LETTER TO THE EDITOR

PROTAC technology as a novel tool to identify the target of lathyrane diterpenoids

KEY WORDS
PROTAC; Lathyrane diterpenoids; Target identification; MAFF; Anti-inflammation

To the Editor:

Proteolysis Targeting Chimera (PROTAC) is an emerging approach to selectively degrading target proteins by utilizing endogenous proteasome. Since PROTACs can degrade target proteins without high affinity, it is natural to speculate that this technology can be used to identify the targets of natural products.

Although a recent study reported the employment of PROTACs to explore the unknown non-kinase target of a multi-kinase inhibitor sorafenib, whether PROTACs can be used to find the potential targets of natural products remains unexplored.

In previous study, we obtained several new lathyrane diterpenoids from Euphorbia lathyris and proved their potent anti-inflammatory activity at low micromole levels with low toxicity. Among them, (2S,3S,4S,5R,9S,11R,15R)-15-acetoxy-3-cinnamoyloxy-5-hydroxyl-14-oxolathyra-6(17),12E-diene (ZCY-001) demonstrated the strongest activity against LPS-stimulated NO release with an IC50 value of 3.0 μmol/L. However, the exact targets and the anti-inflammatory mechanism of lathyrane diterpenoids still need to be elucidated.

V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (MAFF) belongs to small MAFs family proteins (sMAFs), which also include MAFG and MAFK and are basic region leucine zipper (bZIP)-type transcription factors lacking transactivation domain. sMAFs are indispensable partners required by CNC proteins including nuclear factor-erythroid 2-p45-related factor 2 (Nrf2) to positive-transcriptionally activate target genes involved in antioxidative response, while homodimers of sMAFs repress this activation by competitively binding with MARE or ARE.

Herein, lathyrol, with the core scaffold structure of the most active lathyrane diterpenoid ZCY-001 from E. lathyris, was selected to synthesize a PROTAC molecule (ZCY-PROTAC) to identify the targets of lathyrane diterpenoids. We identified MAFF as the target of lathyrol and (2S,3S,4R,5R,7R,9S,11R,15R)-5,15-diacetoxy-3-benzoyloxy-7-hydroxyl-14-oxolathyra-6(17),12E-diene, ZCY020, a similar lathyrane diterpenoids. The findings imply that PROTAC technology was a promising new method for the target identification of natural products.

1. MAFF is a degradation target of ZCY-PROTAC

To synthesize the PROTAC of lathyrane diterpenoid, lathyrol, the core scaffold structure of the most active lathyrane diterpenoid ZCY-001 (Supporting Information Fig. S1A), was selected as the POI ligand. We coupled lathyrol with different length of 3-polyethylene glycol (3-PEG) linkers conjugated to thalidomide and obtained three PROTACs (referred as ZP-1, ZCY-PROTAC and ZP-3, Supporting Information Schemes S1–S3 and Fig. S2A). Compared with others, ZCY-PROTAC showed the strongest inhibitory activity against LPS-stimulated NO release (Fig. 1B and Fig. S2A), and showed only very low toxicity (Fig. S1B). A Tandem Mass Tag (TMT)-based quantitative proteomic approach (Fig. S2B) was applied and according to the plot, MAFF was significantly degraded by ZCY-PROTAC treatment (Fig. 1C). KEGG pathway enrichment analysis showed that significant changed proteins are mostly related with lysosome, ferroptosis, and mitophagy (Fig. S2C).

As shown in Fig. 1D and Fig. S2D, MAFF was significantly degraded by ZCY-PROTAC with dose- and time-dependent manners.
in RAW264.7 and HEK293T cells. The degradation of MAFF was blocked by proteasome inhibitor MG132 and NAE inhibitor MLN4924 (Fig. 1E–1F and Fig. S2E). And lathyrol and thalidomide can both compete with ZCY-PROTAC to block the degradation of MAFF mediated by ZCY-PROTAC (Fig. 1G and Fig. S2F). Single lathyrol or thalidomide showed no degradation effects on MAFF within 48 h in both RAW264.7 and HEK293T cells (Fig. S2G), excluding the influence of the two ligands in ZCY-PROTAC on MAFF degradation. Among the three PROTACs, ZCY-PROTAC exhibited the moderate degradation effect in RAW264.7 cells (Fig. S2H). And among the sMAFs proteins, only the MAFF protein was intensively degraded by ZCY-PROTAC in a dose-dependent manner (Fig. S2I), indicating that ZCY-PROTAC has certain selectivity for the degradation of MAFF. These results strongly demonstrate that the degradation of MAFF mediated by ZCY-PROTAC depends on the ubiquitin–proteasome system, and that MAFF is verified to be the degradation target of lathyrol-based PROTAC.

2. MAFF is the target of lathyrol and ZCYO20

To further confirm that MAFF is a direct target of lathyrol, microscale thermophoresis (MST) assay, surface plasmon resonance (SPR) assay, cellular thermal shift assay (CETSA) and drug affinity-responsive target stability (DARTS) assay were used to evaluate the ability of lathyrol binding to MAFF. As shown in Fig. 1H and Supporting Information Fig. S3A, the equilibrium dissociation constant ($K_D$) values of lathyrol were 20.90 ± 2.34 and 15.5 μmol/L assayed by MST and SPR, respectively. In CETSA assay, lathyrol significantly increased the cellular thermal stability of MAFF protein (Fig. 1I). In DARTS assay, the degradation of MAFF was significantly reduced after lathyrol treatment (Fig. 1I). Besides, we synthesized a fluorescent probe (naphthalimide-conjugated lathyrol), named ZCY-probe (Supporting Information Scheme S4), and the result showed that MAFF protein was located in the nucleus and lathyrol was mainly co-located in the nucleus (Fig. S3B), indicating the direct interaction between lathyrol and MAFF in cells.

ZCY020, a lathyrane diterpenoid sharing the same core scaffold structure with lathyrol, was also tested for its ability to bind with MAFF. First, the ability of ZCY-PROTAC to degrade MAFF was blocked by the addition of ZCY020 (Fig. 1J and Fig. S3C), indicating the interaction between MAFF and ZCY020. Encouragingly, ZCY020 presented an equivalent binding affinity to MAFF with the $K_D$ values in MST and SPR assays were 19.50 ± 1.70 and 16.6 μmol/L, respectively (Fig. 1K and Fig. S3A). The CETSA assay and DARTS assay also confirmed that MAFF is the direct target of ZCY020 (Fig. 1L). These above results confirmed that MAFF is the direct target of lathyrol and ZCY020. Considering the strong anti-inflammatory activity and the large amounts in the seeds of *E. lathyris*, ZCYO20 was selected as the representative lathyrane diterpenoid for further study.

3. ZCYO20 inhibits the formation of MAFF homodimer and promotes the heterodimerization of MAFF and Nrf2

Upon LPS stress, MAFF expression in nucleus increased, while Nrf2 slightly increased and mainly translocated into the nucleus; ZCYO20 significantly promoted the nuclear translocation of Nrf2 and the formation of the heterodimer of MAFF–Nrf2.
(Fig. 2A). LPS stimuli obviously elevated the sumoylation of MAFF, whereas ZCY020 significantly suppressed these effects (Supporting Information Fig. S4A). ZCY020 did not affect the ubiquitination of MAFF (Fig. S4B). Besides, ZCY020 inhibited the binding of MAFF both to itself and to MAFK, but enhanced the interaction of MAFF with Nrf2 in basal RAW264.7 cells (Fig. 2B) and HEK293T cells (Fig. S4C–S4D). These studies revealed that ZCY020 may mediate the positive activation of MAFF–Nrf2 pathway via inhibiting MAFF homodimers and boosting the formation of MAFF–Nrf2 heterodimer.

4. ZCY020 activates MAFF–Nrf2/HO-1 pathway to exert anti-inflammatory activity by targeting MAFF

HMOX1 (HO-1), regulated by Nrf2-in response to oxidative and inflammatory stress, has significant antioxidant and anti-inflammatory effects. ZCY020 significantly up-regulated the expression and nuclear translocation of Nrf2 and the transcription of its target genes including HO-1 (Fig. 2C and Supporting Information Fig. S5A–S5B). Besides, ZCY020 inhibited the production of reactive oxygen species (ROS) stimulated by LPS (Fig. 2D).

In addition, ZCY020 inhibited the phosphorylation of IκBα and the nuclear translocation of NF-κB p65 (Fig. 2E and Fig. SSC), thus inhibited the levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NO production (Fig. S5D). ZCY020 also significantly decreased interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in transcription and expression levels (Fig. S5E). As shown in Fig. 2F and Fig. S5F, ZCY020 evidently increased sequestosome 1 (p62), BCL2 interacting protein 3 (BNIP3) and microtubule-associated protein 1 light chain 3 (LC3)-II (Fig. 2F), whereas decreasing the production of mROS (Fig. S5F). NAC significantly attenuated the increase of p62, BNIP3 and LC3B-II and autophagosome accumulation (Fig. S5G–S5H), further suggesting that ZCY020 promotes mitophagy.

To verify that ZCY020 activates Nrf2 pathway through MAFF–Nrf2 heterodimer by targeting MAFF, an Nrf2 inhibitor ML385 or MAFF shRNA was applied. The promotion on Nrf2, HO-1 expression and the inhibition on ROS (Fig. 2G and Fig. S5I) were significantly reversed by ML385. ML385 also blocked the inhibitory effects on mtROS and the facilitating effects on BNIP3 and LC3B-II of ZCY020 (Fig. 2H and Fig. S5J). Furthermore, MAFF shRNA (Fig. S5K) decreased the effects on HO-1 expression and ROS levels (Fig. 2I), phosphorylation of IκBα (Fig. 2J) and NO release, with the IC50 value increasing to 15.21 ± 0.78 μmol/L (Fig. S5L). MAFF shRNA also hindered the up-regulation of ZCY020 on the expression of p62, BNIP3 and LC3B-II (Fig. 2K). Thus, the above studies indicated that ZCY020 activates the Nrf2/HO-1 pathway to exert antioxidant and anti-inflammatory effects through MAFF–Nrf2 heterodimers by targeting MAFF.

5. ZCY020 shows inhibitory effects on LPS-induced acute lung injury in mice

To further evaluate the anti-inflammation of ZCY020 in vivo, we next assessed the anti-inflammatory effects of ZCY020 on LPS-stimulated acute lung injury (ALI) in mice. ZCY020 administration effectively inhibited the LPS-caused mortality in a dose-dependent manner (Fig. 2L) and ameliorated the histopathological changes in the lungs and livers of ALI mice (Fig. 2M and Supporting Information Fig. S6A). Moreover, ZCY020...
treatment significantly decreased the transcription and expression levels of IL-1β, IL-6 and TNF-α (Fig. S6B), and increased the expression of Nrf2 and HO-1 (Fig. 2N), promoted the transcription of HMOX1, NQO1, GCLC and GCLM (Fig. S6C). Moreover, BNIP3 and LC3B-II were enriched, and the level of p-IκBα was decreased significantly by ZCY020 (Fig. S6D). Together, these results proved that ZCY020 activates the MAFF–Nrf2 pathway exerting anti-inflammatory effects against ALI.

In summary, we creatively employed PROTAC technology combined with quantitative proteomics to observe the changes of proteome before and after, then confirmed that MAFF is a key target protein of lathyrane diterpenoids. Based on this, we verified that lathyrane diterpenoid ZCY020 exerts anti-inflammatory effects through the MAFF–Nrf2 pathway by targeting MAFF in LPS-induced RAW264.7 macrophages and ALI mice, suggesting that the method of PROTAC probes combined with quantitative proteomics, which we call targeted degradomics here, can be a novel and valuable supplementary approach for the target identification of natural products and other drugs, and is worthy of further application and investigation.

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Author contributions

Yanli Wu, Yueying Yang, and Wang Wang performed the major experiments under the supervision of Hua Li and Lixia Chen. Dejuan Sun, Jing Liang and Man Zhu performed some experiments. Yanli Wu and Yueying Yang analysed the data. Yanli Wu and Wang Wang wrote the original draft. Hua Li and Lixia Chen edited the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.07.007.

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