COMMUNICATION

Chemoproteomic fishing identifies arzanol as a positive modulator of brain glycogen phosphorylase†

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The interactome of arzanol was investigated by MS-based chemical proteomics, a pioneering technology for small molecule target discovery. Brain glycogen phosphorylase (bGP), a key regulator of glucose metabolism so far refractory to small molecule modulation, was identified as the main high-affinity target of arzanol. Competitive affinity-based proteomics, DARTS, molecular docking, surface plasmon resonance and *in vitro* biological assays provided molecular mechanistic insights into the arzanol–enzyme interaction, qualifying this positive modulator of bGP for further studies in the realm of neurodegeneration and cancer.

The identification of cellular targets is critical to clarify the mechanism of action of natural products and optimize their activity.[1] In this context,[2] we were intrigued by the structure and broad polypharmacological profile of arzanol (Fig. 1, Panel A), an anti-inflammatory prenylated heterodimeric phloroglucinyl α-pyrone isolated from eternelle (*Helichrysum italicum* L.), a plant of current perfumery relevance also used in herbal medicine.[3] Arzanol shows antioxidant activity *in vitro* and *in vivo*, inhibits NF-κB activation as well as HIV-1 replication in T cells, and, by inhibiting critical enzymes of the inflammatory cascade (PGES-1, 5-LOX and COX-1), also modulates the release of pro-inflammatory mediators (interleukins, TNFα, and PGE₂).[4] The anti-inflammatory activity of arzanol was confirmed in a preclinical setting, providing a rationale for further studies on this compound.[5] Spurred by this multi-target profile of activity, we have explored the interactome of arzanol using a chemical proteomic approach. For this purpose,

a complex protein mixture from HeLa cells under pseudo-physiological conditions was used as the fishing pond, and chromatography and drug affinity responsive target stability[6] were used for target identification.

The experimental strategy used in this research can be sorted out in four different steps: (a) covalent immobilization of arzanol on a solid support, incubation with HeLa cell lysates, and mass spectrometry (MS)-based identification of its target; (b) DARTS experiment followed by immunoblotting to monitor the stability of arzanol interactor over enzymatic proteolysis; (c) competitive affinity chromatography experiments, surface plasmon resonance and molecular docking to identify arzanol binding sites on its target, and (d) evaluation of arzanol bioactivity *in vitro*.

Arzanol was covalently coupled to solid beads capitalizing on the reactivity of its electrophilic phenone carbonyl (Fig. S1, ESI†). An agarose matrix activated with 1,1′-carbonyldiimidazole (CDI) was linked to a TRX (4,7,10-trioxa-1,13-tridecanediamine) spacer to avoid steric interferences during the fishing experiments. The nucleophilic coupling of the PEG amino group to the arzanol phenone carbonyl (Fig. S1, ESI†) afforded an imine, which was next reduced by NaBH₄ (Fig. 1A and Fig. S2, ESI†). The coupling procedure was checked by RP-HPLC analysis and quenched by acetylation, with beads having a final arzanol concentration of 700 nmol per 0.1 mL resin (Fig. S3, ESI†). To distinguish between the specifically bound components and background contaminants, crude HeLa cell lysates were loaded on the arzanol-modified beads or on an empty matrix. After 16 hours of incubation, the anchored proteins were released from the resin and resolved by SDS-PAGE. The gel lanes were cut into 10 pieces, digested by trypsin, and analyzed by mass spectrometry using a nanoflow reversed-phase UPLC MS/MS combined with a Mascot database search.[7]

To identify the macromolecular targets specifically captured by arzanol, the inventory of the identified proteins was compared with that of the control in five independent experiments. Unexpectedly, brain glycogen phosphorylase (bGP), which is

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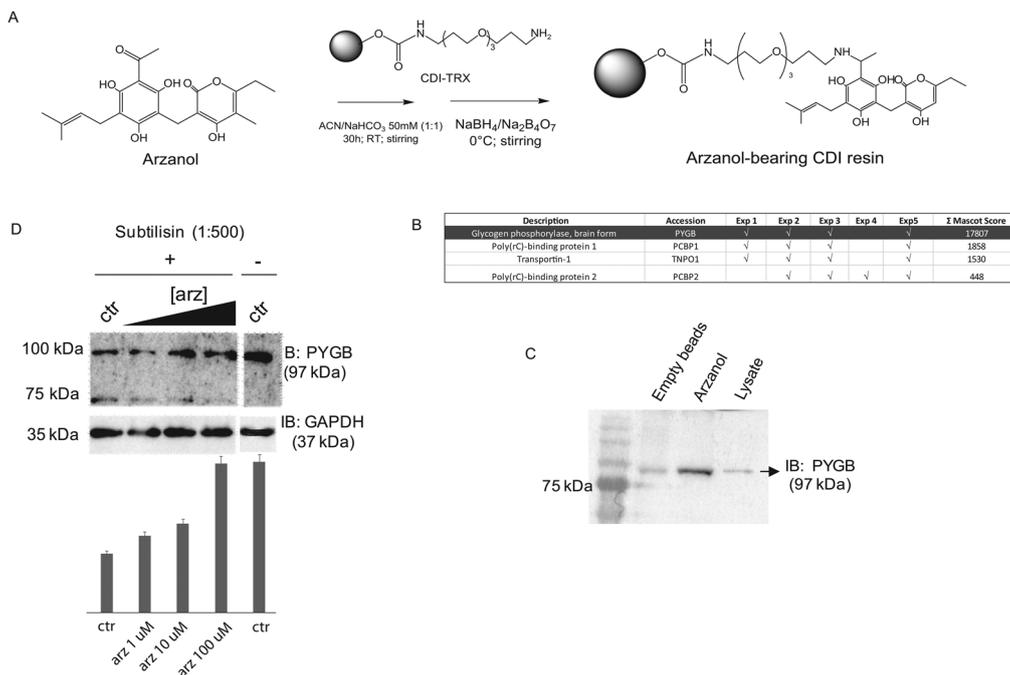
Fig. 1 (A) Chemical structure of arzanol and its reaction on CDI-TRX resin; (B) identified common targets in five independent chemical proteomics experiments reported with the sum of Mascot scores; (C) western blot analysis on the proteins eluted from the control and the arzanol-beads using an antibody against bGP (D) bGP protection to subtilisin upon arzanol interaction: DARTS was performed using HeLa cell lysates treated or not with increasing quantities of unmodified arzanol and subtilisin. GAPDH served as a loading control for densitometric analysis using the software IMAGEJ. The experiment was repeated three times and S.D. is reported in the histogram.

not a protein involved in the inflammatory cascade, was identified as the top arzanol interactor since it is identified in four over five experiments and with the highest Mascot score (Fig. 1B). This protein has never been fished out in our affinity chromatography experiments with other natural compounds[8a] nor reported as a contaminant (http://crapome.org/?q=geneprofiledetail/PYGB/human/1.1).

Besides, since arzanol is endowed with a wide range of pharmacological profiles, a large number of potential hits were expected. A few of them, found in three over five replicates and reported in Table S1 (ESI†), seem to be of interest such as transportin-1, perilipin-2 and -3, VAT-1 homolog and programmed cell death protein 6. Many other hits were found due to a high background content, possibly derived from the nonspecific binding of abundant proteins rather than true affinity as ligands.[8b,c] Thus, an in-depth mechanistic investigation on the interaction of arzanol with bGP has been performed at a molecular level. GPs are key enzymes in glycogen metabolism, promoting the rate-limiting step of its mobilization. Three GP isozymes (muscle, liver, and brain) are known that,[9] while displaying high similarity and catalyze the same reaction, significantly differ in terms of sensitivity to the allosteric activator adenosine monophosphate (AMP). In particular, bGP is strongly but non-cooperatively activated by AMP, which stabilizes its so-called active state (R-state).[10] Immunoblotting with a specific antibody against bGP showed a *bona fide* enrichment of bGP by arzanol fishing baits (Fig. 1C). Next, the DARTS protocol was applied to HeLa cell lysates,[6] which were obtained under gentle

experimental conditions to preserve macromolecular complexes and native protein structures, in the presence and in the absence of different concentrations of arzanol. In this way, the binding of a ligand to its supposed target protein translates into a limited proteolysis, as detected by an immunoblotting semi-quantitative analysis. As shown in Fig. 1D, the presence of increasing concentrations of the unmodified arzanol protects bGP from subtilisin enzymatic action, as expected for a small molecule–macromolecule interaction.

Since bGP is unique within GPs to respond non-cooperatively to AMP activation, the arzanol binding site was investigated by competitively incubating the arzanol-bearing beads with AMP as a probe on bGP. Thus, the arzanol-modified and control matrices were added to HeLa cell lysates containing arzanol or AMP at different concentrations. In this way, the competition between the arzanol-bearing beads and free metabolites for their target was promoted. The surnatants were next discarded, and the affine partners were eluted and revealed by immunoblotting. bGP was specifically eluted from the arzanol-loaded beads, but its binding visibly decreased, in a concentration dependent fashion, when free arzanol or AMP were previously added to the protein mixture (Fig. 2A). These data suggest that arzanol directly interacts with bGP, fitting in the same pocket of the endogenous AMP. Moreover, surface plasmon resonance analysis confirmed a direct interaction between the counterparts with a K_D of 0.32 ± 0.15 µM as calculated by the interpolation of the association and dissociation phases of different arzanol concentrations onto the immobilized bGP (Fig. 2B and Fig. S6, ESI†). In parallel experiments, molecular
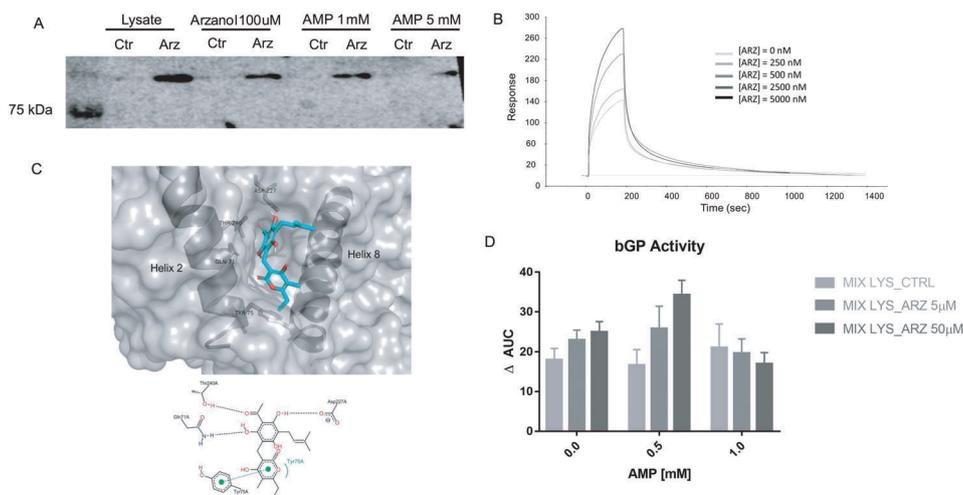
Fig. 2 (A) HeLa cell lysates with or without arzanol at 100 μM or AMP at 1 and 5 mM were incubated with the control (Ctr) or arzanol (Arz)-containing beads. bGP bound to the arzanol beads has been eluted and revealed by immunoblotting. (B) Sensograms obtained from injections of ARZ (0.25–5 μM) on immobilized bGP. (C) Best feasible predicted binding geometry determined by the molecular docking analysis between bGP and arzanol. Residues involved in the complex formation and the helixes containing the AMP binding site were labelled and reported in a stick and cartoon representation, respectively. (D) Effect of arzanol and AMP on the bGP activity in HeLa cell lysates. The "Δ AUC" is the area under the kinetic curve referring to the variation in the absorbance of the sample along a time course of 200 min. S.D. was calculated on three independent measurements.

docking of arzanol into the whole bGP binding sites was performed using the three-dimensional structure of a human protein, whose crystallographic structure has been resolved.[10a] On the basis of the predicted affinity, arzanol showed its best interaction when docked into the AMP binding site (Fig. 2C). The predicted equilibrium dissociation constant ($K_{d,pred}$) related to this binding site was 0.65 ± 0.18 μM. In particular, arzanol interacts with the residues Gln71, Asp227, Thr240 (by H-bonds) and Tyr75 (by an aromatic or π interaction), as shown in Fig. 2C. The latter is also involved in the stabilization of AMP, in accordance with the crystallographic data.[10b] To validate these data, the ability of arzanol to modulate the bGP activity was measured in vitro. HeLa cell lysates, used as a source of active bGP, were incubated in a mixture containing phosphoglucomutase, glucose-1,6-diphosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$. Glycogen breakdown was followed by monitoring the glycolytic NADPH formation at 340 nm.[11] The same experiments were then carried out in the presence of increasing concentrations of arzanol and/or AMP. Since AMP is an allosteric agonist of bGP, the enzyme activity was measured in the presence of AMP, confirming its action on bGP in the HeLa cell lysates (see also Fig. S7, ESI†). A similar concentration-dependent increase of the activity was also observed with arzanol, showing that it can increase the bGP enzyme activity in the absence of AMP, even at the lowest concentrations assayed.

Conversely, modest enzyme inhibition by arzanol was measured in the presence of high concentrations of AMP (Fig. 2D). Taken together, these results are in full accord with the molecular docking and competition experiments, suggesting that AMP and arzanol compete for the same allosteric binding site on bGP, inducing similar conformational changes and promoting the transition to the its active form.

Glycogen is a multi-functional energy source, which is used to control glycemia in the liver and to support contractions in muscles. In brain, glycogen acts as an emergency glucose storage to protect neurons against hypoglycemia and hypoxic stress, being critical for high cognitive processes such as learning and memory consolidation.[12,13] So far, interest in GPs has been mainly focused on their potential for the management of type 2 diabetes since the inactivation of liver GP diminishes blood glucose levels. More recently, their role in the realm of cancer has also been investigated, since glycogen metabolism is altered in many tumors.[14] On the other hand, the inhibition of glycogen mobilization in the brain is known to be associated with impaired cognitive functions and neuro-degeneration, as exemplified by Lafora disease, amyotrophic lateral sclerosis, and Alzheimer's disease.[15] The activation of brain glycogen breakdown represents a potential therapeutic strategy to fight these degenerative conditions,[15] but no selective regulator of bGP had been reported so far. Arzanol is, therefore, endowed with significant potential to spur a medicinal chemistry campaign to explore and optimize its biological space and validate bGP as a novel clinical target.

Conflicts of interest

The authors declare no conflict of interest.

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