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ORIGINAL ARTICLE

Synergistic tumor suppression by a *Perilla frutescens*-derived methoxyflavanone and anti-cancer tyrosine kinase inhibitors in A549 human lung adenocarcinoma

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Abstract Anti-cancer tyrosine kinase inhibitors (TKIs) are effective in many types of cancers including non-small cell lung cancer, while appearance of TKI-resistant tumors suggests a need for the development of their potentiation strategies. We have previously shown that a methoxyflavanone derivative from the Asian medicinal herb *Perilla frutescens* (*Perilla*-derived methoxyflavanone; PDMF) shows a prominent anti-tumor activity against A549 human lung adenocarcinoma. Here we show that PDMF and anti-cancer TKIs (nilotinib, bosutinib, dasatinib, and ponatinib) synergistically suppress proliferation of A549 cells. Flow cytometric analysis indicated that co-stimulation with nilotinib (4 μM) and PDMF induced G_2/M cell cycle arrest in low PDMF doses (10–50 μM), whereas this combination triggered de novo G_1 arrest in higher PDMF dosages (50–125 μM). We also found that co-administration with nilotinib and PDMF significantly suppressed in vivo tumorigenicity of A549 cells in athymic nude mice.

Keywords A549 cells · Lung cancer · Methoxyflavanone · *Perilla frutescens* · Tyrosine kinase inhibitors

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Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (Siegel et al. 2013). In the clinical practice, lung carcinomas are divided into two main histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), where the other accounts for 80% of total lung cancer cases (Meoni et al. 2013). The main characteristic of NSCLC is its high aggressive invasion and metastatic properties. Therefore, the majority of advanced NSCLC cases require receiving combination of chemotherapy, and/or radiation treatment (Molina et al. 2008). Tyrosine kinase inhibitors (TKIs) are now the golden stone of

treatment in NSCLC (Gridelli et al. 2014). These drugs target biological functions that cancer cells are critically reliant on, ultimately causing tumor shrinkage (Krause and Van Etten 2005). However, a serious problem is that many of patients who especially receive the epidermal growth factor receptor (EGFR)-TKI therapy get resistance to the agents (Rosell et al. 2009), suggesting a need for the development of other chemotherapeutic drugs. Thus, there remains a need for strategies to safely increase the effectiveness of TKIs.

Recently, a considerable attention has been given to the identification of new therapeutic agents that show synergistic effect with TKIs as a promising direction to overcome the above-mentioned problems. In general, combination therapies not only potentiate the therapeutic efficacy of each agent alone, but also enable the use of reduced doses (Milano et al. 2009; Nautiyal et al. 2011). In this context, plant flavonoids, especially those from dietary sources, are commonly perceived as non-toxic, well-tolerated, easily available, and inexpensive compounds that can target multiple cellular pathways (Surh 2003; Sak 2012). It has been shown that flavonoids have anti-tumor activities through induction of apoptosis and cell cycle arrest of tumor cells (Szliszka et al. 2008; Kim et al. 2013). In particular, flavanones are a type of flavonoids that exhibit chemo-preventive and anti-cancer properties (Graf et al. 2005). Previous in vitro studies showed that naturally-occurring flavanones have anti-tumor activity against human cancer cells; e.g. naringenin in THP-1 and U937 leukemia cells (Park et al. 2008b; Jin et al. 2009), hesperidin in SNUC4 colon cancer and NALM6 leukemia cells (Park et al. 2008a; Ghorbani et al. 2012), and liquiritigenin in SMMC7721 hepatocarcinoma and HeLa cervical cancer cells (Zhang et al. 2009; Liu et al. 2011). Flavanones also received a considerable attention as these agents may potentiate the cytotoxic effects of chemotherapy and radiotherapy, protect normal cells from therapy-associated toxicity, increase a systemic bioavailability of cytostatic agents, and in some cases, even overcome chemoresistance (Garg et al. 2005; Kuno et al. 2012).

Recently, our group has identified a new class of methoxyflavanone derivative (8-hydroxy-5,7-dimethoxyflavanone) from the Asian dietary herb, *Perilla frutescens* (named *Perilla*-derived methoxyflavanone, PDMF). PDMF selectively suppresses Akt activation to inhibit type I hypersensitivity reactions (Kamei et al. 2017). We also have found that PDMF shows prominent tumor suppressive activity on A549 human adenocarcinoma through induction of p53-driven G_2/M cell cycle arrest and apoptosis (Abd El-Hafeez et al. unpublished data). In the present study, we show a synergistic tumor-suppressive potency of PDMF and anti-cancer TKIs on A549 cells in vitro as well as in vivo.

Materials and methods

Cell cultures and reagents

The A549 human lung adenocarcinoma was obtained from the RIKEN CELL BANK (Tsukuba, Japan), and was cultured in Dulbecco's-modified Eagle's medium (DMEM; Life Technologies, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) in a humidified atmosphere with 5% CO_2 at 37 °C. *Perilla*-derived methoxyflavanone (PDMF; 8-hydroxy-5,7-dimethoxyflavanone) (Kamei et al. 2017) was chemically synthesized by Tokyo Chemical Industry (Tokyo, Japan). Nilotinib, bosutinib, dasatinib, and ponatinib were purchased from LC laboratories (Woburn, MA, USA). Doxorubicin was purchased from Sigma-Aldrich. All chemicals used in this study were of analytical or cell culture grade.

Cell proliferation assay

Cell proliferation was analyzed by BrdU incorporation using a Colorimetric Cell Proliferation ELISA, BrdU Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. A549 cells were seeded at 5×10^3 cells/well and cultured overnight in a 96-well plate. The cells were then treated with sub-optimal doses of PDMF alone (10, 50, 100 or 125 μM), or in combination with sub-optimal doses of Bcr-Abl TKIs (4 μM nilotinib, 1 μM bosutinib, 50 nM dasatinib or 0.5 μM ponatinib) for 24 h. The doses of PDMF and TKIs were determined by a preliminary dose–response study on A549 cells, in which reduction in cell proliferation is limited up to 20%. A549 cells were also treated with either 1 μM doxorubicin as a positive control or dimethyl sulfoxide

(DMSO, vehicle) as a negative control. BrdU was added to a final concentration of 10 μM and cultured for the last 2 h.

Drug interaction analysis

To evaluate whether the anti-tumor effects of the combination of PDMF and TKIs were synergistic, the drug interactions were analyzed based on the combination index method of Chou and Talalay (1984) using a CompuSyn software (ComboSyn, Paramus, NJ, USA). The dose response curves for single agents and their combinations were generated, and the combination index (CI) values were calculated. The resulting CI values of 0.1–0.3 indicate strong synergism, those of 0.3–0.7 for synergism, 0.7–0.85 for moderate synergism, and 0.85–0.90 for slight synergism.

Cell cycle and apoptosis analyses

Cell cycle progression or induction of apoptosis of A549 cells was analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the BD Pharmingen^{TM} FITC-BrdU Flow Kit (BD Biosciences) or the Annexin V/Propidium Iodide (PI) Staining Kit (BioLegend, San Diego, CA, USA), respectively. A549 cells were treated with 10, 50, 100 or 125 μM PDMF alone, or in combination with 4 μM nilotinib. A549 cells were also treated with 8 μM nilotinib alone or 1 μM doxorubicin for positive controls. Quantitative analysis of those flow cytometry data was performed with FlowJo software (FlowJo, Ashland, OR, USA).

Animal experiments

All animal experiments were carried out using protocols approved by the Committee on Animal Experimentation of Hiroshima University, Japan. Four-week-old female BALB/c nude mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan), and kept under specific pathogen-free conditions.

For construction of A549 cell xenograft model, BALB/c nude mice (n = 42) were subcutaneously (*s.c.*) injected with 5×10^6 A549 cells into their dorsal skins. The mice were then randomly divided into seven groups (six mice each), and *s.c.* administered with 40 μl of 0.5% DMSO in PBS (control), 10 μM PDMF (6.4 ng/g murine body weight), 125 μM PDMF (79.7 ng/g body weight), 4 μM nilotinib (4.5 ng/g body weight), 4 μM nilotinib with 10 μM PDMF, 4 μM nilotinib with 125 μM PDMF, or 20 μM nilotinib (22.5 ng/g body weight). Those agents were started to be injected every three days from the next day of the A549 cell transplantation. On day 18, tumor volumes were started to be monitored every three days. Tumor volume was calculated as (wide^2 × length)/2 of formulated tumor (Tomayko and Reynolds 1989). On day 36, mice were sacrificed and their final tumor weights were quantified.

Statistical analysis

Data are represented as mean ± SD. Student's *t* test was performed to determine the statistical significance compared to corresponding controls. Statistical significance was defined as $P < 0.05$ or $P < 0.005$. The data shown in the figures are representative data for three independent experimental results.

Results and discussion

PDMF and anti-cancer TKIs synergistically suppress cell proliferation of A549 human lung cancer cells

A549 human lung adenocarcinoma cells are used as a model of EGFR-TKI-resistant NSCLC cells (Ito et al. 2014; Cao et al. 2016). For those EGFR-TKI-resistant cells, the Bcr-Abl type of TKIs may offer alternative anti-cancer agent, since the EGFR-TKI-resistant A549 cells indeed show a good response to a Bcr-Abl TKI (Zhang et al. 2003), probably because of its cross-reaction to a wide array of oncogenic TKs. For those reasons, here we decided to analyze the combination anti-tumor effect of Bcr-Abl TKIs (nilotinib, bosutinib, dasatinib and ponatinib) and PDMF on A549 cells.

To test whether combination of PDMF and TKIs presents a synergistic anti-cancer effect, A549 cells were stimulated with serial doses of PDMF (10, 50, 100, and 125 μM) and sub-optimal doses of TKIs (nilotinib, bosutinib, dasatinib and ponatinib). BrdU incorporation assay revealed that those combination

regimens resulted in a greater loss of the cell proliferation compared to the individual agents, suggesting a synergistic growth inhibitory effect of PDMF and TKIs on A549 cells (Fig. 1). To further confirm the synergism, interactions between PDMF and TKIs were analyzed with the median-effect principle of Chou and Talalay (1984). The resulting combination index (CI) theorem of the Chou–Talalay analysis offers quantitative definition for additive effect ($CI = 1$), synergism ($CI < 1$), and antagonism ($CI > 1$) in drug combinations. The CI values in the experimental points indicated that all the combination regimens of PDMF and TKIs showed strong synergism (with CI of 0.1–0.3) or synergism (with CI of 0.3–0.7) (Supplementary Table 1), clearly indicating that PDMF and those TKIs synergistically suppress proliferation of A549 cells. Among four TKIs tested, the overall maximal synergism was noted when PDMF was combined with nilotinib (shown underlined in Supplementary Table 1). We therefore selected nilotinib for further analysis.

Co-stimulation with PDMF and anti-cancer TKI (nilotinib) synergistically induces cell cycle arrest at G_1 and G_2/M phases

We next analyzed impact of the PDMF/nilotinib co-stimulation on cell cycle progression of A549 cells. Flow cytometric analysis indicated that low doses of PDMF (10–50 μM) and nilotinib (4 μM) synergistically induced G_2/M cell cycle arrest in A549 cells (Fig. 2a, b). Intriguingly, we found that co-stimulation with higher doses of PDMF (50–125 μM) synergized with nilotinib to induce de novo G_1 cell cycle arrest (Fig. 2a, b), suggesting that the synergistic anti-cancer effect of PDMF and nilotinib is executed by a two-stage cell cycle arrest depending on the PDMF dosages. We also found that the PDMF co-stimulation had no effect on apoptosis induction in A549 cells

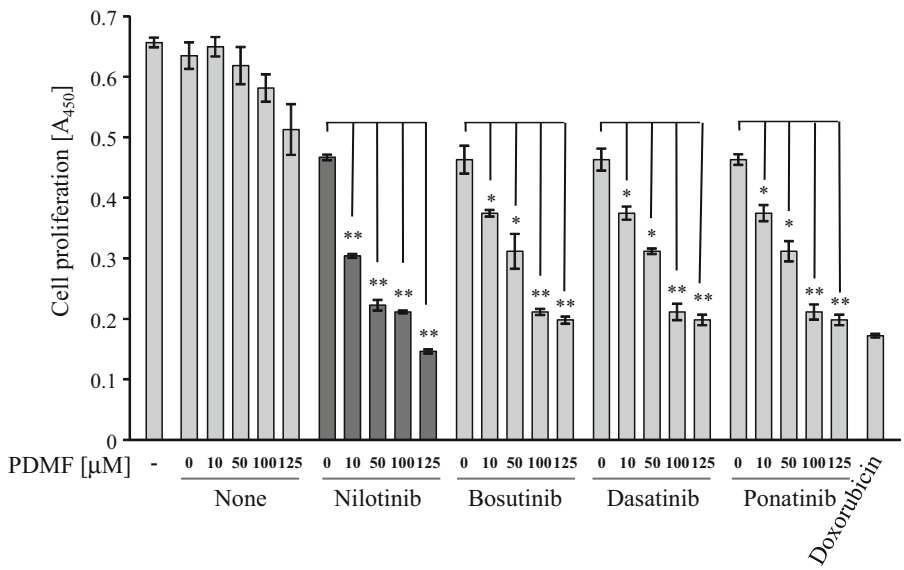

Fig. 1 PDMF and anti-cancer TKIs synergistically suppress proliferation of A549 human lung adenocarcinoma. Inhibitory effects of sub-optimal doses of PDMF (10–125 μM), sub-optimal doses of Bcr-Abl TKIs (4 μM nilotinib, 1 μM bosutinib, 0.05 μM dasatinib, or 0.5 μM ponatinib), and their combinations on the proliferation of A549 cells were determined by BrdU incorporation assay. The sub-optimal doses of PDMF and TKIs were determined by a preliminary dose–response study on A549 cells, in which a reduction in cell proliferation is limited up to 20%. Doxorubicin (1 μM, DNA damaging agent) and dimethyl sulfoxide (DMSO, vehicle) are set as a positive and negative vehicle controls, respectively. Representative data of three independent experiments are shown as mean ± SD of three replicates. $^*P < 0.05$ and $^{**}P < 0.005$ indicate significant differences assessed by Student's *t* test

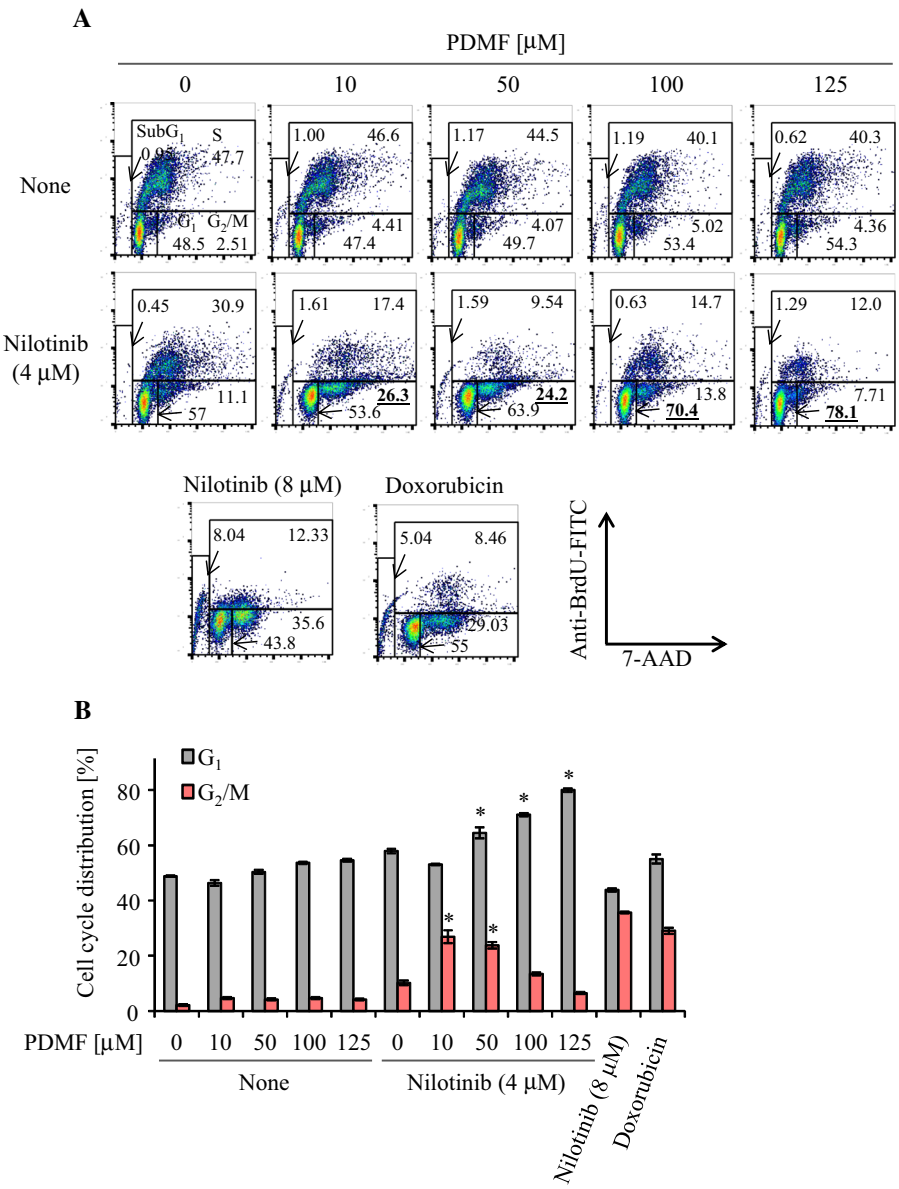

Fig. 2 PDMF synergizes with nilotinib to induce G_1 and G_2/M cell cycle arrest on A549 cells. **a** Representative data are shown for the cell cycle distribution analyzed by flow cytometry. A549 cells were treated with a serial concentration of PDMF (10–125 μM) in the presence or absence of nilotinib (4 μM). 8 μM nilotinib (TKI only at higher dose) and 1 μM doxorubicin are set as positive controls. **b** Quantification of G_1 and G_2/M cell cycle arrest of A549 cells upon co-stimulation with PDMF and nilotinib. The values are the mean ± SD of three different experiments. $^*P < 0.05$ and $^{**}P < 0.005$ indicate significant differences compared with nilotinib only (4 μM)

(Supplementary Fig. 1), suggesting that the synergistic tumor suppressive effect is mainly fulfilled through augmentation of G_1 and G_2/M cell cycle arrest.

Mechanisms underlying the PDMF/nilotinib-driven G_1 (high PDMF doses) and G_2/M (low PDMF doses) cell cycle arrest are currently unknown. Our preliminary data indicate that co-stimulation with a high dose PDMF (125 μM) synergized with nilotinib to induce cyclin-dependent kinase (CDK) inhibitor p21, whereas a low dose PDMF (10 μM) and nilotinib synergistically decreased protein levels of CDK1 and cyclin B1, that form a hallmark cyclin/CDK complex in the G_2/M cell cycle checkpoint (data not shown). Thus, one possible explanation is that the synergistic two-stage cell cycle arrest could be fulfilled through direct up- and down-modulation of those G_1 and G_2/M cell cycle checkpoint molecules. Another critical issue is that a possible anti-cancer effect after more than 24 h remains to be investigated in the present study. Further rigorous analysis is needed to elucidate the precise mechanisms by which the co-stimulation with nilotinib and PDMF fulfills anti-tumor potency.

Co-administration with PDMF and nilotinib suppresses tumorigenicity of A549 cells in athymic nude mice

To further test the synergistic anti-tumor effect of PDMF and nilotinib in vivo, we co-administered PDMF and nilotinib to an A549 cell-xenograft model on athymic nude mice. We found that co-treatment with PDMF and nilotinib significantly impaired tumorigenicity of A549 cells as compared to nilotinib alone or PDMF alone (Fig. 3a); especially, co-administration with 125 μM PDMF markedly suppress in vivo tumor development. The PDMF co-treatment also significantly decreased final A549 tumor weight in a dose-dependent manner (Fig. 3b). These results suggest that co-administration with PDMF and nilotinib to cancer-adjacent sites prevent in vivo tumorigenesis of A549 cells. To further gain insight into the application of PDMF, oral and/or systemic administration study of PDMF and nilotinib should be conducted in the next studies. Another unsolved issue is safety and specificity of the PDMF/nilotinib combination. We preliminary observe that PDMF by itself is less cytotoxic to WI-38 human normal lung fibroblast cells than to A549 cells (Abd El-Hafeez et al., unpublished data), but cancer selectivity as well as safety issue of the combination regimen should be overcome in future studies.

In conclusion, here we show that a *P. frutescens*-derived flavanone derivative (PDMF) synergizes with anti-cancer TKI to augment tumor suppressive potency on A549 human adenocarcinoma in vitro as well as in vivo. Mechanistically, the synergistic anti-tumor effect is fulfilled by the induction of a two-stage

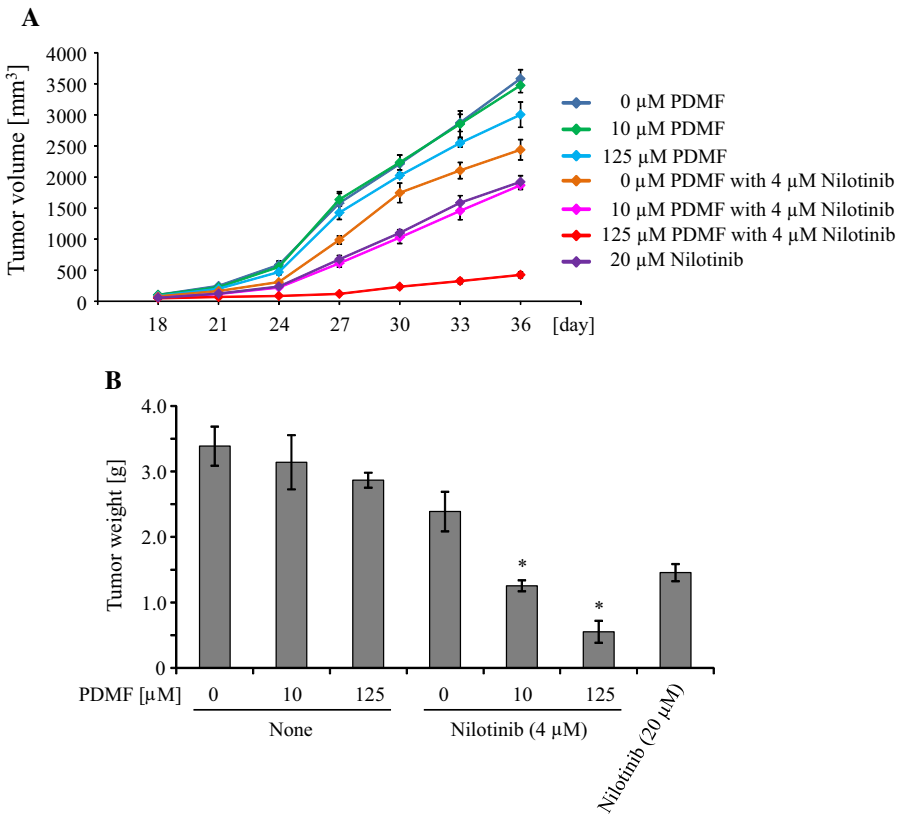

Fig. 3 Co-administration of PDMF and nilotinib impairs in vivo tumorigenicity of A549 cells in athymic nude mice. Six week-old BALB/c nude mice were subcutaneously (*s.c.*) injected with A549 cells (5×10^6 cells/head). Mice were then randomly assigned to seven groups (six mice per group) that were *s.c.* administered with 40 μl of 0.5% DMSO in PBS (control), 10 μM PDMF (6.4 ng/g murine body weight), 125 μM PDMF (79.7 ng/g body weight), 4 μM nilotinib (4.5 ng/g body weight), 4 μM nilotinib with 10 μM PDMF, 4 μM nilotinib with 125 μM PDMF, or 20 μM nilotinib (22.5 ng/g body weight). 18 days after xenotransplantation, tumor volumes were monitored each three days (**a**). On day 36, mice were euthanized and final tumor weights were quantified (**b**). $^*P < 0.05$ indicates significant difference compared with nilotinib only (4 μM)

cell cycle arrest at G_1 and G_2/M phases. Further investigation is required to develop the combinational cancer preventive strategies utilizing PDMF and anticancer TKIs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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