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CaMK phosphatase (CaMKP/POPX2/PPM1F) inhibitors suppress the migration of human breast cancer MDA-MB-231 cells with loss of polarized morphology

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ABSTRACT

CaMK phosphatase (CaMKP/POPX2/PPM1F) is a Ser/Thr protein phosphatase that belongs to the PPM family. Accumulating evidence suggests that CaMKP is involved in the pathogenesis of various diseases, including cancer. To clarify the relationship between CaMKP activity and human breast cancer cell motility, we examined the phosphatase activity of CaMKP in cell extracts. CaMKP activity assays of the immunoprecipitates prepared from the cell extract revealed that cells exhibiting higher motility had higher CaMKP activity, with no significant differences in the specific activity being observed. Two CaMKP-specific inhibitors, 1-amino-8-naphthol-4-sulfonic acid (ANS) and 1-amino-8-naphthol-2,4-disulfonic acid (ANDS), inhibited the migration of highly invasive MDA-MB-231 breast cancer cells without significant cytotoxicity, while an inactive analog, naphthionic acid, did not. Furthermore, the cells lost their elongated morphology and assumed a rounded shape following treatment with ANS, whereas they retained their elongated morphology following treatment with naphthionic acid. Consistent with these findings, ANS and ANDS significantly enhanced the phosphorylation level of CaMKI, a cellular substrate of CaMKP, while naphthionic acid did not. The present data suggest that CaMKP could be a novel therapeutic target for cancer metastasis.

1. Introduction

CaMK phosphatase (CaMKP) is a Mn^{2+}-dependent, calyculin A/okadaic acid-insensitive Ser/Thr protein phosphatase that belongs to the PPM family [1,2]. It was first discovered in rat brain extracts as a dephosphorylating activity toward a phosphorylated peptide surrounding the Thr286 autophosphorylation site of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II [3]. Subsequently, POPX2 was identified as a binding partner of PIX, the p21-activated protein kinase-interacting guanine nucleotide exchange factor, and was proven to be a human ortholog of CaMKP [4]. Since 2004, CaMKP has often been called PPM1F based on the systematic nomenclature for PPM family phosphatases [5].

CaMKP was reported to dephosphorylate and regulate not only protein kinases such as CaMKs but also other proteins involved in cellular signaling and morphogenesis [1,2]. Thus, determination of its physiological roles could provide new insights toward understanding the whole picture of intracellular signal transduction based on protein phosphorylation. Meanwhile, accumulating evidence suggests that CaMKP is involved in the pathogenesis of various diseases, including cancer, heart diseases, and psychiatric disorders [1,2]. Thus, CaMKP has recently received increased

Abbreviations: ANDS, 1-amino-8-naphthol-2,4-disulfonic acid; ANS, 1-amino-8-naphthol-4-sulfonic acid; BSA, bovine serum albumin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKP, CaMK phosphatase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FCS, fetal calf serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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attention as a novel drug target for these diseases. Furthermore, CaMKP-specific inhibitors could become powerful tools for treating these diseases as novel pharmaceutical agents.

Susila et al. [6] were the first to demonstrate that CaMKP is involved in the migration and invasion of human breast cancer cells. Specifically, they demonstrated that knockdown of CaMKP inhibited the motility and invasiveness of breast cancer cells, while overexpression of CaMKP enhanced these properties. These findings were subsequently confirmed by other research groups using different experimental systems [7,8]. Cancer metastasis is a complex process composed of multiple steps, including epithelial−mesenchymal transition, polarity formation, migration/invasion, colonization, and angiogenesis [9], and CaMKP is known to play various roles in these steps [2]. However, most previous studies were based on knockdown or overexpression of CaMKP, and CaMKP activity is known to be regulated by both activators [10,11] and covalent modifications such as phosphorylation [12] and disulfide formation [13]. Furthermore, CaMKP can serve as a scaffolding protein, rather than a phosphatase, in some cases [14]. Therefore, it is important to clarify whether the phosphatase activity of CaMKP is involved in the processes that lead to cancer metastasis. However, there is surprisingly little data available for the relationship between CaMKP phosphatase activity and cancer metastasis.

In the present study, we established an assay system to directly measure the phosphatase activity of CaMKP in human breast cancer cells, and then compared the CaMKP activities among different types of these cells. To further explore the role of CaMKP activity in cancer cell migration, we examined the effects of two CaMKP inhibitors, 1-amino-8-naphthol-4-sulfonic acid (ANS) and 1-amino-8-naphthol-2,4-disulfonic acid (ANDS) [15], on the migration of human breast cancer MDA-MB-231 cells. Our findings suggest that the inhibitors could abrogate the polarized cell morphology by inhibiting endogenous CaMKP, leading to inhibition of cell migration with no significant effect on cell viability.

2. Materials and methods

2.1. Materials

The two CaMKP inhibitors (ANS and ANDS) and an inactive analog (naphthionic acid) were obtained from Tokyo Chemical Industry. A phosphopeptide substrate, pp10 (YGGMHRQET(p)VDC), which contains an amino acid sequence surrounding the autophosphorylation site of CaMKII, was synthesized as described [16–18]. Construction of the pcFLAG-CaMKIα-myc-His_6 plasmid was described previously [18]. An anti-c-Myc antibody was purchased from Fujifilm-Wako. A polyclonal antibody against human CaMKP was raised by immunizing rabbits with an immunogen sequence, CQDLPSSLPEPETQAPPRS, corresponding to amino acid residues 437–454 in human CaMKP. Immunization was carried out as described previously [19], and the antibody was purified by affinity chromatography using immunogen sequence-coupled agarose [20]. An antibody against phospho-CaMKI (Thr177) was prepared as previously described [15]. Human breast cancer cell lines MDA-MB-231 cells were purchased from European Collection of Authenticated Cell Cultures. MCF-7 and T47D cells were obtained from American Type Culture Collection.

2.2. Immunoprecipitation and protein phosphatase assay of endogenous CaMKP

MDA-MB-231, MCF-7, and T47D cells were cultured in growth medium (Dulbecco's modified Eagle's medium [DMEM] [Fujifilm-Wako] containing 2.5 mM glutamine and 100 μg/mL penicillin G and streptomycin supplemented with 10% fetal calf serum [FCS]) in a humidified incubator at 37 °C under 5% CO_2. Breast cancer cells at 70% confluency in 9-cm dishes were detached with 1 mL of 0.25w/v% trypsin-1 mmol/L EDTA 4Na solution (Fujifilm-Wako) and suspended in 7 mL of DMEM containing 10% FCS. After centrifugation (1000 rpm for 5 min), the cell pellets were washed with 10 mL of phosphate-buffered saline (PBS), resuspended in 1 mL of PBS, placed in 1.5-mL tubes, and centrifuged again (1000 rpm for 5 min). Following resuspension of the cell pellets in 200 μL of homogenization buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, and cOmplete Mini, EDTA-free protease inhibitor cocktail [Roche; 1 tablet/10 mL]), the cells were disrupted by sonication (30 s × 5 times) in a Model W-225 sonicator (Heat Systems Ultrasonics). The disrupted cells were centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatant was ultracentrifuged at 75,000 rpm for 1 h at 4 °C. The resulting supernatant was used as the cell extract for the protein phosphatase assay after determination of the protein concentration.

The cell extract (185 μg protein) was placed in a 1.5-mL tube and homogenization buffer was added to a final volume of 100 μL. Next, 1 μL of anti-CaMKP antibody or negative control anti-GOT1 polyclonal antibody (Aviva Systems Biology) was added and incubated for 30 min on ice, followed by addition of 10 μL of SureBeads Protein G Magnetic Beads (Bio-Rad) and incubation for 1 h at 4 °C. The magnetic beads were washed three times with 1 mL of Tris-buffered saline (TBS) and resuspended in 10 μL of CaMKP dilution buffer (50 mM Tris-HCl pH 7.5, 0.05% Tween 20, 1 mM DTT). The bead suspension was analyzed by the *in vitro* phosphatase assay or SDS-PAGE followed by western blotting.

The protein phosphatase assay was carried out using the pp10 phosphopeptide as a substrate. The reaction mixture (50 μL) comprised 50 mM Tris-HCl pH 7.5, 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 20 μM pp10, 0.1 μM calyculin A, and bead suspension (5 μL). The phosphatase reaction was initiated by addition of the substrate and allowed to proceed at 30 °C for 3 h. The reaction was terminated by addition of 5 μL of 2 M HCl, and the magnetic beads were removed by magnetic separation. The amount of inorganic phosphate released from the substrate into the mixture was determined by the malachite green assay as described previously [17].

2.3. Transwell migration assay

Breast cancer cells at 70% confluency in 9-cm dishes were detached with 0.25w/v% trypsin-1 mmol/L EDTA 4Na solution and resuspended in growth medium. After centrifugation (1000 rpm for 5 min), the cell pellets were washed with PBS and resuspended in serum-free DMEM. Next, 2.5×10^5 cells in 500 μL of serum-free medium were seeded onto cell culture inserts (8-μm pore size) in 24-transwell dishes (Becton Dickinson). The lower chamber was filled with 500 μL of DMEM containing 1% FCS, and CaMKP inhibitors or naphthionic acid which had been dissolved in 10% dimethyl sulfoxide (DMSO) were added to a final concentration of 0–30 μM (0.1% DMSO). The same concentration of CaMKP inhibitors or naphthionic acid was added to the culture insert, and the cells were allowed to migrate for 22 h in a humidified incubator at 37 °C under 5% CO_2. Non-migrated cells were removed from the upper surface of the insert membranes using a cotton swab. Cells that had migrated to the lower surface of the insert membranes were fixed and stained using a Diff Quick Staining Kit (Sysmex). The numbers of migrated cells were counted in 4 or 5 different fields for each insert. Each experiment was performed in triplicate.

2.4. Cytotoxicity assay

MDA-MB-231 cells (2.5×10^4; 100 μL) were cultured for 24 h in growth medium in a 96-well plate at 37 °C under 5% CO_2. After two rinses with 100 μL of PBS, the cells were cultured in 100 μL of serum-free DMEM containing 0–30 μM CaMKP inhibitors or naphthionic acid for 22 h at 37 °C under 5% CO_2. After further washing with 100 μL of PBS, 100 μL of DMEM containing 0.2% FCS and 10 μL of CCK-8 (Dojindo) were added and the cells were incubated at 37 °C under 5% CO_2. The absorbance at 450 nm was measured every 1 h for 3 h using a microplate reader (Thermo Type 357). The absorbance change per hour was calculated from the slope of the time course, and the absorbance change relative to the control was calculated.

For trypan blue staining, cells were cultured in a 96-well plate with 100 μL of serum-free DMEM containing 0–30 μM CaMKP inhibitors or naphthionic acid for 22 h at 37 °C in 5% CO_2, and then stained with 0.5% trypan blue solution (Nacalai Tesque) for 1 min. After removal of the trypan blue solution, PBS (200 μL) was added and the cells were observed under a microscope (Olympus Model CK2). Cell viability was calculated by counting living and dead cells.

2.5. Fluorescence staining for morphological observation

MDA-MB-231 cells cultured to 70% confluency in growth medium in 9-cm dishes were detached with 0.25w/v% trypsin-1 mmol/L EDTA 4Na solution and suspended in growth medium. After centrifugation (1000 rpm for 5 min), the cell pellets were washed with PBS and resuspended in serum-free DMEM. Cells (2.5×10^4) were seeded on cover glasses coated with 0.1% polylysine in 24-well plates. A CaMKP inhibitor or naphthionic acid (10 μM) was added to each well and the cells were cultured for 22 h at 37 °C under 5% CO_2. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For cell permeabilization, 0.1% Triton X-100 in TBS was added and the cells were incubated for 10 min. After three washes with TBS, the cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and washed with PBS. To stain F-actin, the cells were incubated with Alexa Fluor 488-phalloidin (1:40; Invitrogen) for 1 h at room temperature. Images were taken using a fluorescence microscope (Keyence Model BZ-9000).

2.6. Detection of phosphorylated CaMKI in MDA-MB-231 cells

MDA-MB-231 cells (2×10^5) in a 12-well plate were transfected with the pcFLAG-CaMKIα-myc-His_6 plasmid (1 μg/well) using Lipofectamine 3000 (Invitrogen) and cultured for 48 h in accordance with the manufacturer's instructions. After two washes with serum- and antibiotic-free DMEM, the cells were incubated in serum- and antibiotic-free DMEM containing CaMKP inhibitors or naphthionic acid (30 μM) for 24 h at 37 °C under 5% CO_2. The medium was completely removed and immediately replaced with 100 μL of 1 × SDS-PAGE sample buffer. The samples were denatured by boiling for 5 min and subjected to SDS-PAGE followed by western blotting analysis.

2.7. Statistical analysis

Welch's *t*-test and one-sample *t*-test were conducted using Microsoft Excel (Microsoft). Tukey's test was carried out using an online service (http://www.gen-info.osaka-u.ac.jp/MEPHAS/steel.html). P-values are indicated on each figure as *$p < 0.05$ (or 0.017 after the Bonferroni correction) and ****$p < 0.0001$.

2.8. Other analytical procedures

SDS-PAGE was carried out using the method of Laemmli [21]. Western blotting analysis was performed essentially as described [20], except that a PVDF membrane was used instead of a nitrocellulose membrane. For western blotting after immunoprecipitation, Clean-Blot™ IP detection Reagent (Thermo Scientific) was used to detect native antibodies only on the membrane without interference from denatured IgGs contaminating the immunoprecipitated samples. The membrane was visualized by an enhanced chemiluminescence detection procedure using chemiluminescent horseradish peroxidase substrates (SuperSignal West Femto or SuperSignal Weat Dura; Thermo Scientific). Quantitation of the band intensity was carried out using ImageJ software. Protein concentrations were determined using Advanced Protein Assay Reagent (ADV01; Cytoskeleton) with BSA as the standard.

3. Results

3.1. CaMKP activity in extracts of human breast cancer cell lines

To explore the possible role of CaMKP activity in cancer cell migration and invasion, we examined the CaMKP activity in breast cancer cell lines by immunoprecipitation followed by a malachite green phosphatase assay. Using MDA-MB-231 cells with high expression of CaMKP, we established appropriate assay conditions for detection of CaMKP activity in cell extracts. Western blotting analysis of a crude cell extract with the anti-CaMKP antibody used in the study showed a single prominent band for CaMKP (Fig. S1). The band almost disappeared when the cells were transfected with a siRNA targeting CaMKP, validating the specificity of the antibody against CaMKP (Fig. S1). Under the experimental conditions used, efficient immunoprecipitation of CaMKP protein from the cell extract was confirmed by western blotting (Fig. 1A). The phosphatase activity in the immunoprecipitate was readily detected by the malachite green phosphatase assay using pp10 as the substrate. The observed phosphatase activity was inhibited to 32.4% of the control in the presence of the CaMKP-specific inhibitor ANDS at 10 μM, confirming that the activity was due to CaMKP. Using this assay system, we compared the CaMKP activities in cell extracts from cancer cell lines with different motilities. Under the assay conditions used, MDA-MB-231 cells exhibited high motility (~10 and 5-fold higher than MCF-7 and T47D cells, respectively). MCF-7 cells showed low motility, and T47D cells had slightly higher motility than MCF-7 cells (Fig. 1B). Overall, cells with higher motility exhibited higher CaMKP activity (Fig. 1C). These data suggest that the phosphatase activity of CaMKP has a role in the regulation of cancer cell motility.

Given that CaMKP activity can be regulated by various mechanisms [10–13], we investigated whether the differences in CaMKP activity arose from differences in CaMKP protein expression or differences in CaMKP activation. For this, we examined the amounts of CaMKP protein in the immunoprecipitates by western blotting. We found that the amount of immunoprecipitated CaMKP protein was roughly proportional to the observed CaMKP activity (Fig. 1A, D). The normalized CaMKP activity based on the amount of CaMKP protein, corresponding to the specific activity, showed no significant differences among the three cancer cell lines (Fig. 1E). Therefore, the differences in CaMKP activity observed in the cell extracts can be explained by differences in expression of CaMKP protein rather than differences in its activation.

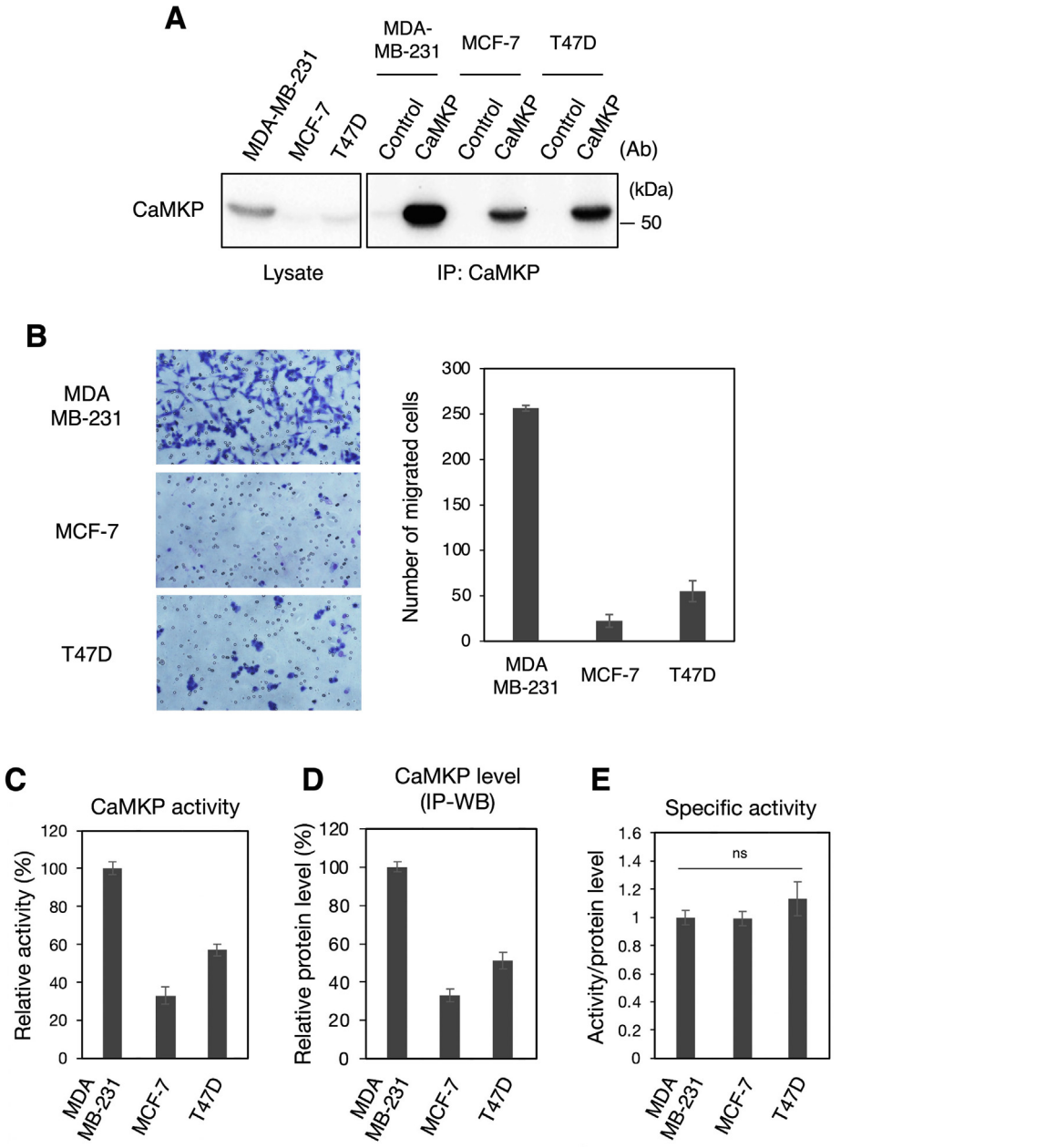
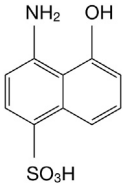
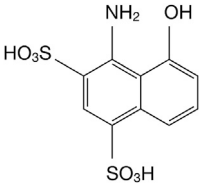
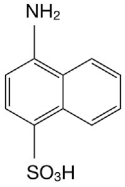


Fig. 1. CaMKP activities in human breast cancer cells with different motilities. (A) Western blotting analysis of crude cell lysates and immunoprecipitates prepared from the indicated breast cancer cells using an anti-CaMKP antibody. As a negative control for the immunoprecipitation, an anti-GOT1 polyclonal antibody was used. (B) Transwell migration assay of the indicated cancer cells. Migrated cells were stained using a Diff Quick Staining Kit and counted in four different fields in each insert. Representative images of migrated breast cancer cells are shown (left). The numbers of migrated cells are shown as means ± SE of three independent replicates (right). (C) CaMKP activities in immunoprecipitates prepared from the indicated breast cancer cells. Phosphatase activities of CaMKP in the immunoprecipitates were assessed by the malachite green phosphatase assay using pp10 as a substrate. (D) CaMKP protein levels in the immunoprecipitates estimated by western blotting analysis. The relative amounts of immunoprecipitated CaMKP shown in the right panel of (A) were quantified using ImageJ software. The data are shown as means ± SE of three independent experiments. (E) Specific activities of CaMKP detected in the immunoprecipitates. The relative phosphatase activity/protein levels of CaMKP were calculated from the data shown in (C) and (D). Summarized data for the means ± SE of three independent experiments are shown. No significant differences in the specific activities denoted as ns were determined by a Tukey's test ($p > 0.05$).

3.2. Effects of CaMKP inhibitors on the migration of MDA-MB-231 cells

If the phosphatase activity of CaMKP is a critical determinant for enhanced motility of cancer cells, the cells should exhibit reduced motility when their CaMKP activity is suppressed by inhibitors. To test this hypothesis, we employed ANS and ANDS (Table 1) as specific inhibitors of CaMKP activity that have no effect on PPM1A or calcineurin [15]. Using MDA-MB-231 cells, which had the highest CaMKP activity and the highest motility among the cancer cell lines in the present study, we examined the effects of the inhibitors on cancer cell motility by transwell migration assays. We found that ANS and ANDS both significantly inhibited the migration of the cancer cells, while the inactive analog naphthionic acid did not (Fig. 2A). Next, we examined the effect of the inhibitors on cell viability using two different assays: CCK-8 assay [22] and trypan blue staining [23]. The former assay is based on detection of reducing activity associated with cell metabolism, while the latter

Table 1
CaMKP inhibitors used in this study.

Name	Chemical structure	CaMKP inhibition	IC_{50} from [15]
1-Amino-8-naphthol-4-sulfonic acid (ANS)		Active	3.3 ± 0.2 μM
1-Amino-8-naphthol-2,4-disulfonic acid (ANDS)		Active	6.4 ± 0.8 μM
4-Amino-1-naphthalensulfonic acid (naphthionic acid)		Inactive	>40 μM

assay is based on the principle that only cells with intact membranes can effectively exclude the dye. No significant cytotoxicity was observed for the two inhibitors in either cell viability assay at the concentrations used for the migration assay (Fig. 2B and C), confirming that the inhibition of cell migration was not caused by toxicity of the inhibitors. Thus, it was concluded that treatment of human breast cancer cells with ANS or ANDS caused a marked reduction in cell migration, again suggesting that the phosphatase activity of CaMKP is crucial for the regulation of cancer cell motility.

3.3. Effect of CaMKP inhibitors on the polarity of MDA-MB-231 cells

Jurmeister et al. [7] showed that siRNA-mediated knockdown of CaMKP expression led a loss of cell polarity. Meanwhile, cell polarity is an important factor for cell migration [9]. To gain a better understanding of how the CaMKP inhibitors abrogated the cancer cell migration, we examined the effects of the inhibitor ANS on the cell polarity. Under control conditions, MDA-MB-231 cells had an elongated morphology (Fig. 3A, left). Following treatment with 10 μM ANS, the cells lost their elongated morphology and assumed a rounded shape (Fig. 3A, middle), resembling the morphology after siRNA-mediated knockdown of CaMKP expression [7]. In contrast, the cells retained their elongated morphology following treatment with the inactive analog naphthionic acid (Fig. 3A, right). The quantified data are summarized in Fig. 3B. The findings suggest that the inhibition of cell migration observed after CaMKP inhibitor treatment at least partially arises through a loss of cell polarity.

3.4. Inhibition of cellular CaMKP activity by the CaMKP inhibitors

To confirm that the CaMKP inhibitors acted on cellular CaMKP, we examined the effect of the inhibitors on the phosphorylation level of CaMKIα, a CaMKP substrate in the cells. Given that the inhibitors suppressed CaMKP activity in the cells, the phosphorylation level of CaMKI should be increased. MDA-MB-231 cells transiently expressing CaMKIα were treated with 30 μM ANS, ANDS, or naphthionic acid, and subjected to western blotting analysis for phosphorylated CaMKI (Fig. 4). The phosphorylation level of CaMKI was significantly enhanced (~3 fold) by treatment with either ANS or ANDS compared with the control DMSO. In contrast, the inactive analog naphthionic acid did not significantly enhance the phosphorylation level of CaMKI. Therefore, we concluded that ANS and ANDS were able to inhibit the cellular CaMKP activity in MDA-MB-231 cells, thereby inhibiting the cell migration. These findings indicate that pharmacological manipulation of CaMKP activity could be effective for inhibiting the migration of cancer cells.

4. Discussion

There is accumulating evidence for the involvement of CaMKP in the pathogenesis of various diseases. Among these diseases, cancer metastasis is of particular clinical importance, and numerous studies have demonstrated pivotal roles for CaMKP in cancer cell metastasis and invasion [1,2,6,7]. CaMKP expression was reported to be 7–8-fold higher in highly invasive MDA-MB-231 cells compared with non-invasive MCF-7 cells [6], suggesting that enhanced expression of CaMKP protein has a critical role on cancer cell motility. However, these studies were mainly based on genetic studies such as CaMKP knockdown or overexpression, and little attention has been paid to the phosphatase activity of CaMKP. Since CaMKP activity was shown to be activated or inactivated by various mechanisms [10–13], it is important to examine CaMKP activities in human breast cancer cells with different cell motility. In the present study, we directly examined the CaMKP activities in human breast cancer cell lines by an enzymatic assay. Overall, the data showed that cancer cells with higher motility exhibited higher CaMKP activity. No significant differences in the specific activity were observed among the three different human breast cancer cell lines examined in the present study (Fig. 1). Therefore, it is concluded that the high CaMKP activity observed in the cancer cells with high motility and invasiveness arises from high expression of CaMKP protein. Uncovering the mechanisms that control CaMKP expression will be critical for future studies on cancer metastasis.

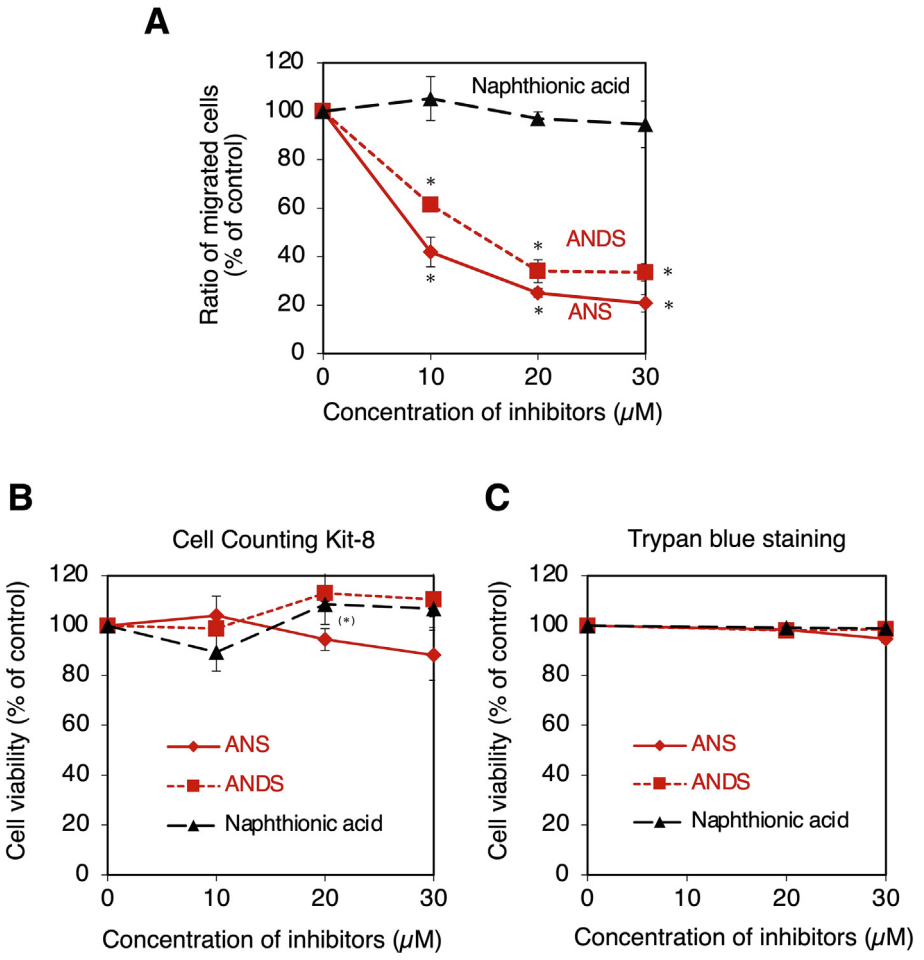


Fig. 2. Inhibition of human breast cancer cell motility by CaMKP inhibitors. (A) Effect of CaMKP inhibitors on the migration of MDA-MB-231 cells. The migration of MDA-MB-231 cells in the presence of 0, 10, 20, or 30 μM CaMKP inhibitors or naphthionic acid was measured by transwell assays. (B) Effect of CaMKP inhibitors or naphthionic acid on the cell viability of MDA-MB-231 cells measured with a Cell Counting Kit-8 assay. (C) Effect of CaMKP inhibitors or naphthionic acid on the cell viability of MDA-MB-231 cells assayed by trypan blue staining. Data are shown as means ± SD of three independent replicates. The asterisks denote significant differences determined by one-sample *t*-tests ($p < 0.017$ after the Bonferroni correction; hypothesized value = 100).

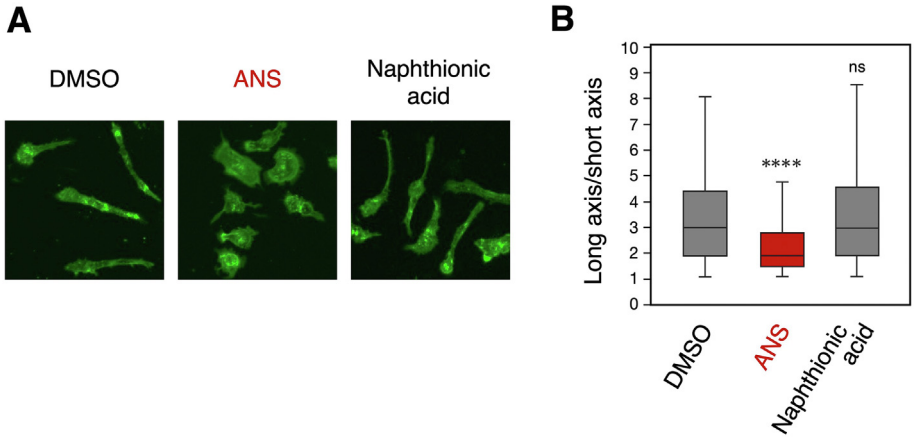


Fig. 3. Effect of a CaMKP inhibitor on the morphology of MDA-MB-231 cells. (A) Morphologies of MDA-MB-231 cells in the presence of the indicated compounds. Cells were seeded on polylysine-precoated cover glasses in a 24-well plate and cultured for 22 h in DMEM containing 10 μM ANS, naphthionic acid, or DMSO (vehicle). After fixation, the cells were stained with Alexa Fluor 488-phalloidin. Representative images for three independent experiments are shown. (B) Quantification of the images shown in (A). Cell morphology was quantified by measuring the long and short axes of the cells using ImageJ software. Box plots represent data from three independent replicates, with >1200 cells measured for each. ****$p < 0.0001$, significant difference versus the DMSO control determined by Welch's *t*-tests. ns: not significant, $p > 0.025$ after Bonferroni correction.

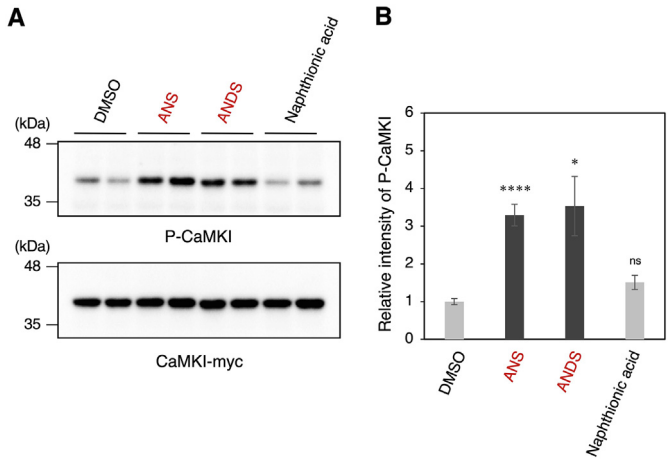


Fig. 4. Effects of the CaMKP inhibitors on the phosphorylation of CaMKI overexpressed in MDA-MB-231 cells. (A) MDA-MB-231 cells were transfected with pcFLAG-CaMKIα-myc-His_6 and cultured for 48 h. The cells were further cultured in serum- and antibiotic-free medium containing ANS, ANDS, or naphthionic acid (30 μM) for 24 h. Lysates of the cells (20 μL) were analyzed by western blotting using anti-phospho-CaMKI (P-CaMKI, upper panel) and anti-c-Myc (CaMKI-myc, lower panel) antibodies. Duplicate samples from independent wells are shown. (B) Relative intensities (P-CaMKI/CaMKI-myc) in the lysates. Data are shown as the means ± SE of three independent experiments. ****$p < 0.0001$ and *$p < 0.017$, significant difference versus the DMSO control determined by Welch's *t*-tests after Bonferroni correction. ns: not significant, $p > 0.017$.

From the viewpoint of clinical medicine, it is important to regulate the activity of a target enzyme by pharmacological manipulation. Involvement of CaMKP in the invasion and metastasis of several types of cancer cells was suggested by genetic studies [6,7]. These findings led us to examine the effect of two CaMKP-specific inhibitors, ANS and ANDS, on the migration of human breast cancer MDA-MB-231 cells. Our data clearly showed that CaMKP inhibitors were effective for suppressing the motility of these metastatic human breast cancer cells (Fig. 2A). Although the specificity and potency of CaMKP inhibitors will require improvement for future studies, it was most likely that the CaMKP inhibitors used in the present study directly inhibited the cellular CaMKP activity, because they inhibited the dephosphorylation of cellular CaMKI while the inactive analog did not (Fig. 4). During the preparation of this manuscript, Grimm et al. [24] reported that another type of CaMKP-specific inhibitor, Lockdown^{Pro}, suppressed the invasion of human cancer cells, including MDA-MB-231 cells. Taking these data into consideration, CaMKP inhibitors may be promising inhibitors of metastasis with novel mechanisms for inhibition of cell motility. The molecular mechanisms for how these compounds inhibit cancer cell motility or invasion remain to be clarified. Given that CaMKP was reported to play important roles in the regulation of centrosome positioning [25], actin remodeling [4], and focal contact formation [26], which are important for regulation of cell polarity, it is likely that inhibition of CaMKP at least partially affects these processes in the cells, leading to the observed inhibition of cell migration. The finding that the CaMKP inhibitors caused loss of the polarized cell morphology (Fig. 3) further supports our hypothesis.

It should be noted that the inhibitors exhibited no significant cytotoxicity at the concentrations that caused marked inhibition of cell migration (Fig. 2B and C). Similarly, Grimm et al. [24] reported that the newly-developed CaMKP-specific inhibitor Lockdown^{Pro} exhibited no significant cytotoxicity at concentrations up to 30 μM. These properties could be advantageous for the control of cancer invasion and metastasis without severe side effects. For terminal care of older patients in particular, better quality of life is more important than curative chemotherapy for complete remission of cancer. Very recently, we found that certain alkyl gallates with well-established safety profiles as food additives specifically inactivated CaMKP through protein carbonylation [18]. Thus, improving the specificity and potency of CaMKP inhibitors/inactivators while retaining lower cytotoxicity will be a key challenge for future studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.11.064.

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