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8-(Tosylamino)quinoline inhibits tumour progression through targeting phosphoinositide-3-kinase/Akt pathway

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We examined whether 8-(tosylamino)quinoline (8-TQ), a structural analogue of BAY 11-7082, is able to modulate various tumourigenic responses using various *in vitro* and *in vivo* experimental conditions. 8-TQ exhibited the strongest suppressive activity on the proliferation of C6, A431, HeLa and MDA-MB-231 cells with IC_{50} values ranging from 10 to 30 μM. According to the analysis of level of active caspase-3, and morphologies of C6, HeLa and MDA-MB-231 cells, it was revealed that 8-TQ is able to induce apoptosis. Furthermore, this compound strongly diminished the invasion of MDA-MB-231 cells, the migration of HeLa cells, and the new generation of blood vessels under non-toxic conditions. Reduction of the phospho-form levels of intracellular signalling enzymes by 8-TQ strongly indicated that molecular signalling machineries composed of phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase-1 (PDK1)/Akt and extracellular-signal-regulated kinase (ERK) could be targeted by 8-TQ treatment. Indeed, the specific inhibitors (LY294002 and U0126) of PI3K/PDK1/Akt and ERK showed similar anti-cancer properties to 8-TQ. Finally, 8-TQ intraperitoneally injected suppressed the increase of tumour volume up to 40% compared to vehicle-treated control. Taken together, our results clearly suggest that 8-TQ might have applications as a novel anti-cancer drug or may be served as a lead compound to be further optimized.

1. Introduction

A great amount of effort has been invested in the development of novel anti-cancer drugs by exploring novel target enzymes such as c-Abl and c-Src and their specific inhibitory drugs such as Gleevec and SKI-606 (Golas et al. 2003; Hiwase et al. 2008). Nonetheless, it is accepted that the development of selective and non-toxic compounds with anti-cancer activity is not an easy task.

Recently developed anti-cancer drugs have been shown to suppress various signalling enzymes important for cancer cell survival (Choy et al. 2005). Oncogenic tyrosine kinases such as Src and several survival- or proliferation-controlling kinases such as phosphatidylinositide-3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1), and Akt (protein kinase B) are representative signalling components that are markedly activated in various cancer cells to maintain a high cellular proliferation rate (Cheng et al. 2008; Warmuth et al. 2003).

By screening anti-cancer drugs, we have found that a derivative of BAY11-7082 is a representative IκBa Kinase (IKK) inhibitor that actively suppresses various inflammatory cytokines (Mendes Sdos et al. 2009) and the induction of heme oxygenase-1 (Min et al. 2011) and intercellular cell adhesion molecule-1 (ICAM-1) expression (Lee et al. 2004), and displays anti-proliferative activity (White and Burchill 2008). Although this compound was originally reported to have anti-inflammatory effects in diseases such as arthritis (Pierce et al. 1997), its strong cytotoxicity raised the possibility that such compounds could be developed as anti-cancer drugs. Therefore, in this study we further characterised the anti-cancer activity of BAY11-7082 derivatives using various tumourigenic models including cell proliferation, invasion, migration, and angiogenesis and also explored their anti-cancer mechanism.

2. Investigations, results and discussion

The proliferation of C6, A431, MDA-MB-231, and HeLa cells was suppressed by 8-(tosylamino)quinoline (8-TQ; Fig. 1) in a dose-dependent manner with IC_{50} values ranging from 10

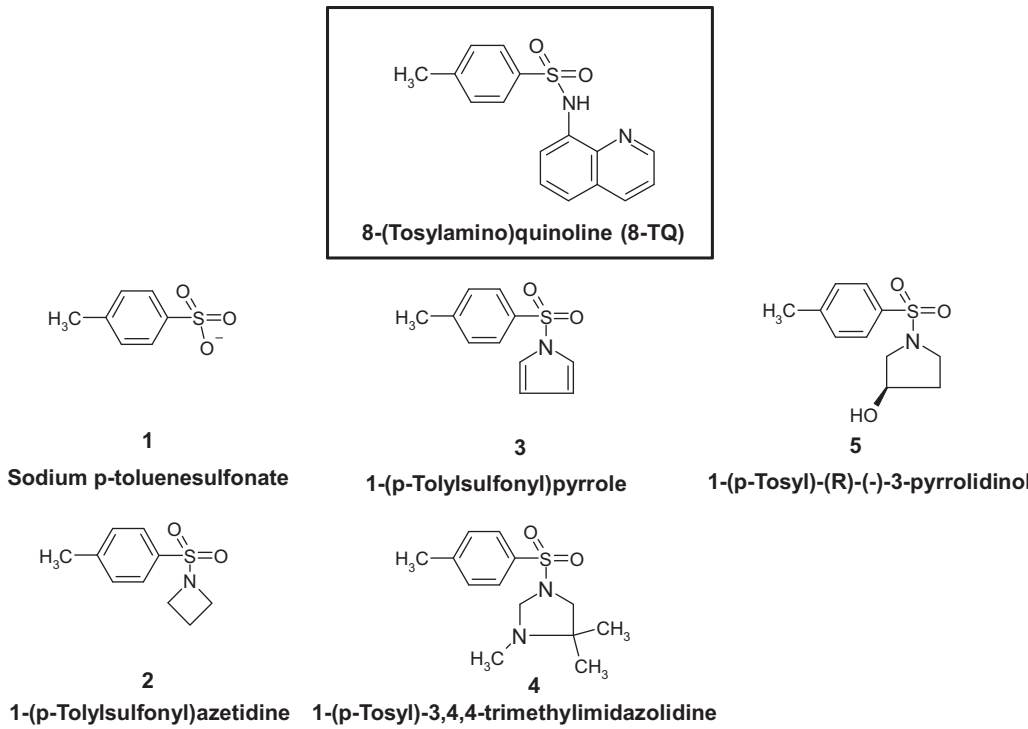

Fig. 1: Chemical structure of 8-TQ and its chemical analogues

to 30 μM (Fig. 2A). The inhibition of cell viability by this compound was accompanied by the induction of apoptosis as assessed increased levels of active caspase-3 (Fig. 2B), and morphological changes of C6, HeLa, and MDA-MB-231 cells (Fig. 2C). None of the other structurally similar compounds that are currently available displayed this inhibitory activity except for BAY11-7082 (Fig. 2D), an IKK inhibitor (Mori et al. 2002), indicating that specific functionality associated with a certain chemical group is required for anti-cancer activity. Interestingly, 8-TQ at concentrations up to 5 μM significantly reduced the invasiveness of MDA-MB-231 cells stimulated by PMA (Fig. 3A), although there was no inhibition of matrix metalloproteinase-2 (MMP-2) and -9 activities, as assessed by zymographic analysis, at concentrations up to 40 μM (Fig. 3B). Furthermore, this compound also significantly suppressed the migration activity of HeLa cells (Fig. 3C) at concentrations that only weakly inhibited cell viability (less than 20% inhibition) (Fig. 2A). Finally, 5 μM 8-TQ also suppressed new blood vessel formation in a chorioallantoic membrane (CAM) assay (Fig. 3D). Therefore, our data strongly suggest that 8-TQ exhibits anti-cancer activity by blocking multiple steps of tumourigenesis.

The fact that this compound suppresses various processes at the same time led us to explore the potential inhibitory mechanism that affected a pathway commonly involved in multiple steps of tumourigenesis (e.g., oncogenic protein-mediated signalling) (Chung et al. 2009). To this end, we focused on intracellular signalling events critical in cell survival, proliferation, invasion, and migration. Intriguingly, 8-TQ dose-dependently decreased the phosphorylation levels of PDK1 and Akt (Fig. 4A, left panel) and also suppressed extracellular signal-regulated kinase (ERK) phosphorylation (Fig. 4A, right panel), indicating that signalling pathways involving these enzymes could be targets of its anti-cancer activity. We next investigated whether these signalling cascades are involved in various tumourigenic responses using specific inhibitors. As Fig. 4B shows, the viability of C6 cells was reduced by treatment with PP2, a Src kinase inhibitor, and LY294002 (LY), a PI3K inhibitor (Geltz and Augustine 1998), but not by U0126, an MEK inhibitor (Duncia et al. 1998), implying that Src and PI3K are important components in maintaining cell survival and proliferation functions. In contrast, U0126 and LY294002 strongly inhibited the migration of HeLa cells and invasion of MDA-MB-293 cells (Figs. 4C and 4D), implying that these two pathways are required for migration and invasion activities of cancer cells. Interestingly, 8-TQ also blocked the formation of the molecular complex between PI3K and Akt, as assessed by immunoprecipitation and western analyses (Fig. 4E). Indeed, a number of papers have reported the role of PI3K/Akt and MEK/ERK pathways in migration and the metastatic potential of cancer cells originated from the colon, breast, and intestines (Meier et al. 2005).

Finally, we investigated whether this compound could function as an anti-cancer drug *in vivo* using an animal model involving implantation of murine T-cell lymphoma RMA cells in mice (Adams et al. 2007). Implanted RMA cells grew well to increase the tumour size in a time-dependent manner. As expected, 8-TQ reduced the size of the tumour by up to 40%, suggesting that this compound might be effective in curing the *in vivo* generated tumour. Although the curative activity of 8-TQ was not complete, we expect that this compound might show stronger preventive activity against tumour generation. Moreover, the fact that this compound did not induce lethality or obvious changes in the animals' body or organs (data not shown) indicates that it could be developed as a non-toxic anti-cancer drug.

In conclusion, we have demonstrated that 8-TQ strongly suppresses various *in vitro* and *in vivo* tumourigenic responses such as proliferation, invasion, migration, and angiogenesis by inhibiting the PI3K/Akt pathway. Although our data indicate *in vivo* efficacy and a good toxicity profile, further preclinical studies of its preventive activity, the inhibitory mode of action on its enzymatic target, and long-term toxicity are needed.

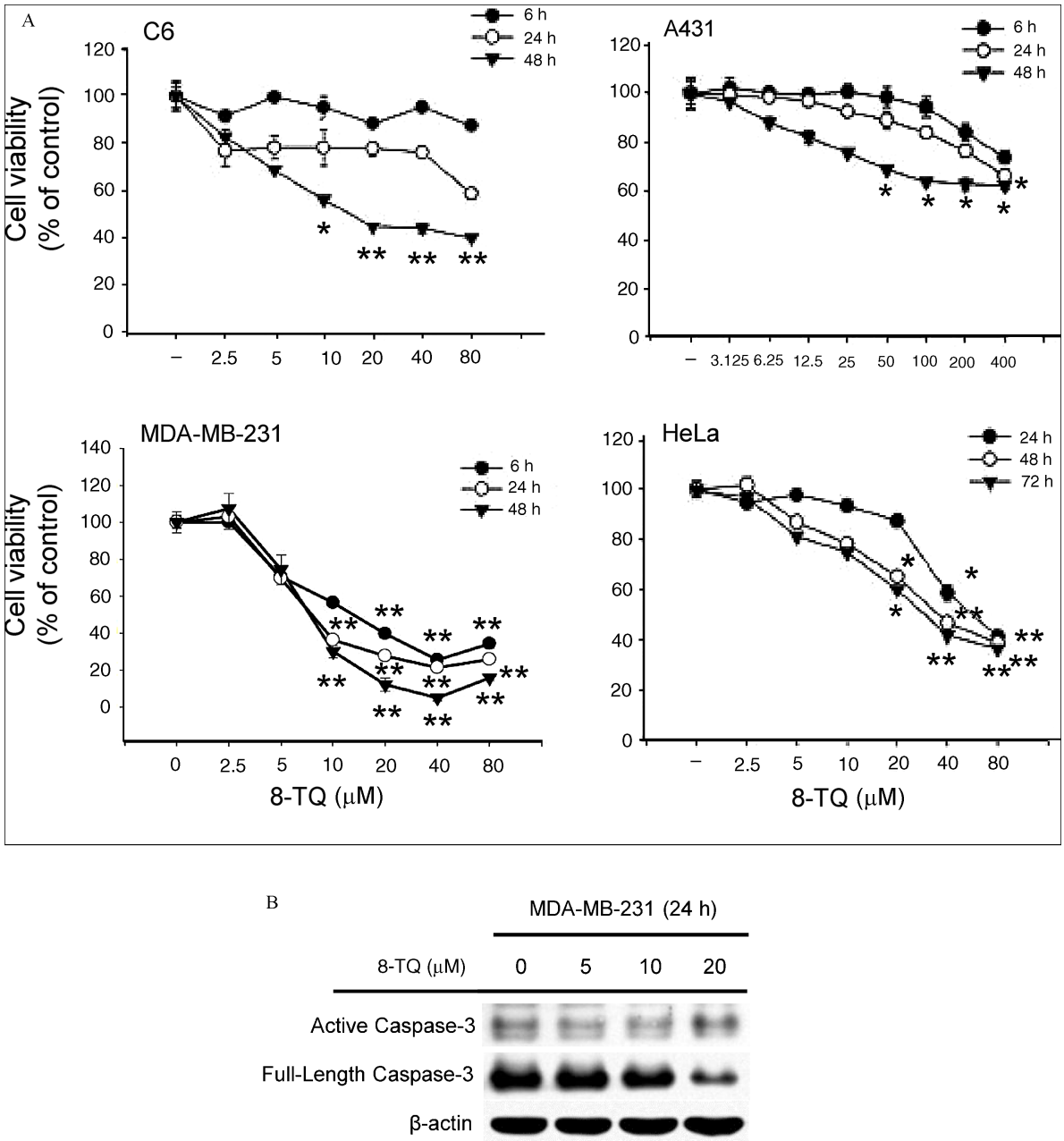

Fig. 2: The effects of 8-TQ and its chemical analogues on the proliferation of C6, A431, HeLa, and MDA-MB-231 cells and the anti-proliferative mechanism of 8-TQ. (A and E) Cells (1×10^6 cells/ml) were incubated with 8-TQ or its chemical analogues for the indicated times. Cell viability was determined by a conventional MTT assay as described in the Experimental section. (B) MDA-MB-231 cells (5×10^6 cells/ml) were incubated with 8-TQ for 24 h. Active, full-length caspase-3, and β-actin were identified using specific antibodies by Western blotting. (C) C6, HeLa, and MDA-MB-231 cells (5×10^5 cells/ml) were incubated with 8-TQ for 24 h. Cytoskeletal (left panel) and morphological (right panel) changes were examined by staining with rhodamine phalloidin and imaging by confocal or inverted microscopy. * $p < 0.05$ and ** $p < 0.01$ compared to normal group

3. Experimental

3.1. Materials

8-TQ, its derivatives (**1** to **5**), phorbol-12-myristate-13-acetate (PMA), and staurosporin were obtained from Sigma (St. Louis, MO, USA). The purity of these compounds was greater than 95% based on HPLC analysis. LY294002, PP2, and U0126 were obtained from Calbiochem (La Jolla, CA, USA). Rat C6, HeLa, A431, and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Antibodies to phospho- or total forms of Src (Y416), PI3K (p85), PDK1, Akt, ERK, p38, JNK, full length caspase-3, and β-actin were purchased from Cell Signalling (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2. Cell culture

RMA, HeLa, A431, C6, and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) or RPMI1640 with 10% foetal bovine serum (HyClone) and 100 U/ml penicillin/streptomycin (HyClone) at 37 °C in a humidified atmosphere with 5% CO_2.

3.3. Cell proliferation assay

Cell viability and proliferation were assessed by a conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide] assay as reported previously. Cells (5×10^4 cells/well) were incubated with various concentrations of test compounds (8-TQ, PP2, LY294002, and U0126) for the indicated times and further incubated with MTT solution (0.5 mg/ml) for an additional 4 h at 37 °C. The absorbance of the samples was measured at 490 nm with a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA).

3.4. Evaluation of morphological changes by confocal microscopy

C6 and HeLa, cells were plated at a density of 2×10^5 cells/well in 12-well plates containing sterile cover slips and grown at 37 °C for 12 h. The cells

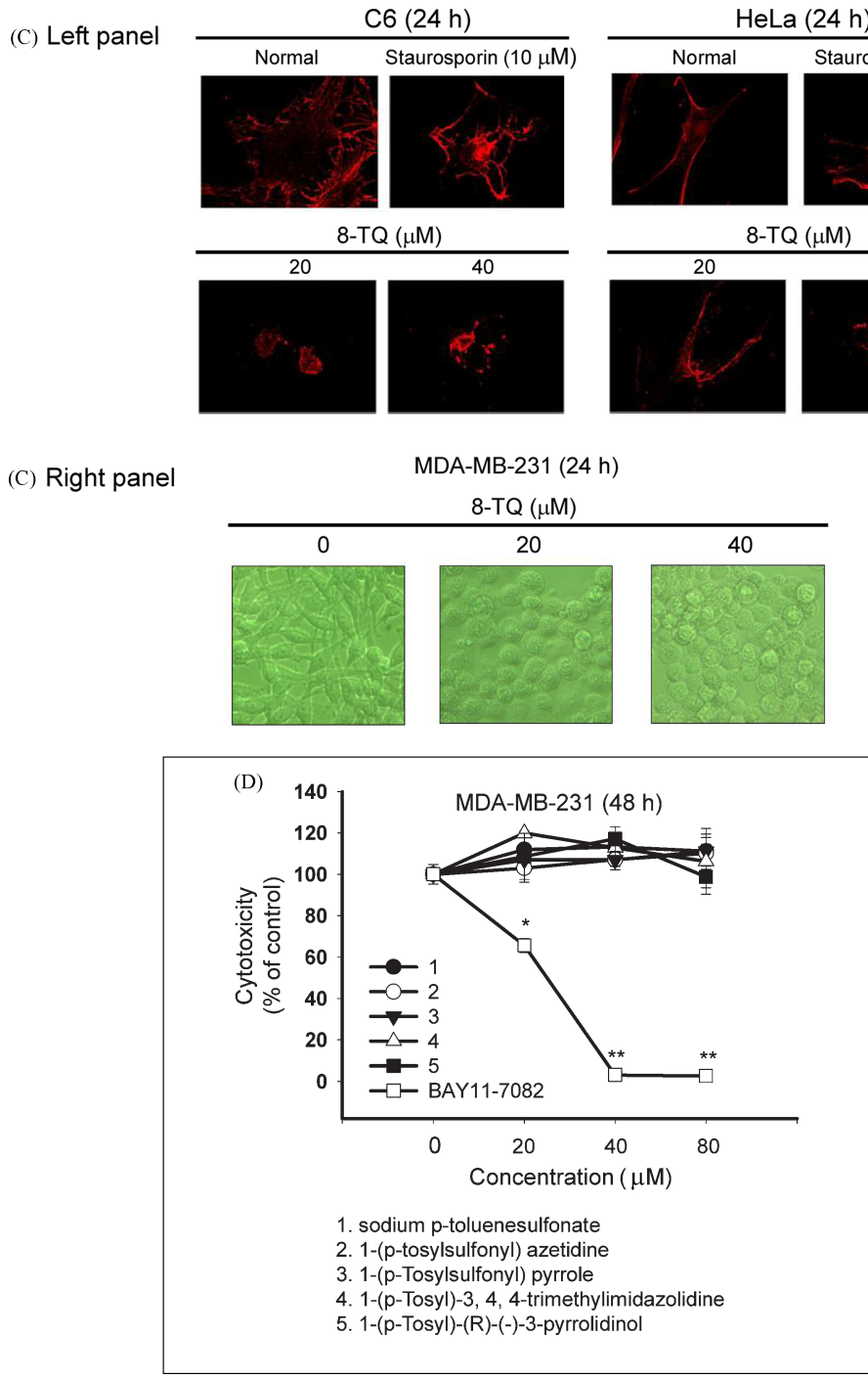

Fig. 2: (*Continued*)

were treated with 8-TQ or staurosporin, washed twice with PBS that was prewarmed to 37 °C, and fixed on the cover slips by incubation in 3.7% formaldehyde for 7 min. The cells were then washed three times with PBS. The coverslips were blocked with 1% BSA for 1 h at room temperature with shaking. For staining of the cytoskeleton, rhodamine phalloidin (1:250; Molecular Probes) in 1% BSA was added, followed by incubation for 1 h in the dark. The coverslips were then washed three times with PBS and mounted onto slides using fluorescent mounting medium (DakoCytomation, Carpentaria, CA, USA). Intensity change in actin cytoskeleton staining was visualised with an Olympus LX70 FV300 confocal microscope (Olympus, Japan).

3.5. Invasion assay

The invasive activity of MDA-MB-231 cells was measured using a Matrigel-coated plate as reported previously (Kim et al. 2004; Koh et al. 2010). After incubation with 8-TQ in the presence or absence of PMA (100 nM) for 24 h, migrated cells were fixed with 4% formaldehyde, stained with haematoxylin and eosin, and counted.

3.6. MMP-2 and -9 activities

MMP-9 activity was measured by gelatin zymography as reported previously (Kim et al. 2004). Conditioned media from cells that were pretreated with 8-TQ in the presence or absence of PMA for three days were concentrated with Centriprep YM-10 (Millipore, MA, USA) at 4 °C. Concentrated proteins (10 μg) were mixed with non-reducing sample buffer containing 0.5 M Tris (pH 6.8), 5% SDS, 20% glycerol, and 1% bromophenol blue at a 1:1 ratio and electrophoresed directly on 10% SDS–polyacrylamide gels (SDS–PAGE) impregnated with 1.5 mg/ml gelatin (Sigma, MO, USA) under non-reducing conditions. After electrophoresis, gels were washed for 1 h at room temperature in a 2.5% (v/v) Triton X-100 solution to remove the SDS, transferred to zymogram development solution (50 mM Tris–HCl, pH 7.6, 5 mM $CaCl_2$), and incubated for 18 h at 37 °C. Gels were stained for 30 min with 0.1% (w/v) Coomassie brilliant blue G250 in 45% (v/v)

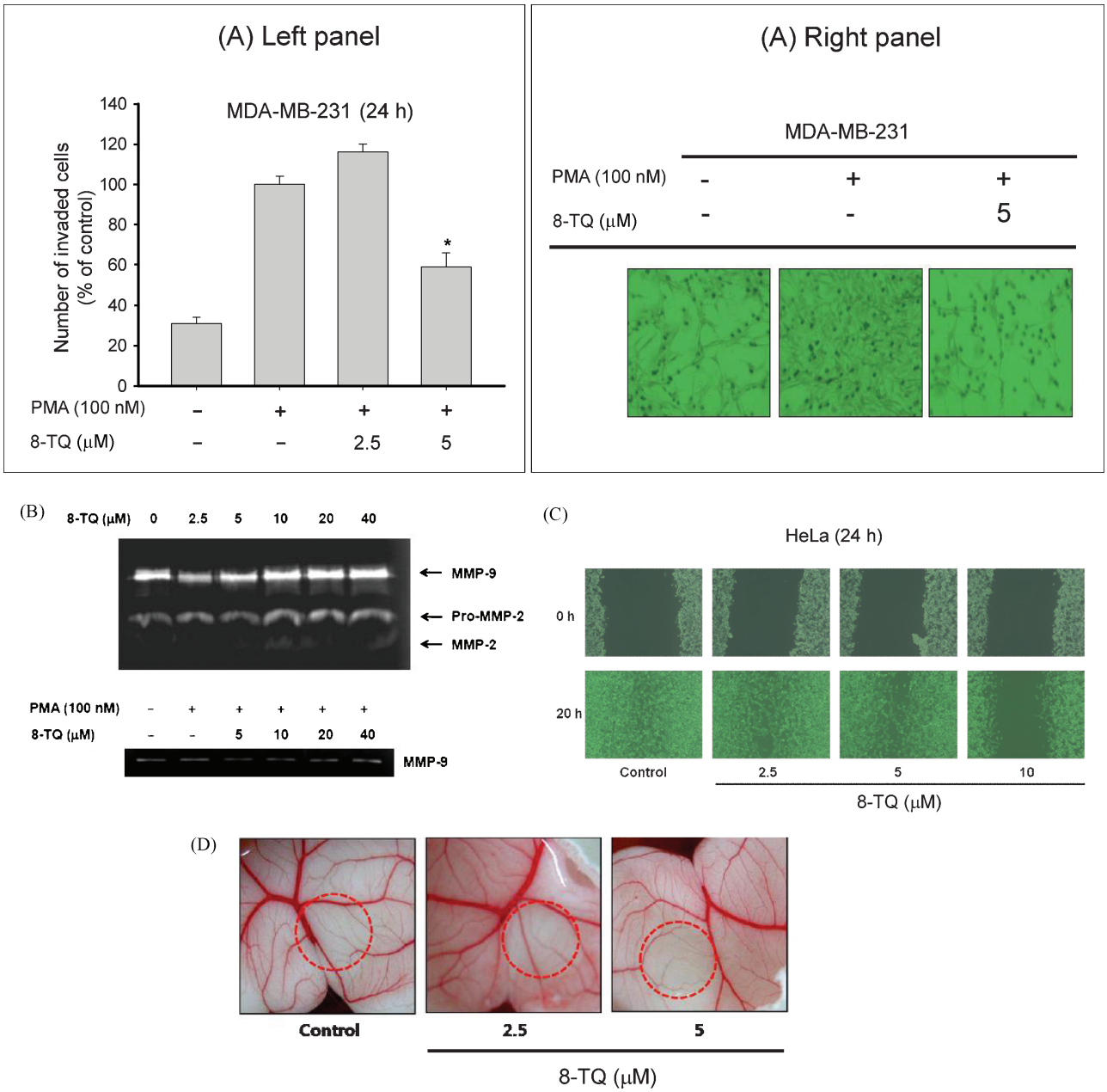

Fig. 3: The effect of 8-TQ on cell invasion, migration, MMP-9 expression, and angiogenesis. (A) Cells (1×10^6 cells/ml) were incubated with 8-TQ in the presence or absence of PMA for 24 h and subjected to a Matrigel invasion assay. Invaded cells were counted with a cell counter and photographed with a digital camera. (B) The effect of 8-TQ on the activity and expression of MMP-9 in PMA-treated MDA-MB-231 cells (5×10^5 cells/ml) was assessed by gelatin zymography (upper) and RT-PCR (lower). (C) HeLa cells (1×10^6 cells/ml) were incubated for 24 h and then scratched with a yellow pipette tip and further incubated in the presence of 8-TQ for an additional 24 h. Photographs were taken with a digital camera. (D) The anti-angiogenic activity of 8-TQ was evaluated by a CAM assay as described in the Experimental section.
* $p < 0.05$ compared to control group

methanol/10% (v/v) acetic acid and destained in 10% (v/v) acetic acid/20% (v/v) methanol. Areas of lysis were observed as white bands against a black background.

3.7. Wound healing assay

HeLa cells were grown to a confluent monolayer in 60-mm plates. A scratch to 8-TQ-treated HeLa cells was introduced by scraping the monolayer with a p200 pipette tip as previously described (Park et al. 2011; Zhao et al. 2006). Images were acquired using an Inverted Phase Microscope (Olympus, Tokyo, Japan).

3.8. Ex vivo chorioallantoic membrane (CAM) assay

Effects on angiogenesis were examined using the chorioallantoic membrane (CAM) assay as reported previously (Kim et al. 2004). Chick embryos were grown using an established window culture method. Fertilised chicken eggs (Pulmuwon, Suwon, Korea) were kept in a humidified incubator (Johnsam, Korea) at 37 °C. After incubation for 3.5 days, approximately 3 ml of albumin were aspirated with a 22-gauge hypodermic needle though a small hole drilled at the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed using forceps, and the shell membrane on the floor of the air sac was peeled away. At the stage of a 4.5-day-old chick embryo, a thermanox coverslip (Nunc, Naperville, IL, USA) loaded with 8-TQ was applied to the CAM. Two days later, an appropriate volume of a 10% fat emulsion (10% Intralipose, Korea Green Cross, Suwon, Korea) was injected into the embryo chorioallantois using a 22-gauge needle and the eggs were observed under a microscope.

3.9. Preparation of cell lysates, immunoprecipitation, and Western blotting (WB)

MDA-MB-231 or C6 cells (5×10^6 cells/ml) treated with 8-TQ for 12 h or 24 h were washed three times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM

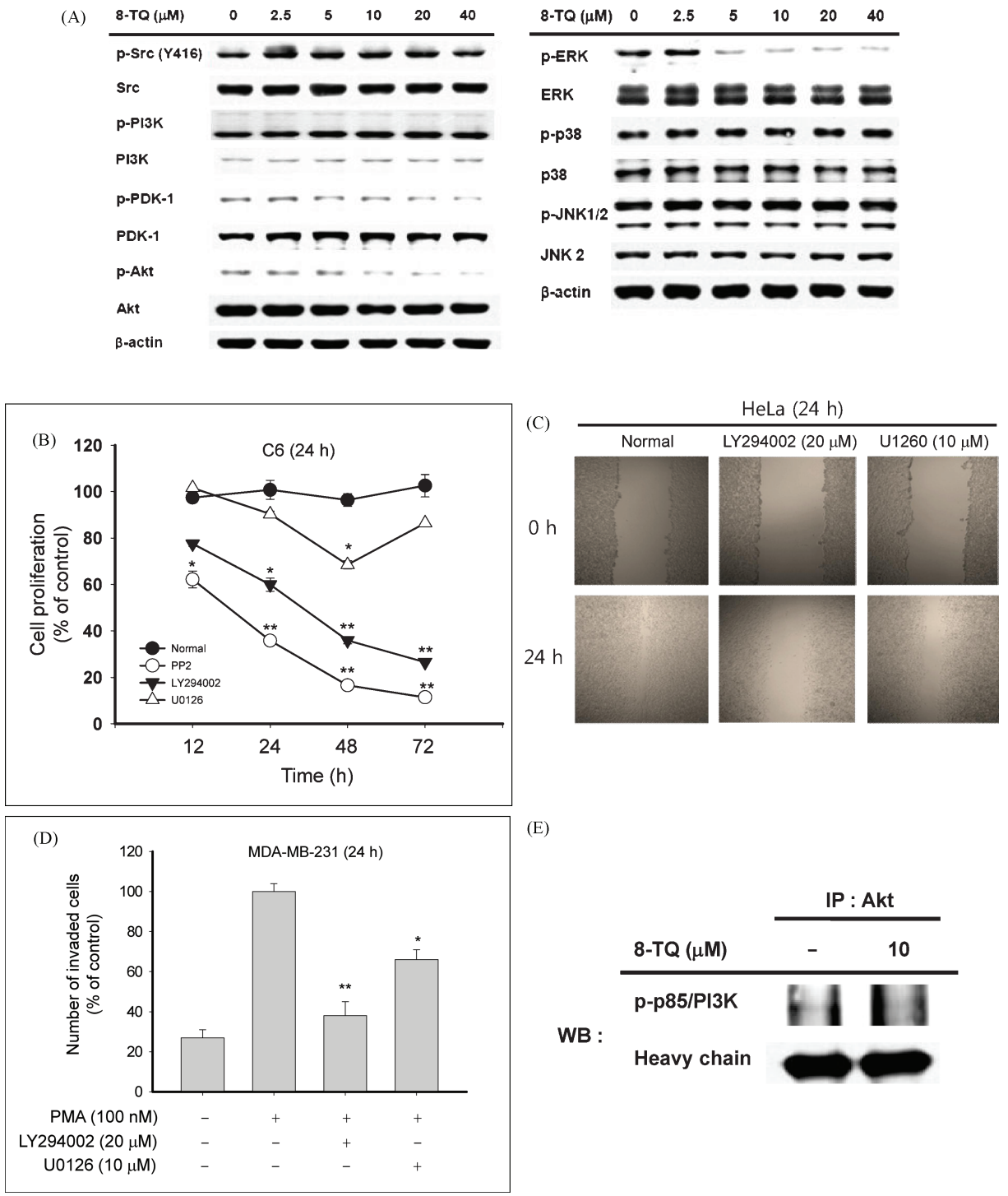

Fig. 4: The effect of 8-TQ on signalling events involved in cell proliferation, migration, and invasion. (A) MDA-MB-231 cells (5×10^6 cells/ml) were incubated with 8-TQ for 12 h. The levels of Src, PI3K (p85), PDK1, Akt, p38, ERK, JNK, and β-actin were identified with antibodies specific for total protein or phospho-protein by Western blotting. (B) C6 Cells (1×10^6 cells/ml) were incubated with LY294002, U0126, or PP2 for indicated times. Cell viability was determined by a conventional MTT assay as described in the Experimental section. (C) HeLa cells (1×10^6 cells/ml) were incubated for 24 h and then scratched with a yellow pipette tip and further incubated with LY294002 (LY) and U0126 (U0) for an additional 24 h. Photographs were taken with a digital camera. (D) MDA-MB-231 cells (1×10^6 cells/ml) were incubated with 8-TQ in the presence or absence of PMA for 24 h and subjected to a Matrigel invasion assay. Invaded cells were counted with a cell counter. (E) The effect of 8-TQ on the formation of a cell survival signalling complex composed of PI3K (p85) and Akt. MDA-MB-231 cells (5×10^6 cells/ml) were treated with 8-TQ for 12 h and subjected to immunoprecipitation and immunoblotting analyses. * $p < 0.05$ and ** $p < 0.01$ compared to normal or control group

sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM PMSF) for 30 min with rotation at 4 °C (Shen et al. 2011). The lysates were clarified by centrifugation at 16,000 g for 10 min at 4 °C and stored at –20 °C until needed.

Cell lysates containing equal amounts of protein (500 μg) from MDA-MB-231 cells (1×10^7 cells/ml) treated with or without 8-TQ for 12 h were precleared with 10 μl protein A-coupled sepharose magnetic beads (50% v/v) (Elpis Biotech, Daejeon, Korea) for 1 h at 4 °C. Precleared samples were incubated with 5 μl anti-Akt antibody overnight at 4 °C. Immune complexes were mixed with 10 μl protein A-coupled Sepharose magnetic beads (50% v/v) and rotated for 3 h at 4 °C.

Whole cell lysates were analysed by Western blotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidenedifluoride (PVDF) membranes by electroblotting. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4 °C, washed three times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibody. The levels of

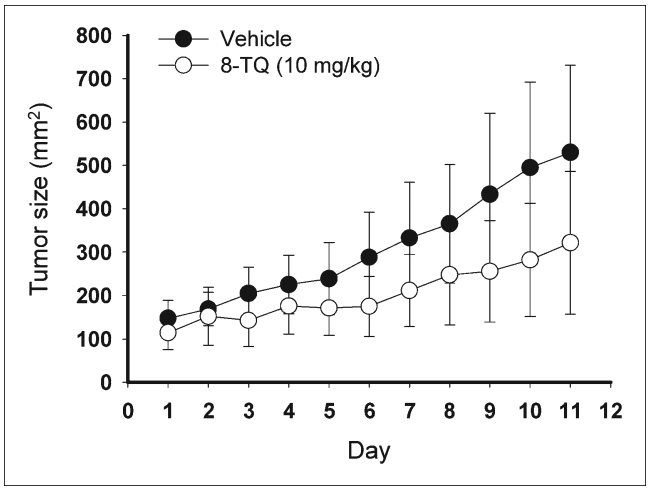

Fig. 5: Effect of 8-TQ on the suppression of tumour growth in the mice transplanted with RMA cells. RMA cells (5×10^7) in 100 μl PBS were subcutaneously transplanted into dorsal lesions of mice. After formation of the tumour mass, 8-TQ was intraperitoneally injected at a dose of 10 mg/kg/day. Tumour size was measured using callipers every day for 11 days

total and phosphorylated full length caspase-3, Src, PI3K (p85), PDK1, Akt, ERK, p38, JNK and β-actin were visualised using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK), as reported previously (Yoo et al. 2011).

3.10. In vivo anti-cancer activity

C57BL/6 male mice (four weeks old, weighing ~20 g) were obtained from DBL (Chungbuk, Korea) and maintained in plastic cages under conventional conditions. Water and pellet diets (Samyang, Daejeon, Korea) were available *ad libitum*. Studies were performed in accordance with guidelines established by the Kangwon University Institutional Animal Care and Use Committee. RMA cells (5×10^7) in 100 μl PBS were subcutaneously administrated into dorsal lesions of the mice. After formation of a tumour mass, 8-TQ was injected intraperitoneally at a dose of 10 mg/kg/day. An equal volume of vehicle (DMSO in PBS) was injected into the control group. The tumour size was measured using callipers every day for 11 days.

3.11. Statistical analysis

Data are presented as the mean ± S.E.M of at least four (Figs. 2A, 2E, 3A Left panel, 4B, and 4D) or three (Fig. 5) independent experiments performed with three samples or six mice and were subjected to the statistical analysis. The other results are shown as one representative experiment with a total of three. The results of relative intensity were calculated from these three samples. For statistical comparison, these results were analyzed using analysis of variance/Scheffe's post-hoc test and Kruskal-Wallis/Mann-Whitney test. A $P < 0.05$ was considered a statistically significant difference. All statistical tests were carried out using the computer program SPSS (SPSS Inc., Chicago, IL).

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