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Identification of Annexin A2 as a target protein for plant alkaloid matrine

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Matrine is a plant alkaloid and a major active component in Chinese medical herb *Sophora flavescens*. Matrine has shown potent anti-cancer activities but with unknown molecular target and mechanism. Using the photo-affinity labeling approach, for the first time, Annexin A2 was identified as a direct-binding target of matrine in cancer cells.

Matrine is a natural alkaloid (Figure 1a) and a major active component in the roots of Chinese medical herb *Sophora flavescens*, which has been widely used in traditional Chinese medicines to treat inflammation, dysentery, eczema, hepatitis, cardiac diseases, and other diseases for centuries.[1-5] Interestingly, matrine itself has shown potent anti-cancer activities.[3] It has been found that matrine induced apoptosis in cancer cells via upregulation of Bax[6] and downregulation of Bcl-2,[7, 8] recovery of tumour suppressor miR122a,[9] and suppression of β-catenin/survivin pathway.[10] Matrine also suppresses the invasion and migration of cancer cells through down-regulation of the NF-κB,[11] EGFR/Akt/MMP-9,[12] TGF-β/Smad,[13] Ras/ERK,[13] and Wnt signalling pathways.[14] In the modern era, as a component of natural herbal medicine, there have been many examples of using matrine as an adjuvant therapy in combination with chemotherapeutic or targeted anti-cancer agents in clinical cancer treatment, such as with cisplatin[15] and sorafenib.[16] However, despite its potent anti-cancer activity and the fact that it has been used for clinical cancer treatment for decades, the direct binding target(s) of matrine and the underlying molecular mechanism still remain elusive, which has become a significant obstacle for the further development and applications of matrine as a clinical drug.

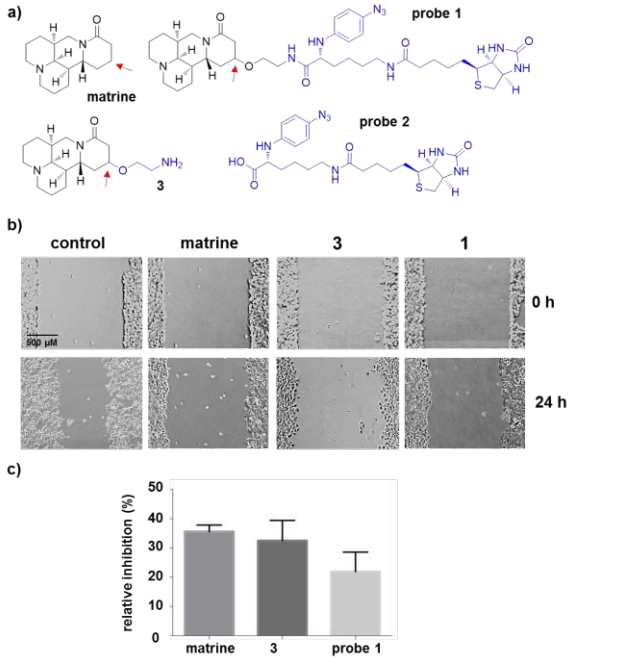

Fig. 1 a) Chemical structures of matrine, PAL probe **1**, control probe **2**, and a matrine analogue **3**; b) Images of wound-healing assay of Hep3B cells treated with matrine, **1**, and **3**, respectively. 3) Relative inhibition of cell migration in 2). Cells were treated with 10 μM of each compound for 24 hours. Percentage of inhibition was calculated relative to untreated cells (control). See the Supplementary Information for details.

Photo-affinity labelling (PAL) is a powerful method widely used for the labelling and capture of proteins.[17-28] PAL establishes stable covalent bonds between protein and the ligand by photo-crosslinking, thereby allowing straightforward characterization of protein-ligand interactions. PAL has been successfully implemented in identifying the target of many bioactive small molecules, proteins, and nucleic acids.[19-31] Here in this report, for the first time, we identified Annexin A2 as a direct target for matrine using the PAL approach.

Typically, a PAL probe is composed of a binding ligand as the bait, a photo-crosslinker for protein capture, and an affinity tag

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for imaging or target enrichment and isolation. It is imperative that these add-on structures do not interfere with target binding. Previous studies have shown that modifications at the β-position of the amide carbonyl group slightly improved the anti-inflammatory activity of matrine (red arrow; Figure 1a).[32] Therefore, we reason that modification at this site may also be acceptable for retaining the anti-cancer activity of matrine. In order to test this, we prepared a PAL probe (probe **1**; Figure 1a) containing a matrine structural motif, a phenylazide photo-crosslinker, and a biotin tag. Phenylazide has been extensively used in PAL with excellent crosslinking efficiency[25] and the biotin tag is for the enrichment of captured proteins. In addition, a control probe (probe **2**) without the matrine motif was also prepared to control for non-specific crosslinking. The synthetic routes of **1** and **2** are shown in the Supplementary Information (Figure S1).

We first tested the anti-migration activity of matrine, probe **1,** and a matrine analogue with a 2-aminoethoxy group at the same modification site (**3**; Figure 1a and S1)[33] in a wound-healing assay with Hep3B cells. Hep3B is a type of hepatocellular carcinoma (HCC) cells that are prone to migration and invasion,[34-36] but also are sensitive to matrine treatment.[7, 9, 10] Hep3B cells were treated with matrine, probe **1**, and analogue **3**, respectively, for 24 hours at 10 μM (Figure 1b). The inhibition on cell migration rate was quantified and compared with untreated cells. As shown in Figure 1c, matrine reduced migration rate by ~35% and analogue **3** has a similar activity (~32%). The inhibitory activity of probe **1** is lower than matrine (~22%), but it is still effective. This result suggests that the modification at the β-position of the carbonyl group is acceptable and **1** is a suitable PAL probe for target identification.

Next, we carried out affinity pulldown experiments with these probes. Hep3B cell lysates (6.0 mg/mL) were incubated with **1** or **2** (10 μM) at 4 °C for 12 hours. Then the mixture was irradiated for 1 min at 365 nm, before streptavidin beads were added to isolate captured proteins. In order to thoroughly remove non-specific background, a stringent washing condition of 1% SDS (x 10) was used. Captured proteins were then eluted and analysed by electrophoresis. As shown in Figure 2a, two protein bands at 45-55 kD that are specific to probe **1** were detected. These bands were excised, subjected to in-gel trypsin digestion, and then analysed by MALDI-TOF/TOF-MS (Figure S2). Eventually, they were identified as Annexin A2 (lower band) and moesin (upper band).

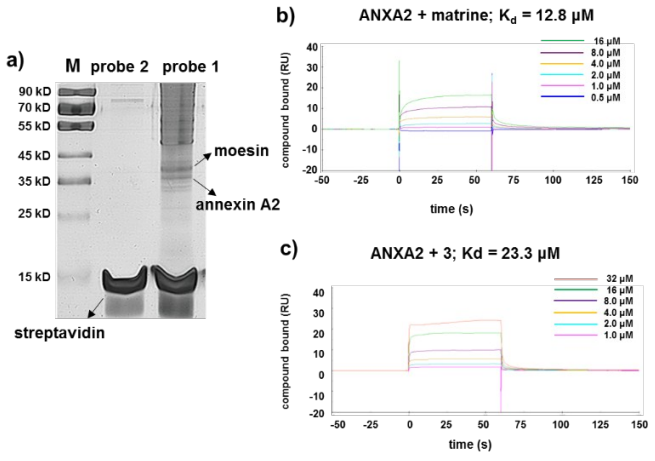

Fig. 2 a) After affinity pulldown with probes **1** and **2**, captured proteins were eluted from streptavidin beads and analysed by 12% SDS-PAGE. M: molecular weight ladder. The dark bands at ~15 kD are streptavidin proteins leaked from beads during elution. b) SPR sensorgram for ANXA2-matrine binding; c) SPR sensorgram for ANXA2-**3** binding. See the Supplementary Information for details.

Annexin A2 (ANXA2) is a pleiotropic calcium-dependent phospholipid binding protein widely distributed in various eukaryotic cells. It orchestrates a myriad of biological processes such as cell migration, endocytosis, fibrinolysis, ion channel formation, and cell-ECM (extracellular matrix) interactions.[37-44] Dysregulation and abnormal expressions of ANXA2 have been linked to cancers and have profound effects on tumour invasion and metastasis.[38-40, 44-46] For instance, studies have shown that ANXA2 promotes migration and invasion of HCC[44, 45, 47] and glioma cells.[48] It is therefore reasonable to consider ANXA2 as a target for matrine to put forth its anti-cancer activity.

Moesin is a protein involved in mitotic spindle functioning through stabilizing cell shape and microtubules at the cell cortex, so that it plays a direct and important role in cell proliferation.[49-51] However, previous studies have shown that much higher concentration of matrine (>4 mM) is required to inhibit the proliferation of cancer cells;[7, 52] therefore, in this study, we focused our investigation on ANXA2.

We determined the binding affinity of matrine with ANXA2 and moesin with surface plasmon resonance (SPR; Figure 2b). Matrine behaved as a typical small molecule ligand for ANXA2 with fast on and off rate and the K_d value was determined as 12.8 μM, and the K_d of analogue **3** with ANXA2 is 23.3 μM (Table S1). Interestingly, neither matrine or **3** showed detectable affinity for moesin (Figure S3). The capture of moesin by probe **1** may be due to its high expression level in Hep3B cells.[53]

Since ANXA2 is associated with tumour invasion and metastasis,[38, 44] wound-healing assay was used to study the inhibitory effect of matrine on cell migration in relation to ANXA2. First, we confirmed that matrine could inhibit the migration of Hep3B cells in a dose-dependent manner (Figure 3a and 3b). In the presence of an ANXA2 antibody, significantly slower rate of migration was also observed, but the migration rate was not further reduced by matrine addition (Figure 3c; ANXA2 columns), suggesting the antibody competes for ANXA2 binding with matrine. In contrast, with a control antibody IgG, cells migrated at similar rate to untreated cells, and matrine was again able to inhibit cell migration dose-dependently (IgG columns). Furthermore, we knocked down the expression of ANXA2 by RNA interference (RNAi) using a plasmid encoding siRNAs (small interfering RNAs) targeting ANXA2 transcripts (Figure S4 and S5). Similar to antibody blocking, ANAX2-knocked-down cells showed significantly lower rate of migration, and the addition of matrine did not further reduce migration rate (Figure 4d; SH columns). With a control plasmid, cells migrated at much higher rate and are again sensitive to matrine addition (NC columns). Compared with the ANXA2 antibody, RNAi knock-down showed less degree of inhibition,

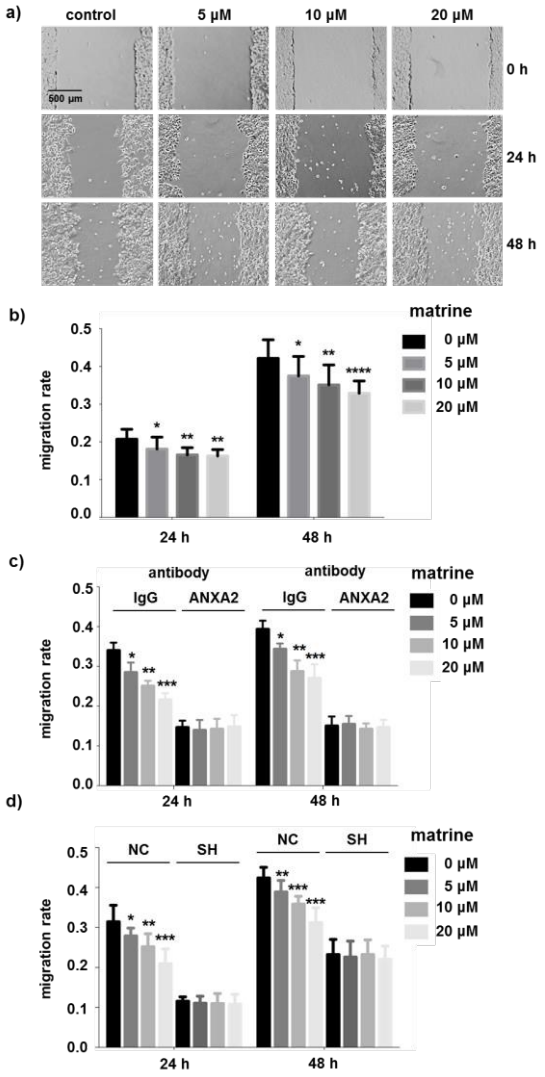

Fig. 3 a) Images of wound-healing assay. Hep3B cells were treated with matrine at various concentrations; b) Relative migration rate quantified from a); c) Relative migration rate with either an IgG antibody or an ANXA2 antibody (2.0 μg/mL; Santa Cruz); d) Relative migration rate of Hep3B cells transfected with either a control plasmid (NC) or a plasmid targeting ANXA2 (SH), then treated with matrine. See the Supplementary Information for details and Figure S6-S7 for images of c) and d).

which may be due to that antibody directly blocks ANXA2, while RNAi might trigger some kind of feedback mechanism in the cell to compensate for the loss of ANXA2 protein. Collectively, these data suggest that ANXA2 is functionally associated with cell migration and matrine may exert its anti-migration activity via binding and inhibiting the ANXA2 protein.

The molecular mechanism of ANXA2 in regulating cell migration has been well studied. Two ANXA2 molecules associate with the *C*-terminal domain of two p11 proteins and form a heterotetrameric complex.[41] This complex acts as a receptor for plasminogen (PLG) and can proteolytically cleaves plasminogen to plasmin (PN).[42, 43] ANXA2-mediated PLG-to-PN conversion complements the classic fibrin-based mechanism and plays an important role during cancer cell migration and invasion.[54] We reason that the binding of matrine to ANXA2 may be able to reduce the conversion of PLG to PN, thereby inhibiting cell migration. Therefore, as a further validation of the

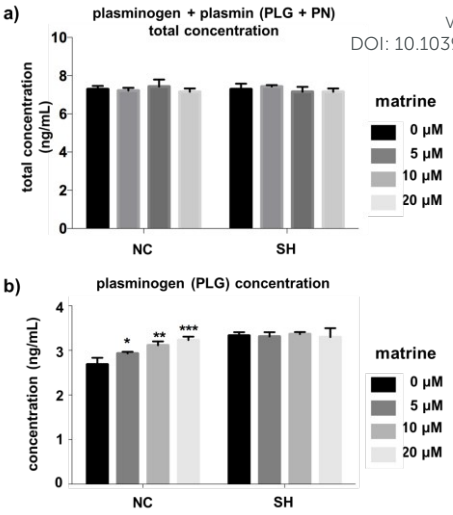

Fig. 4 ELISA measurements of a) the total concentrations of PLG and PN, and b) PLG concentrations of Hep3B cells transfected with either a control plasmid (NC) or a plasmid targeting ANXA2 (SH), then treated with matrine at different concentrations.

functional connection between matrine, ANXA2, cell migration, Hep3B cells were transfected either with the plasmid targeting ANXA2 or with a control plasmid, and then matrine was added at various concentrations. Next, using an ELISA assay, the total concentration of PLG+PN and the concentration of PLG alone were determined, respectively. For cells transfected with the control plasmid, the total concentration of PLG and PN remained roughly the same upon matrine addition, but the concentration of PLG alone increased with the increase of matrine concentration, indicating matrine did inhibit PLG-PN conversion dose-dependently (Figure 4, NC columns). In contrast, when cells were transfected with the anti-ANXA2 plasmid to knock down ANXA2, both the total concentration of PLG+PN and the PLG concentration remained mostly unchanged, suggesting that the PLG-PN conversion was not significantly affected by matrine with ANXA2 being knocked-down (Figure 4, SH columns). Collectively, these results have demonstrated that matrine inhibits the proteolytic activity of ANXA2 and results in lower level of the PLG-to-PN conversion, thereby inhibiting the migration of Hep3B cells.

Conclusions

In summary, for the first time, we identified Annexin A2 as a directly binding target for matrine, a major active component in Chinese herb medicine and a promising anti-cancer agent. Although the binding affinity of matrine with ANXA2 is modest, the antibody blocking and RNAi knockdown experiments have demonstrated that ANXA2 inhibition may be the mechanism underlying the anti-migration activity of matrine. The inhibition of ANXA2-mediated PLG-PN conversion by matrine has corroborated this conclusion. Currently we are conducting more in-depth investigation to elucidate more details of the mechanism underlying the anti-cancer activities of matrine.

Bioactive components in traditional Chinese medicines are usually considered to have multiple targets and have complex

modes of actions. Although only two major proteins (Annexin A2 and meosin) were identified in this study, other targets may exist but have eluded our detection (e.g. having low cellular abundance or not able to bind probe **1**). We are currently implementing different target identification approaches in order to explore other potential targets of matrine.[55, 56]

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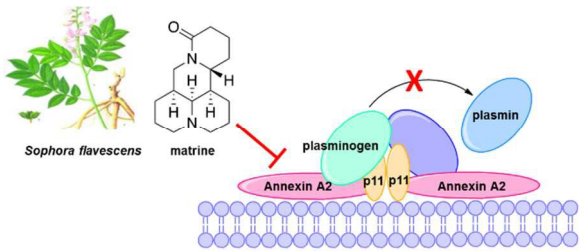

The cellular target of matrine is identified.