MedChemComm

RESEARCH ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Med. Chem. Commun.*, 2018, **9**, 1826

Assessment of a bifendate derivative bearing a 6,7-dihydro-dibenzo[*c*,*e*]azepine scaffold as a potential anti-metastatic agent†

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Received 11th June 2018,
Accepted 17th September 2018

DOI: 10.1039/c8md00294k

rsc.li/medchemcomm

Multidrug resistance (MDR) and metastasis are major causes of mortality in patients with cancer. We recently reported a bifendate derivative bearing a dibenzo[*c*,*e*]azepine scaffold (**4i**) as a P-gp and BCRP-medicated MDR reversal agent. As a continuation of the previous research, its ability to inhibit cancer metastasis was investigated in MDA-MB-231 cells in the present work. Wound-healing and chamber migration assays showed that **4i** could significantly attenuate the migration of MDA-MB-231 cells. Additionally, **4i** obviously suppressed the invasive activity of MDA-MB-231 cells, thus displaying potential anti-metastasis activity. Preliminary mechanism studies indicated that the anti-metastasis activity of **4i** was associated with the inhibitory effect on the activity and expression of MMP-2 and MMP-9. These results, together with the previous findings, suggest that compound **4i** could be a promising lead for the development of novel anti-cancer agents with anti-MDR and metastatic activities.

Introduction

Multidrug resistance (MDR) is regarded as a major obstacle to successful chemotherapy in cancer patients.[1–3] One of the most widespread mechanisms of MDR is the overexpression of ATP-binding cassette (ABC) transporters at the plasma membrane,[4–6] which can efflux various chemotherapeutic drugs out of cancer cells, thus significantly reducing intracellular drug concentration and consequent drug insensitivity. The most extensively characterized ABC transporters include ABCB1 (P-glycoprotein, P-gp), ABCG2 (BCRP) and ABCC1 (MRP1).[7–9] Theoretically, inhibiting the overexpression and/or activity of ABC transporters is a promising strategy to overcome MDR of cancer cells.

Besides MDR, metastasis is also a leading cause of mortality in patients with cancer.[10,11] When metastasis occurs, the survival prospect of patients becomes considerably worse, resulting in approximately 90% death in cancer patients. Therefore, prevention or suppression of metastasis has become a severe challenge in the field of cancer research, and the development of efficient anti-metastatic drugs is thus of vital significance in the management of cancer. Actually, cancer metastasis involves an extremely complex sequence of events, including detachment from the primary site, migration, invasion into the surrounding tissue, intravasation, entering and traveling through the circulation, extravasation and establishment of new foci of malignancy at a secondary site.[12,13] Among them, the principal steps are migration and invasion, which play significant roles in the progression of cancer metastasis. Therefore, blocking cancer cell migration and invasion is regarded as a potential therapeutic strategy for the treatment of cancer metastasis.[14]

Recently, we have described a series of bifendate derivatives bearing a dibenzo[*c*,*e*]azepine scaffold as novel anti-MDR agents. Among them, compound **4i** (Fig. 1) can potently reverse P-gp and BCRP-mediated MDR *via* blocking P-gp and BCRP efflux function.[15,16] Interestingly, Sun *et al.* found that bifendate displayed potential anti-metastatic activity by inhibiting the adhesion and invasion of cancer cells with high metastasis potential at non-cytotoxic concentrations.[17]

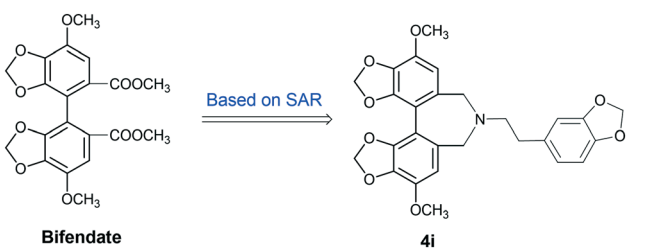

Fig. 1 Chemical structures of bifendate and compound **4i**.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8md00294k

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In addition, recent research showed that schisandrin B, a natural product with an alkoxyl biphenyl scaffold, could suppress cancer migration and invasion by inhibiting the epithelial–mesenchymal transition.[18] With these in mind and considering that compound **4i** possessed a similar alkoxyl biphenyl scaffold to bifendate and schisandrin B, we determined whether compound **4i** could attenuate the migration and invasion of cancer cells. Additionally, the preliminary anti-metastatic mechanism of **4i** was also investigated in the present work.

Results and discussion

Anti-migratory activities of 4i

As mentioned above, cell migration is a critical step in cancer metastasis.[19,20] Therefore, we firstly determined the effects of compound **4i** on the motility properties of MDA-MB-231 cells by a wound-healing assay and a chamber migration assay, respectively.[21] The lead compound bifendate, and the well-documented anti-metastatic compounds baicalein and **LG500** were selected as positive control group sets.[22,23] As shown in Fig. 2, compound **4i** can significantly inhibit the migration of MDA-MB-231 cells across the wounded space at doses below IC_{50} (the cytotoxicity of **4i** against MDA-MB-231 cells was determined by the MTT assay, and the IC_{50} value was 88 μM). Meanwhile, bifendate showed little inhibitory effect on the migration of MDA-MB-231 cells even at the highest dose (45 μM). Notably, the anti-migration activity of **4i** is comparable to that of the positive controls baicalein and **LG500** under our experimental conditions.

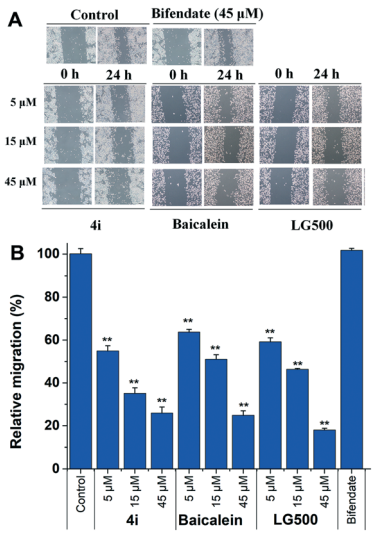

Fig. 2 Inhibitory effect of **4i** on MDA-MB-231 cell migration *in vitro*. (A) **4i** inhibits the migration of cells across the wounded space. The distance of the wound edge was measured before and after the treatment. Baicalein and **LG500** were selected as positive control group sets. Image magnification: ×100. (B) Quantification of the relative migration. Relative migration (%) was identified by dividing the migration distance of MDA-MB-231 cells treated with **4i**, baicalein, **LG500** or bifendate by that of the control group. $^{**}P < 0.01$ *vs.* control group.

Subsequently, the inhibitory effect on cell mobility of **4i** was confirmed by the chamber migration assay. As shown in Fig. 3, **4i** can result in an obvious delay in cell migration up to 32.9% and 47.5% at the dose of 15 and 45 μM. These results suggested that **4i** displayed potent anti-migratory activity.

Anti-invasive activities of 4i

Cell invasion is another critical step in cancer metastasis.[24] We next determined the anti-invasive activities of **4i** by an invasion assay. As shown in Fig. 4, in the absence of **4i**, MDA-MB-231 cells displayed high invasive capability, for the cells were able to penetrate through the Matrigel-coated filters completely (Fig. 4A, control group). In sharp contrast, **4i** can significantly decreased the number of MDA-MB-231 cells that penetrate through the Matrigel-coated filters, and the inhibitory rates of invasion were up to 54.4% and 71.2% at the dose of 15 and 45 μM, respectively, indicating that **4i** could markedly suppress the invasive activity of MDA-MB-231 cells.

Effect of 4i on the activity and protein expression of MMP-2 and MMP-9

The mechanism underlying cancer metastasis is complex, which involved a series of metastatic cascades. Plenty of biomolecules and signal transduction pathways play significant roles in the progression of cancer metastasis. Among them, matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, play critical roles in the invasion and metastasis of malignant cells.[25,26] To probe the potential anti-invasive mechanisms of **4i**, we determined the effect of **4i** on the activity and protein expression of MMP-2 and MMP-9 in MDA-MB-231 cells. Firstly, gelatin zymography was carried out to examine the effect of **4i** on the activity of MMP-2 and MMP-9. As shown in Fig. 5A, the activities of MMP-2 and MMP-9 were reduced in a dose-dependent manner after treatment with **4i** in MDA-MB-231 cells. Quantification analysis showed that **4i**, at the highest concentration (45 μM), could decrease the MMP-2 and MMP-9 activity to 56.9% and 30.8%, respectively. Subsequently, western blot analysis was used for evaluating the effect of **4i** on the expression of MMP-2 and MMP-9. As shown in Fig. 5B, **4i** could dose-dependently suppress the protein expression of MMP-2 and MMP-9 in MDA-MB-231

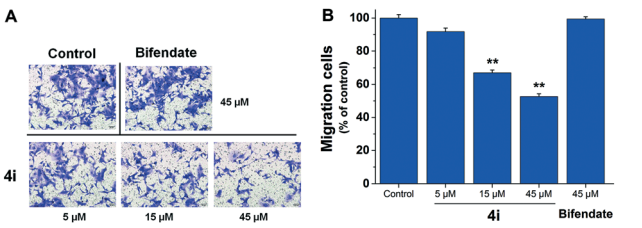

Fig. 3 Effect of **4i** on the mobility of MDA-MB-231 cells *in vitro*. (A) **4i** inhibits cell migration. Image magnification: ×200. (B) Quantification of the migrated cells. Migrated cells (% of control) were identified by dividing the number of migrated MDA-MB-231 cells treated with **4i** or bifendate by that of the control group. $^{**}P < 0.01$ *vs.* control group.

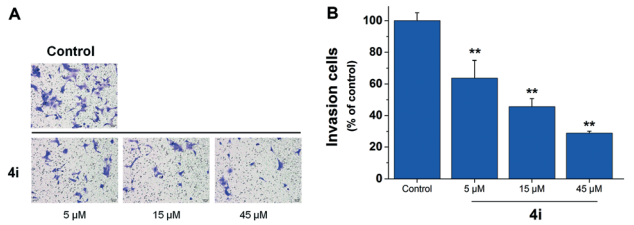

Fig. 4 Effect of **4i** on the invasion of MDA-MB-231 cells *in vitro*. (A) **4i** inhibits cell invasion. The invaded cells were measured by crystal violet staining. Image magnification: ×200. (B) Quantification of the invaded cells. Invaded cells (% of control) were identified by dividing the number of invaded MDA-MB-231 cells treated with **4i** by that of the control group. $^{**}P < 0.01$ *vs.* control group.

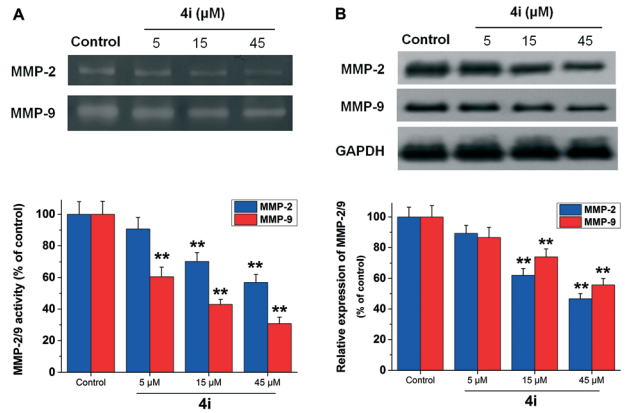

Fig. 5 Effects of **4i** on the activity and expression of MMP-2 and MMP-9 in MDA-MB-231 cells. (A) **4i** inhibits the activity of MMP-2/9 in MDA-MB-231 cells. MMP-2/9 activity was determined by the gelatin zymography assay. (B) **4i** suppresses the protein expression of MMP-2/9. $^{**}P < 0.01$ *vs.* control group.

cells. These data suggested that the anti-metastatic activity of **4i** might be associated with the inhibitory effect on MMP-2 and MMP-9 activity and expression.

Materials and methods

Chemicals and reagents

Compound **4i** was synthesized as previously described,[15] and dissolved in dimethyl sulfoxide (DMSO) as a stock solution (stored at −20 °C). The stock solution was diluted with the medium before each experiment. The controls were treated with the same amount of DMSO (0.1%) in the corresponding experiments. Fibronectin and Matrigel were purchased from BD Biosciences (Bedford, MA). A Transwell chamber (3428) and a six-well cell culture plate (3516) were purchased from Corning Incorporated (Cambridge, MA). Antibodies to MMP-2 (KG22546) and MMP-9 (KG22551) were obtained from Jiangsu keyGEN Biotechnology Co., Ltd. (Jiangsu, China). Baicalein was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). All other chemicals and agents were obtained from commercial sources and are of analytical grade.

Cell lines and cell culture

Due to their highly metastatic and invasive capacity, MDA-MB-231 cells (a human breast carcinoma cell line) were selected as model cells in this study. The MDA-MB-231 cells were purchased from the Cell Bank of the Shanghai Institute of Cell Biology (Shanghai, China). The MDA-MB-231 cells were cultured in Leibovitz's L15 medium (Gibco, Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA), 100 unit per mL penicillin and 100 μg mL^{-1} streptomycin (Beyotime Institute of Biotechnology, Haimen, China) at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

Colorimetric MTT assay

The MDA-MB-231 cells (10^4 per well) were seeded in 96-well plates for 24 h and then exposed to different concentrations of compound **4i**. After incubation for 72 h, the colorimetric MTT assay was executed and the cell growth inhibitory rate (%) was calculated according to a previous method.[27] IC_{50} was taken as the concentration that caused 50% inhibition of cell growth and was calculated by the Logit method.

Wound-healing assay

The MDA-MB-231 cells were seeded in 6-well plates until confluence. The monolayer of cells was wounded by scraping with a sterile 200 μL pipette tip (Axygen, Union city, CA). After wounding, the cells were washed with PBS twice to remove detached cells and then incubated with **4i**, baicalein, **LG500** (5, 15, or 45 μM), or bifendate (45 μM), respectively. The plates were photographed at 0 h and 24 h with an inverted microscope (Olympus) equipped with a digital camera (Nikon, Japan, Tokyo) to determine the relative distance of cell migration, and five randomly chosen fields were analyzed for each well.

Chamber migration and invasion assays

In the chamber migration assay, a Transwell system was utilized. The MDA-MB-231 cells with or without compound **4i** (5, 15, or 45 μM, 24 h) or bifendate (45 μM, 24 h) were suspended in serum-free medium, and then seeded into the upper compartment, while the medium containing 10% fetal bovine serum was added into the lower compartment. After incubation for 4 h, the non-migrated cells on the upper side of the membrane were removed with a cotton swab. The migrated cells on the bottom surface were fixed with 100% methanol and stained with 0.5% crystal violet. The migrated cells were quantified by manual counting and three randomly chosen fields were analyzed for each group.

Invasion assays were performed with chambers as described previously.[21] The assay was performed essentially as the chamber migration assays above except that the Transwell chambers were initially coated with Matrigel (40 μg/100 μL per chamber) at 37 °C for 1 h. After incubation for 24 h,

the invaded cells were stained and analyzed as conducted above.

Gelatin zymography assay

The activity of MMP-2 and MMP-9 in MDA-MB-231 cells was determined by the gelatin zymography assay. Briefly, the cells were treated with or without compound **4i** (5, 15, or 45 μM) in serum-free medium for 24 h, and then the supernatants were collected. The gelatin zymography assay was performed according to the previous method.[21] After electrophoresis, the gels were washed twice with 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3 and 2.5% Triton X-100 (v/v) for 30 min, followed by brief rinsing in washing buffer without Triton X-100. The gels were then incubated at 37 °C for 36 h in 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.05% NaN_3, pH 7.5. The digestion was terminated and the gels were stained with 0.1% Coomassie Brilliant Blue R250 followed by destaining with 10% acetic acid and 10% methanol. After treatment, enzyme-digested regions were observed as white bands against a blue background. The zones of enzymatic activity were seen as negatively stained bands.

Western blot analysis

The cells seeded in six-well plates were treated with various concentrations of **4i** (5, 15, or 45 μM) for 24 h, then collected and lysed. Western blot analysis was conducted according to previous methods.[21] The membranes were exposed to the BCIP/NBT alkaline phosphatase color-developing reagent for 15 min. All the blots were stripped and reprobed with polyclonal anti-GAPDH to verify equal protein loading.

Statistical analysis

Differences in the parameters between two cell groups were analyzed by a two-tailed Student's *t* test. $P < 0.05$ was considered as statistically significant.

Conclusions

In summary, MDR and metastasis are major causes of cancer chemotherapy failure. Earlier studies showed that **4i** exhibited a significant inhibitory effect on P-gp and BCRP efflux function, thus displaying potent anti-MDR activity. In the present work, the anti-metastatic activities of **4i** were investigated by wound-healing, chamber migration and invasion assays. Excitingly, the data showed that **4i** could significantly inhibit the migration and invasion of MDA-MB-231 cells, thus displaying potential anti-metastatic activity. Preliminary mechanism studies indicated that the anti-metastatic activity of **4i** was possibly related to its inhibitory effect on MMP-2 and MMP-9 activity and expression. Notably, a new synthetic compound **2H7** bearing the same 6,7-dihydro-dibenzo[*c*,*e*]azepine scaffold as **4i** also displayed potent anti-migration activity against MDA-MB-231 cells (see the ESI†). These results, together with the anti-MDR results, suggested that compound **4i** might be a promising lead for the development of novel anti-MDR and metastatic agents.

Conflicts of interest

The authors declare no competing interest.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 81703359 and 81402789), the Natural Science Foundation of Jiangsu Province (Grant No. BK20171179), the Foundation for Jiangsu Six Talent Peaks Program (Grant No. 2017-YY-039), the Jiangsu Provincial 333 High-level Talents Cultivation Project (2016) and the Science and Technology Project of Xuzhou City (Grant No. KC16SG254). We thank Associate Professor Chenglin Li (Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou, China) for providing compound **LG500**.

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