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COMMUNICATION

Cellular target identification of Withangulatin A using fluorescent analogues and subsequent chemical proteomics

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Withangulatin A(WA) has been reported to possess potent antitumor activity. However, its possible mechanism and direct proteomic targets remain unknown. Herein we report the subcellular localization of WA by designing and synthesizing its fluorescent analogues with coumarin moieties. Furthermore, sarco/endoplasmic reticulum calcium-ATPase (SERCA)2 was identified as the potential target of WA for its antitumor activity by chemical proteomics.

As an important source for anticancer drug discovery, in the last few decades, natural products and their derivatives have gained much attention.[1-3] *Physalis angulata* L., known as a traditional Chinese medicine, has been used for treatment of diseases such as tumors, hepatitis, rheumatism and nephronia for a long time.[4] Various withanolides that have great bio-activities have been isolated from this species.[5, 6] Withangulatin A(WA) is one of the withanolides isolated from *P. angulata* L[7] and exhibits potent antitumor, anti-inflammatory and immunosuppressive activity.[8-10] In spite of numerous studies have been undertaken to elucidate the anticancer mechanisms of WA, such as changing cellular morphology,[11] inhibiting topoisomerase II activities and suppressing general protein synthesis,[7] its molecular targets and detailed mechanisms remain elusive, which largely hampered the further development of WA for cancer therapy.

To overcome these obstacles, recently a number of chemical biology approaches have been developed to tackle these challenges. Among these methods, fluorescent probe that combined natural products and fluorescent organic dyes has become a powerful tool for visualizing the subcellular location, monitoring changes in cellular morphology and exploring biological phenomenon at many levels.[12, 13] Whereas, a major drawback of this method is that it is hard for fluorescent imaging alone to detail the direct target and explain the deeper behaviour of bioactive molecules. At the same time, activity-based protein profiling (ABPP) and affinity-based probe (A*f*BP) has been widely applied in target profiling,[14, 15] and clickable probes are widely preferred to discover biological target of bio-active molecules as it can make probes more cell-permeable with less perturbation to the native binding and can accurately reflect the drug's actions in the in vivo physiological environment.[16, 17] However, the toxicity of copper(I) hindered the usage of this method in living system.[18] Furthermore, sometimes it is hard for us to accurately identify the exact protein that can clarify the mechanism of active natural products from hundreds of proteins. In order to identify the proteomic target of WA more efficiently, herein we first synthesized a series of new fluorescent analogues based on WA to explore its subcellular location. Subsequently WA was conjugated with Epoxy-activated Sepharose 6B beads to pull down the target protein of WA. With the help of fluorescent WA probes, we can rule out non-specific binding proteins and improve accuracy in the proteome-wide identification of molecular targets.

It is important for fluorescent probes not to affect the activity when natural product is linked to fluorophore. Like many bioactive

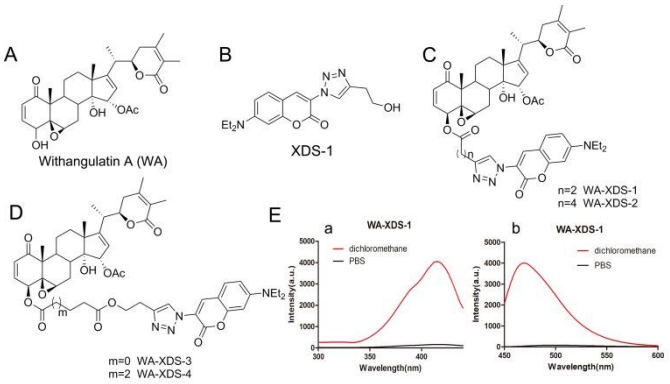

Fig. 1 Chemical structures of (A) WA; (B) XDS-1; (C) WA-XDS-1, WA-XDS-2 and (D) WA-XDS-3, WA-XDS-4; (E) Excitation (a) and emission (b) spectra of WA-XDS-1 in dichloromethane (DCM) and PBS.

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withanolides, WA has an α,β-unsaturated ketone group in ring A (Fig. 1A), which is considered as an essential group to maintain its activity[19] and the modification on 4-position hydroxyl of WA did not diminish or even improve the anticancer activity.[20, 21] With this in mind, fluorescent probes were designed and synthesized by connecting 4-hydroxyl group of WA with a coumarin moiety through linkers of various lengths. Specifically, we chose high fluorescent N,N-dialkyl-7-aminocoumarin (Scheme S1) as a fluorescent moiety because it is relatively small and has no toxicity.[22] Furthermore, as an environment-sensitive fluorophore with "off-on" mechanism, N,N-dialkyl-7-aminocoumarin is suitable of detecting hydrophobic interaction between WA and its target proteins.[23]

Fluorescent probes were synthesized from WA in five or six steps. 3-azido-7-diethylaminocoumarin was synthesized first based on previously published procedures.[24] Then in order to identify the influence of linkers to the activity of WA, four different lengths of linkers were designed to combine WA with the coumarin moiety by click reactions between acetylenic and azide groups. Finally, four WA-based fluorescent probes were synthesized (Fig. 1C and Fig. 1D) and XDS-1, a negative control probe without WA moiety, was also synthesized to exclude the possibility that the uptake and localization of the probes were driven by fluorescent moiety itself (Fig. 1B). The detailed synthetic procedures are provided in Schemes S1-S4 and Fig.S1-S9. Upon finishing of synthesis, we investigated the cytotoxic activity of four new fluorescent probes and XDS-1 against several human cancer cell lines using Cell Counting Kit-8 (CCK-8) assay. WA was also tested for comparison and the data were summarized in Table S1. WA-XDS-1 had the greatest cytotoxic activity versus WA and the anticancer activity of WA-XDS-4 was weaken, indicating a long linker was harmful to maintaining the cytotoxic activity. Meanwhile, XDS-1 had no cytotoxic activity until 100 μM. Then the environment-sensitive property of these fluorescent probes was tested. Spectral analysis showed that all the probes showed obvious stokes shift (~60nm)

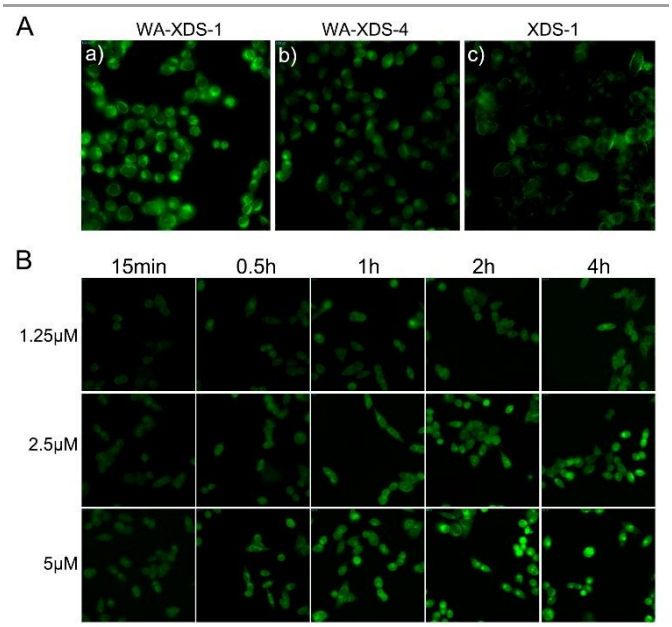

Fig. 2 (A) Uptake of probes in MDA-MB-231 cells: (a) 5 μM WA-XDS-1 for 1h; (b) 5 μM WA-XDS-4 for 1h; (c) 100 μM XDS-1 for 1h. (B) Time- and concentration-dependent uptake of WA-XDS-1 in MDA-MB-231 cells.

with maximum excitation at 410nm and maximum emission at 470nm, and the influence of solvent on fluorescence spectra was obvious. Compared with the excitation and emission spectra of fluorescent probes in dichloromethane, the aqueous PBS solution quenched the fluorescence gravely (Fig. 1E and Fig. S10), indicating that these fluorescent conjugates were suitable to detect the change in micro-environmental polarity with a turn-on property when the probes were interacting with target proteins.

The uptake properties of four fluorescent derivatives and XDS-1 were detected by MDA-MB-231 cells (Fig. 2A and Fig. S11). Bright fluorescence inside MDA-MB-231 cells was observed when probes were incubated with 5 μM of WA-XDS-1 for 1h. Affected by the long linker which decreased the cytotoxic activity, WA-XDS-4 showed the weakest fluorescence among four probes. Moreover, naked coumarin dye (XDS-1) showed no significant cellular location even at a concentration of 100 μM after 2h staining, verifying that it was WA moiety that led the uptake and localization of fluorescent probes in MDA-MB-231 cells. Then the uptake of WA-XDS-1 was investigated based on time and concentration to optimize experimental condition. It was best for MDA-MB-231 cells to be incubated with 2.5 μM of WA-XDS-1 for 1h, the higher concentration or longer time was harmful to cells and may change cellular morphology (Fig. 2B).

To further detail the subcellular localization of WA, we co-stained fluorescent derivatives trained MDA-MB-231 cells with a panel of organelle-specific dyes (Fig. 3A-3C and Fig. S12-S14). Compared with MitoTracker (mitochondria specific dye) and LysoTracker (lysosomes specific dye), the co-localization of probes with ER-Tracker (endoplasmic reticulum specific dye) matched much better, which indicated ER was the main organelle that WA worked in,

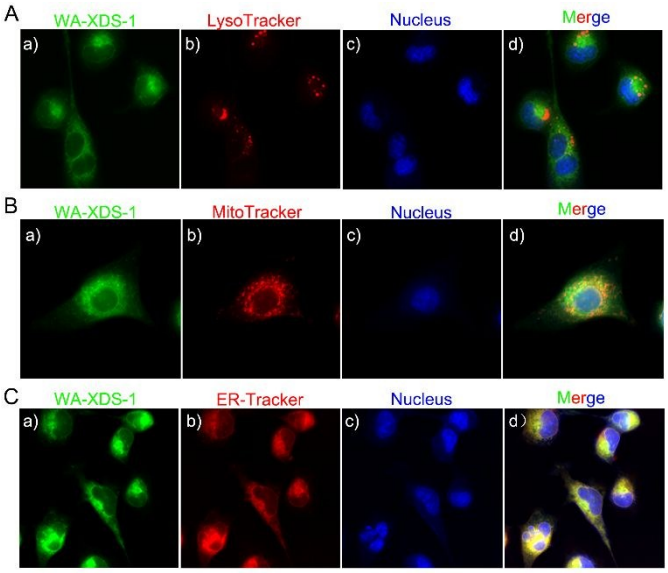

Fig. 3 (A) Co-stain of WA-XDS-1 with LysoTracker in MDA-MB-231 cells: (a) 2.5 μM of WA-XDS-1; (b) 50 nM of LysoTracker; (c) Reddot (nuclei specific dye); (d)overlay of three images. (B) Co-stain of WA-XDS-1 with MitoTracker in MDA-MB-231 cells: (a) 2.5 μM of WA-XDS-1; (b) 100 nM of MitoTracker; (c) Reddot; d) overlay of three images. (C) Co-stain of WA-XDS-1 with ER-

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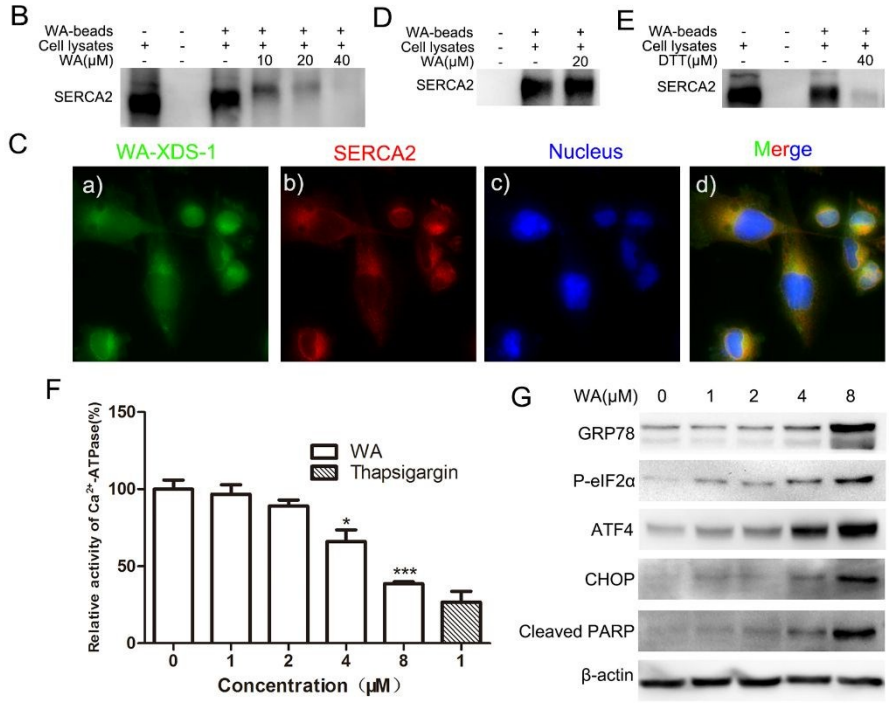
Tracker in MDA-MB-231 cells: (a) 2.5 μM of WA-XDS-1; (b) 1 μM of ER-Tracker; (c) Reddot; (d) overlay of three images.

Fig. 4 (A) Proteomic analysis of proteins interact specifically with WA-conjugated beads. (B) WA-conjugated beads were incubated with MDA-MB-231 cell lysates in the absence or presence of WA with different concentrations at 4 °C for 12 h, then the proteins bound to WA beads were detected by immunoblotting. (C) The co-localization of WA-XDS-1 and SERCA2 in MDA-MB-231 cells: (a) 2.5μM of WA-XDS-1; (b) immunofluorescence against SERCA2; (c) 500nM of PI (nuclei specific dye); (d) overlay of three images. (D) MDA-MB-231 cell lysates were pre-incubated with WA beads for 2 h and then further incubated with or without 20μM of WA at 4 °C for 12 h for competitive binding. (E) WA conjugated beads were pre-incubated with or without 40μM of DTT for 2 h and then further incubated with MDA-MB-231 cell lysates at 4 °C for 12 h. (F) MDA-MB-231 cells were incubated with WA of various concentrations and thapsigargin for 24h and lysed, then measured the activities of Ca^{2+}-ATPase. (G) MDA-MB-231 cells were incubated with various concentrations of WA for 24h and lysed, then the protein levels of GRP78, phosphorylated eIF2α, ATF4, CHOP and Cleaved PARP were detected by immunoblotting.

A

ID	Protein	PSMs
P13639	Elongation factor 2	25
Q13509	Tubulin β-3 chain	20
P13645	Keratin, type I cytoskeletal 10	14
F8VPD4	CAD protein	11
P30050	60S ribosomal protein L12	9
Q5TFE4	5'-nucleotidase domain-containing protein 1	9
P05141	ADP/ATP translocase 2	9
Q05639	Elongation factor 1-alpha 2	8
P27348	14-3-3 protein theta	8
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	7
P25398	40S ribosomal protein S12	7
Q8TD16	Protein bicaudal D homolog 2	7
Q9ULT8	E3 ubiquitin-protein ligase HECTD1	7
Q16576	Histone-binding protein RBBP7	7
P04080	Cystatin-B	6
C9JXB8	60S ribosomal protein L24	6
P30048	Thioredoxin-dependent peroxide reductase, mitochondrial	6
F8VVM2	Phosphate carrier protein, mitochondrial	6
P25705	ATP synthase subunit alpha, mitochondrial	6
B3KRM2	Serine/threonine-protein phosphatase	5
O15269	Serine palmitoyltransferase 1	5
P18031	Tyrosine-protein phosphatase non-receptor type 1	4



revealing a great possibility that the target protein of WA was located in ER.

Upon the identification of the subcellular location of WA, next we performed pull-down/LC-MS experiments using affinity-based profiling to identify the targets of WA. WA-conjugated sepharose beads were prepared as affinity reagent based on previous report[25] and MDA-MB-231 cell lysates were incubated with the beads to capture the target proteins, then the complex was resolved by boiling and the proteins interacted with WA-beads were resolved by SDS/PAGE followed by silver staining and further identified using LC-MS/MS analysis(Fig.S15). Fig. 4A represents different proteins found to interact specifically with WA-conjugated sepharose. According to the discovery of fluorescent probes that ER was the main organelle that WA played a role in, we concentrated on ER located proteins which could possibly clarify the anticancer mechanism of WA. As the first protein that located in ER with the highest score of peptide spectrum matches (PSMs), SERCA2, known to play an important role in calcium transport, was finally identified as a potential target of WA.

To verify the interaction between WA and SERCA2, we incubated WA beads with MDA-MB-231 cell lysates in the absence or presence of different concentrations of WA as competition substance and the protein was detected by Western blot (Fig. 4B). It was obvious to discover SERCA2 was pulled down by WA beads and it can be reversed by excess amount of WA concentration-dependently. Immunofluorescence experiment was also performed and the co-localization matched well (Fig. 4C and Fig.S16). To further confirm the interaction between WA and SERCA2. in vitro pull-down assay and biolayer interferometry (BLI) test with recombinant protein were then carried out. Consistent with the result of initial pull-down assay, WA-beads successfully co-precipitated with purified SERCA2 protein and excess amount of WA weaken the interaction (Fig.S17). Moreover, WA showed direct binding with the recombinant SERCA2 protein in BLI analysis with K_D= 10.3μM (Fig.S18).

Considering the α, β-unsaturated ketone group in ring A of WA is a bioactive group, which is easy to act with sulfhydryl in cysteine via the Michael addition reaction, whether WA could covalently bind to SERCA2 was further investigated. WA-conjugated sepharose beads were pre-incubated with MDA-MB-231 cell lysates, and then the lysates were further treated with an excess amount of WA as competition. Post-competition of WA failed to prevent the binding between WA beads and SERCA2 (Fig. 4C), which meant an irreversible interaction between WA and SERCA2. Then we used dithiothreitol (DTT), which also have sulfhydryl, to detect its influences on the association of SERCA2 with WA-conjugated

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sepharose beads. DTT remarkably abolished the interaction between beads and SERCA2 (Fig. 4D), suggesting that WA might covalently bind to the sulfhydryl of cysteine on SERCA2.

As the main pump that transports Ca^{2+} from cytoplasm to ER, SERCA dependent calcium transport is important to maintain the calcium homeostasis and keep the concentration of Ca^{2+} in ER four orders of magnitude higher than that in cytosol.[26] Various cancer cells exhibit dysregulated Ca^{2+} signaling[27] and overexpression of SERCA activates cell survival pathways through the effect on cellular Ca^{2+} peak and oscillations in various cancers,[28-30] which make it a potential target for antitumor therapy.[31] To test the effects of WA on SERCA activity, MDA-MB-231 cells were incubated with WA and the Ca^{2+}-ATPase activity was measured using Ca^{2+}-ATPase kit. The result indicated WA significantly inhibited the Ca^{2+}-ATPase activity in a dose-dependent manner (Fig. 4E), and 8 μM of WA inhibited the Ca^{2+}-ATPase activity by 62%.

It is reported the inhibition of SERCA activity often results in an imbalance of calcium levels in cancer cells, which causes unfolded protein response (UPR), ER stress and eventually apoptosis.[32] Thus the effects of WA were tested on the ER stress-associated markers and apoptotic signalling pathway in MDA-MB-231 cells. WA treatment significantly caused a markedly enhanced expression of GRP78, ATF4, P-eIF2α and CHOP, as well as the cleavage of PARP in a dose-dependent manner (Fig. 4F). These results indicated WA could act against cancer cell through inhibiting SERCA activity and inducing ER stress-associated apoptosis.

In conclusion, we developed four new WA-based fluorescent probes to explore its subcellular distribution and found ER was the main organelle WA located in, through chemical proteomics-based target identification and molecule/cell-based validation, we elucidated that WA targets SERCA2, inhibits its activity and finally causes ER stress-associated apoptosis in MDA-MB-231 cells. This work highlighted a new strategy to find the cellular target of bioactive natural product more accurately through the combination of affinity-based proteome profiling and fluorescent imaging and could help to understand the antitumor activity of WA and the related derivatives.

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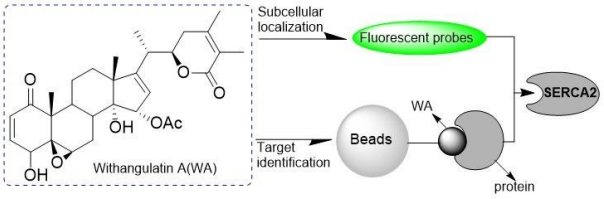

Find the target of Withangulatin A with the combination of fluorescent probes and chemical proteomics.

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