous studies have reported that mTOR regulates cell growth, i.e. cell size in tumour as well as normal cells *via* 4E-BP-1 and S6K1/S6.[17] Indeed, mTOR inhibitors, including CCI-779 and WYE-125132, have been shown to reduce tumour cell size *in vitro* and *in vivo*.[22,23] NVP-BEZ235, a dual inhibitor of PI3 K and mTOR, was also reported to reduce tumour cell size in a mouse model of tuberous sclerosis.[24] In regard to PI3K inhibitors, LY294002 reduced cell size *in vitro*,[17] but LY294002 is not a specific inhibitor of class I PI3Ks, but inhibits a number of protein kinase such as casein kinase 2 and DNA-PK.[10,25] In this study, we clearly demonstrate that a specific PI3K inhibitor ZSTK474 reduces tumour cell size *in vivo* along with a decrease in the level of phosphorylated 4E-BP1 and S6. Our findings suggest that ZSTK474-induced tumour regression is caused in part by tumour cell size reduction. However, further studies are needed to establish the functional involvement of cell size reduction in the efficacy of the antitumour activity of PI3K inhibitors.

In summary, using the Fucci system we have demonstrated that a PI3K inhibitor ZSTK474 induces G1 arrest, but not apoptosis, *in vivo* along with tumour suppression even in tumours carrying a gain-of-function mutation in the *PIK3CA* gene. Moreover, we show that G1 arrest and inhibition of tumour growth induced by ZSTK474 last at least three months as long as ZSTK474 is daily administered to the recipient mice. In addition, we found that ZSTK474 reduces cell size both *in vivo* and *in vitro*. ZSTK474 has entered Phase I clinical trials in U.S. in January 2011, and we expect that ZSTK474 could be used as a maintenance therapy for patients with advanced cancers in future clinical trials.

Conflict of interest statement

Takao Yamori has a research fund from Zenyaku Kogyo Co., Ltd., which is the proprietary company of ZSTK474; H. Yoshimi is an employee of Zenyaku Kogyo Co., Ltd.

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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.ejca.2011.10.006.

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