Electronic Supplementary Material (ESI) for ChemComm.

Chemoproteomic Fishing Identifies Arzanol as a Positive Modulator of Brain Glycogen Phosphorylase.

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Analysis of Arzanol reactivity towards amine containing 4,7,10-Trioxa-1,13-tridecanediamine

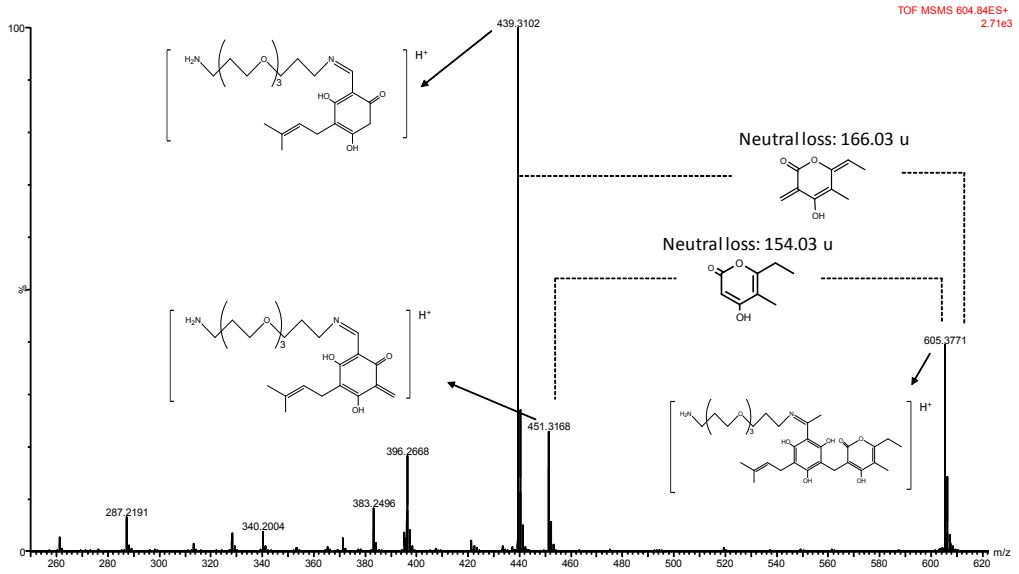

Figure S1: MS/MS fragmentation of the ion at m/z of 605.37 obtained upon the reaction of Arzanol with TRX. The daughter peaks at m/z of 451.32 and 439.312 were both attributed to fragments containing the TRX bound to the methyl carbonyl thought a stable imine formation. This carbonyl is the most reactive, as expected.

Immobilization of Arzanol on CDI-Agarose Beads

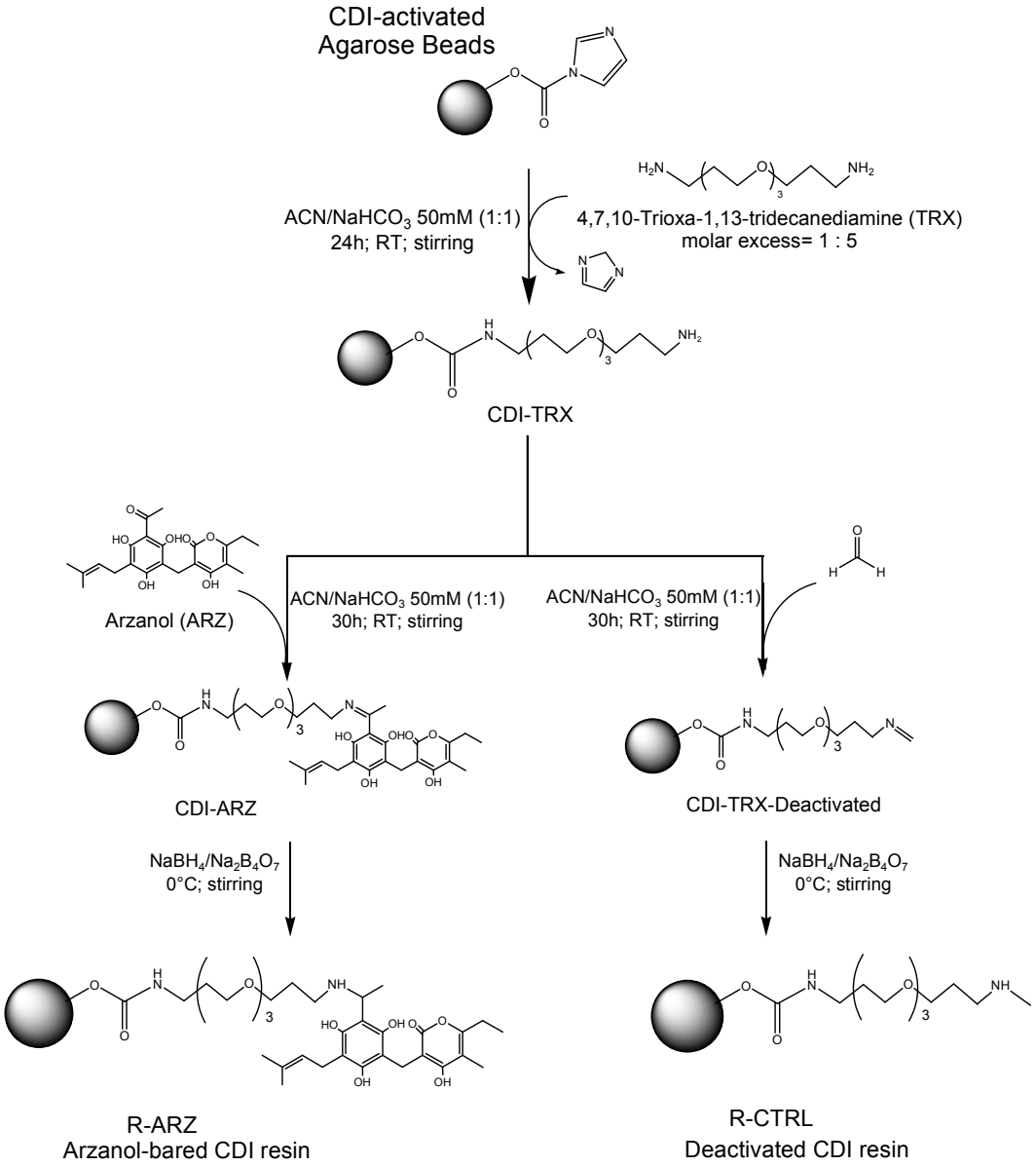

Figure S2: Full reaction scheme of Arzanol immobilization on CDI beads modified by PEG (on the left) and of empty beads for control experiments.

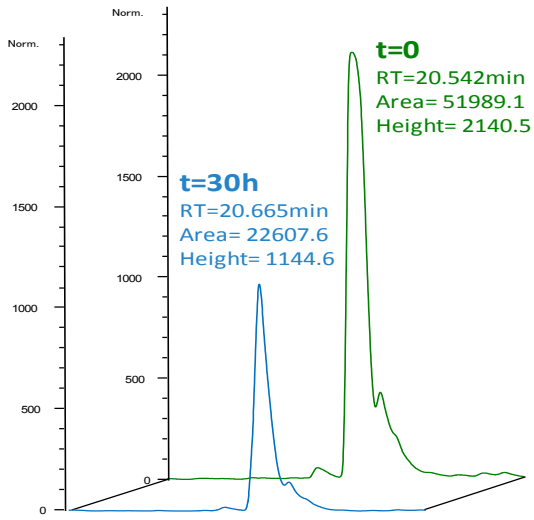

Figure S3: HPLC profile of free Arzanol before and after coupling reaction on CDI-TRX resin. After 30 h of reaction, around 60% of Arzanol has been covalently immobilized on the resin.

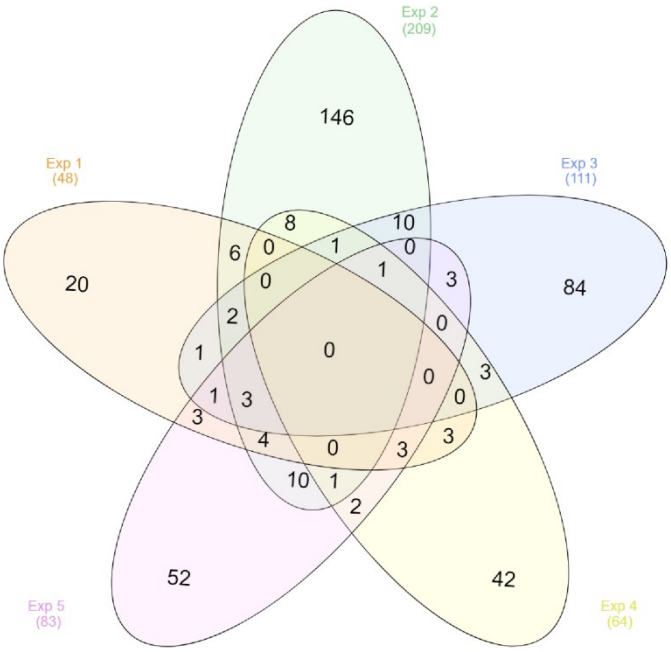

Figure S4: Venn diagram (http://www.interactivenn.net/) showing the intersection of the five sets of all Arzanol putative protein partners identified in the five different experiments (with Mascot score ≥ 60) and not detected in the corresponding control experiments.

Table S1. Proteins Identified in at least 4 or 3 of the 5 Independent Fishing Experiments and Not Detected in the Corresponding Control Experiments, with a Mascot Score higher than 60.

Description	Accession	Exp 1	Exp 2	Exp 3	Exp 4	Exp5	Σ Mascot Score
Glycogen phosphorylase, brain form	PYGB	√	√	√		√	17807
Poly(rC)-binding protein 1	PCBP1	√	√	√		√	1858
Transportin-1	TNPO1	√	√	√		√	1530
Poly(rC)-binding protein 2	PCBP2		√	√	√	√	448
Tubulin alpha-1C chain	TBA1C	√	√	√			22754
Tubulin beta chain	TBB5	√	√	√			11134
Actin, alpha cardiac muscle 1	ACTC	√	√			√	5110
Perilipin-3	PLIN3	√	√			√	3278
Beta-actin-like protein 2	ACTBL	√	√			√	3057
Heterogeneous nuclear ribonucleoprotein R	HNRPR	√	√			√	1404
Synaptic vesicle membrane protein VAT-1 homolog	VAT1	√			√	√	1277
Programmed cell death protein 6	PDCD6	√			√	√	1223
Sodium/potassium-transporting ATPase subunit alpha-1	AT1A1	√			√	√	260
Perilipin-2	PLIN2		√		√	√	259

Score: The probability that the observed match is not a random event

MATRIX SCIENCE **MASCOT Search Results**

Protein View: PYGB_HUMAN

Glycogen phosphorylase, brain form OS=Homo sapiens GN=PYGB

Database: SwissProt
Score: 12136
Nominal mass (M_r): 97319
Calculated pI: 6.40
Taxonomy: Homo sapiens

Sequence similarity is available as an NCBI BLAST search of PYGB_HUMAN against nr.

Search parameters

MS data file: C:\Users\Mascott\Desktop\Federica\E21_ARZANOLO_2
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M), Phospho (ST)

Protein sequence coverage: 59%

Matched peptides shown in ***bold red***.

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  1 MAKPLTDSEK RKQISVRGLA GLGDVAEVRK SFNRHLHFTL VKDRNVATPR
 51 DYFFALAHTV RDHLVGRWIR TQQHYYERDP KRIYYLSLEF YMGRTLQNTM
101 VNLGLQNACD EAIYQLGLDL EELEEIEEDA GLGNGGLGRL AACFLDSMAT
151 LGLAAYGYGI RYEFGIFNQK IVNGWQVEEA DDWLRYGNPW EKARPEYMLP
201 VHFYGRVEHT PDGVKWLDTQ VVLAMPYDTP VPGYKNNTVN TMRLWSAKAP
251 NDFKLQDFNV GDYIEAVLDR NLAENISRVL YPNDNFFEGK ELRLKQEYFV
301 VAATLQDIIR RFKSSKFGCR DPVRTCFETF PDKVAIQLND THPALSIPEL
351 MRILVDVEKV DWDKAWEITK KTCAYTNHTV LPEALERWPV SMFEKLLPRH
401 LEIIYAINQR HLDHVAALFP GDVDRLRRMS VIEEGDCKRI NMAHLCVIGS
451 HAVNGVARIH SEIVKQSVFK DFYELEPEKF QNKTNGITPR RWLLLCNPGL
501 ADTIVEKIGE EFLTDLSQLK KLLPLVSDEV FIRDVAKVKQ ENKLKFSAFL
551 EKEYKVKINP SSMFDVHVKR IHEYKRQLLN CLHVVTLYNR IKRDPAKAFV
601 PRTVMIGGKA APGYHMAKLI IKLVTSIGDV VNHDPVVGDR LKVIFLENYR
651 VSLAEKVIPA ADLSQQISTA GTEASGTGNM KFMLNGALTI GTMDGANVEM
701 AEEAGAENLF IFGLRVEDVE ALDRKGYNAR EYYDHLPELK QAVDQISSGF
751 FSPKEPDCFK DIVNMLMHHD RFKVFADYEA YMQCQAQVDQ LYRNPKEWTK
801 KVIRNIACSG KFSSDRTITE YAREIWGVEP SDLQIPPPNI PRD
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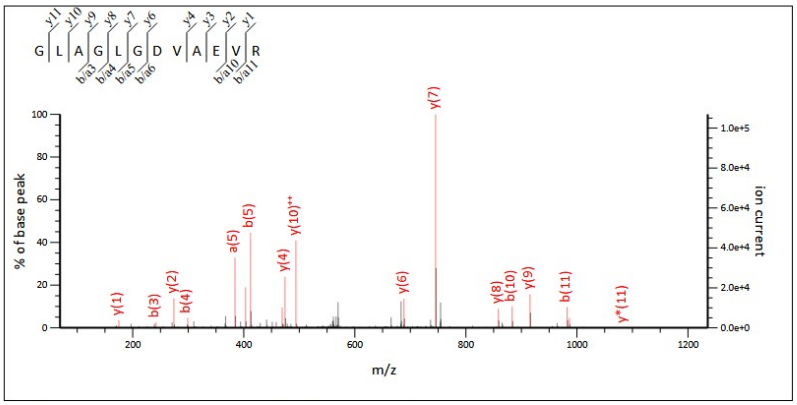
MATRIX SCIENCE Mascot Search Results

Peptide View

MS/MS Fragmentation of **GLAGLGDVAEVR**
Found in **PYGB_HUMAN** in **SwissProt**, Glycogen phosphorylase, brain form OS=Homo sapiens GN=PYGB PE=1 SV=5

Match to Query 786: 1155.623266 from(578.818909,2+) intensity(25335494.0000) rtinseconds(2814.0776) index(2346)
Title: 20171130_E21_ARZ2.3007.3007.2 File:"20171130_E21_ARZ2.raw", NativeID:"controllerType=0 controllerNumber=1 scan=3007"
Data file C:\Users\Mascott\Desktop\Federica\E21_ARZANOLO_20171128\20171130_E21_ARZ2.mgf



MATRIX SCIENCE Mascot Search Results

Peptide View

MS/MS Fragmentation of **NLAENISR**
Found in **PYGB_HUMAN** in **SwissProt**, Glycogen phosphorylase, brain form OS=Homo sapiens GN=PYGB PE=1 SV=5

Match to Query 87: 915.477452 from(458.746002,2+) intensity(205446.6094) rtinseconds(1409.7842) index(879)
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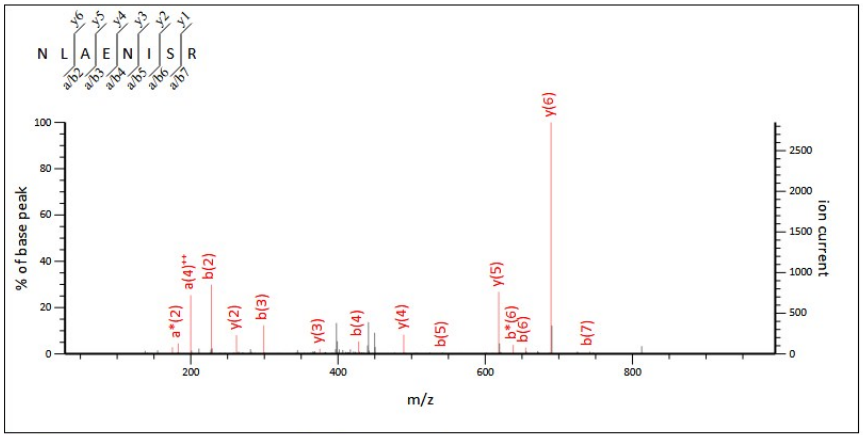
Data file C:\Users\Mascott\Desktop\Federica\E21_ARZANOLO_20171128\20171130_E21_ARZ2.mgf



MATRIX SCIENCE **Mascot Search Results**

Peptide View

MS/MS Fragmentation of **TCFETFPDK**
Found in **PYGB_HUMAN** in **SwissProt**, Glycogen phosphorylase, brain form OS=Homo sapiens GN=PYGB PE=1 SV=5

Match to Query 738: 1143.489354 from(572.751953,2+) intensity(38037488.0000) rtinseconds(2423.0313) index(1809)
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Data file C:\Users\Mascott\Desktop\Federica\E21_ARZANOLO_20171128\20171130_E21_ARZ2.mgf

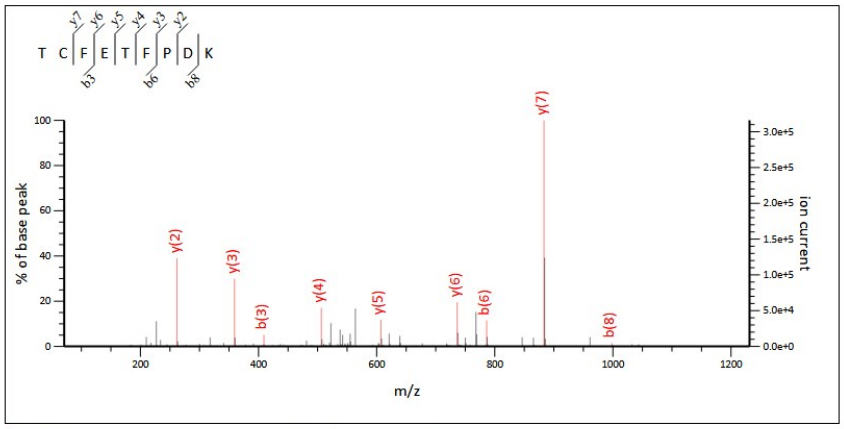
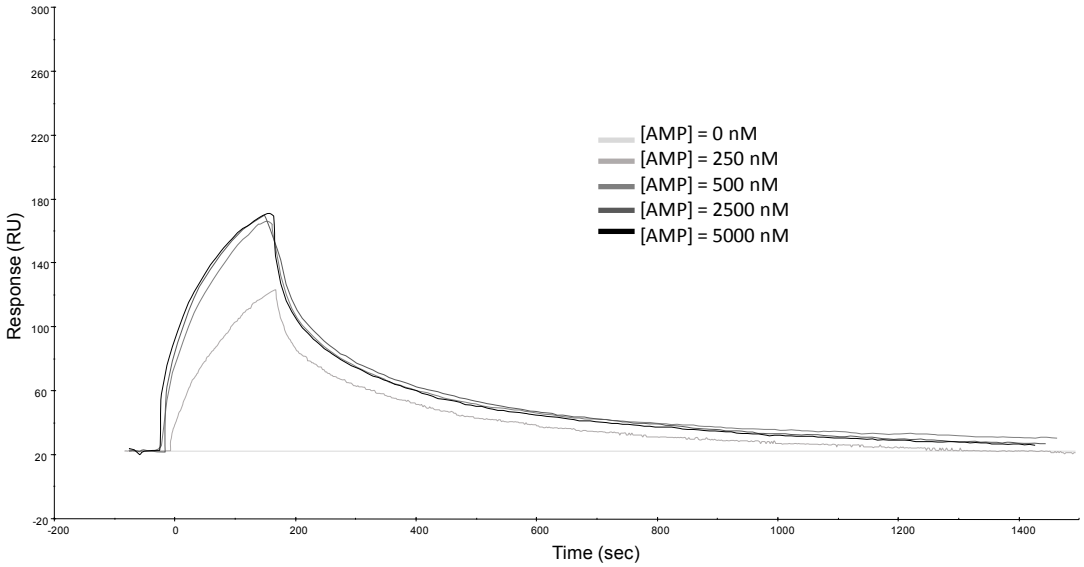


Figure S5: Example of MASCOT Search Results for bGP identification followed by tandem mass spectra of three different peptides of the protein.



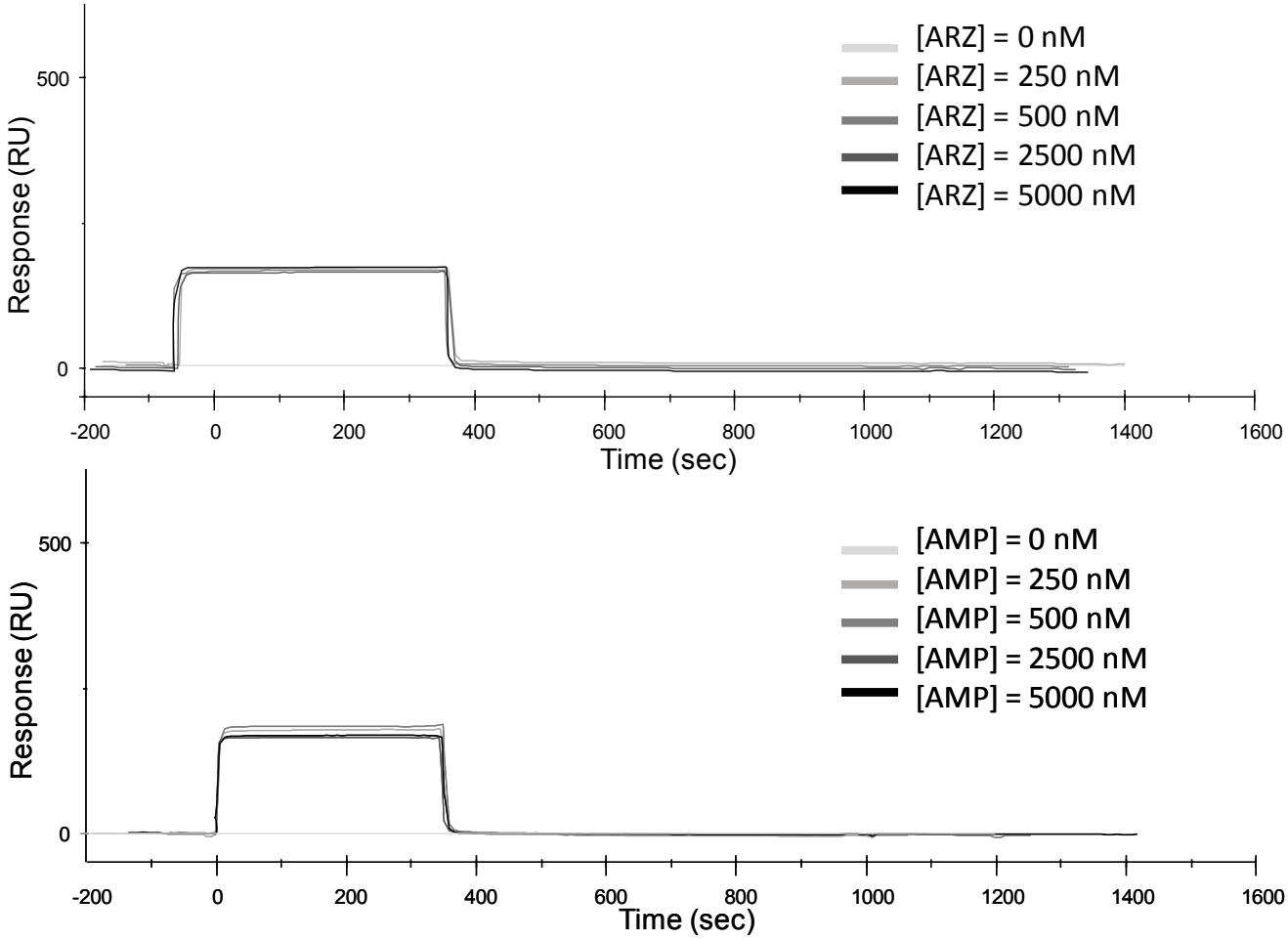


Figure S6: Surface plasmon resonance (SPR) analysis: the upper panel shows the sensorgrams obtained when free AMP was put in contact with the immobilizer bGP. Clear association and dissociation phases were visible and the Langmuir 1:1 analysis gave a K_D of 8.34±5.69 nM in between the counterparts. Since AMP is a known ligand of bGP, these data validates our SPR analysis confirming that bGP is in a native folded state. The middle and lower panels show the lack of interaction between arzanol and AMP on a control immobilizied protein as lysozyme, respectively.

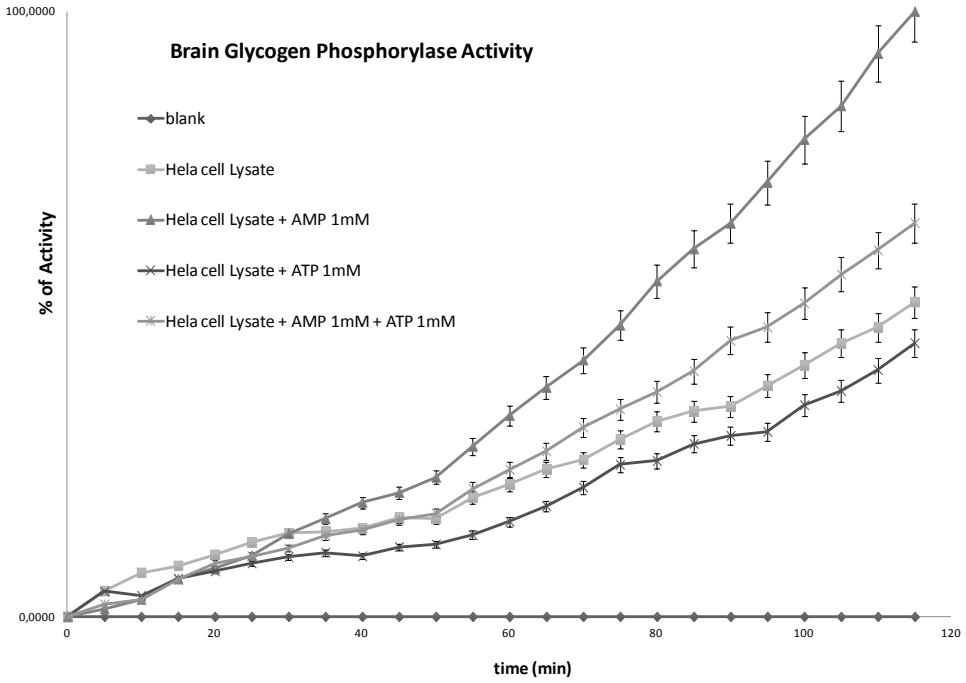


Figure S7: bGP activity as percentage over time. An *in vitro* kinetic assay has been carried out evaluating glycogenolytic activity of bGP in presence of different concentrations of ATP (a know inhibitor of bGP) and/or AMP (a known activator of bGP). Lysate from HeLa cells has been treated with an enzymatic mixtures containing glycogen, NADP, alpha-D-glucose 1,6-bisphosphate, glucose-6-phosphate dehydrogenase, and phosphoglucomutase and variations in the absorbance at 340 nm have been measured. Results easy show that the HeLa lysate has a certain glycogenolytic activity due to active bGP (see squares); this activity is enhanced adding AMP (see triangles) and it is decreased adding ATP (see crosses), as expected. Adding both AMP and ATP at the same concentration, AMP reveals to be a stronger activator en respect of ATP as inhibitior (see stars). 100% of bGP activity has been arbitrarily put at bGP + 1mM AMP after 115 min of reaction. Standard deviation has been reported for three independent measurements.

Experimental Procedures

Analysis of Arzanol most reactive carbonyl

Arzanol has been treated with 4,7,10-Trioxa-1,13-tridecanediamine (TRX) in solution. Briefly, 2 µL of a solution of Arzanol (10 µg/µL in 100% DMSO) has been added to 18 µL of ACN (0,5% TEA) containing TRX in molar excess (1:100) and the obtained solution has been kept in orbital agitation (400 RPM, RT). The reaction has been monitored after 3h by LC and MS analysis. The composition of the raw reaction mixture has been analysed by RP-HPLC (Agilent 1260 Infinity equipped with a Phenomenex C-18 Jupiter column 5 µm, 300 Å, 150x2mm) at flow of 200 µL/min from 10% to 95% buffer B in 15 minutes (buffer A: H_2O 0,1% TFA, buffer B: 95% ACN, 5% H_2O, 0,07% TFA) and the major chromatographic peak (RT=20min) has been investigated by ESI-Q-TOF analysis: the fragmentation of the base peak at 605.37 m/z, corresponding to the $[M+H]^+$ of the imine product from ARZ + TRX, unambiguously confirmed that the most reactive carbonyl of Arzanol is the extra-ring acetyl group (Fig. S1).

Immobilization of Arzanol on CDI-Agarose Beads

After proper washing and equilibration steps as reported on product sheet, 400 µL of Pierce™ CDI-activated Resin (1,1´-carbonyl diimidazole activated 6% crosslinked beaded agarose; Thermo Scientific) has been resuspended in 400 µL of 50% ACN 50% $NaHCO_3$ (50mM) containing 100 µmol of 4,7,10-Trioxa-1,13-tridecanediamine (TRX at 1:5 molar excess respect to the reactive CDI groups on the beads). The coupling proceeded for 24h (RT, constant rotation) and the formation of the carbamate groups have been verified by the positiveness of the Kaiser test (Kaiser test kit, Sigma Aldrich) due to the presence of the free primary amino-groups of TRX bearing the beads. The beads have been washed and reconditioned in the coupling buffer (50% ACN 50% NaB_4O_7 containing 2% TEA), then they have been divided into two aliquots of 200 µL each. 200 µL of solid matrix have been resuspended in 100 µL of coupling buffer containing 2.5 µmol of Arzanol to covalently bond the small molecule to the spacer arm bearing the beads. The aliquot left has been used as negative control incubating it with the coupling buffer without the molecule (Fig. S2). The reaction has been kept in constant rotation (RT) and after 30h the concentration of Arzanol decreased of around 60%, as shown in Fig.S3: around 700 nmol of the small molecule has been covalently bonded to each 100 µL of the resin support through the spacer diamine arm. Both the aliquots of 200 µL of solid matrix, bared or not with Arzanol, were re-suspended in
200 µL of a solution of formaldehyde (1:500 molar excess respect to the loading capacity of the resin) in $Na_2B_4O_7$ 20mM with the purpose of deactivate the free amine groups on the beads. To avoid the possibility of an equilibrium reversion, the obtained imines have been reduced to amine groups suspending the beads in a 1M solution of $NaBH_4$ in $Na_2B_4O_7$ 20mM (0°C, 30min, static position, Fig. S2). The obtained aliquots of Arzanol-bared beads and control matrix have been used in the following affinity chromatography purification steps.

Identification of Arzanol interactome by affinity chromatography and mass spectrometry

HeLa cells have been mechanically lysed in PBS pH 7.4 (137 mM NaCl, 2,7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) containing 0,1% Igepal and a protease inhibitor cocktail (Sigma Aldrich). Protein concentration has been determined by Bradford spectrophotometric assay.[1]
After washing steps with PBS, Arzanol-bared beads and control matrix have been suspended in lysate solutions containing about 800 µg of proteins each and the incubation has been carried on overnight (4°C, constant rotation). Resins have been washed and the proteins have been eluted by treatment (95°C, 5 mins) with Laemmli buffer (100 mM Tris pH 6,8, 4% SDS, 0.2% Bromophenol Blue, 20% glycerol, 2% β-mercaptoethanol). The obtained protein mixtures have been separated by 1D-SDS-PAGE (12% polyacrilamide), stained by Coomassie Blue and then digested *in situ* by trypsin. Briefly, exceeded pieces of gel have been destained and disulfuric bonds between cysteines in the proteins have been reduced by DTT (6.5 mM in AmBic 50mM, 60°C, 1h) and then carboamidomethylated by IAM (10mM in AmBic 50mM, RT, dark, 30mins). A solution of trypsin (12 ng/ul) has been used to digest proteins in gel meshes (overnight, 37°C) and peptide mixtures have been extracted with 100% ACN and dried in SpeedVac.[2] Peptides have been resuspended in FA 10% and submitted to a nanoUPLC-nanoESI-MSMS analysis. The nanoAcquityLC System (Waters Corp. Manchester, U.K.) was equipped with a binary pump and a BEH C-18 column (1,7 µm, Waters Corp. Manchester, U.K.) interfaced with the nanoESI of the hybrid mass spectrometer LTQ-Orbitrap XL (Thermo Scientific). 5 µL of each sample have been injected and separated in 55 mins by a linear gradient from 15% to 50% of buffer B (buffer A: 95% H_2O, 5% ACN, 0.1% FA; buffer B: 95% ACN, 5%H_2O, 0.1% FA; flux: 280nl/min). Most intense doubly and triple charged peaks have been fragmented and the raw peak lists have been submitted to MASCOT Daemon software (SwissProt 2017_01; 553,474 sequences; 198,069,095 residues). All the experiments have been performed in 5 independent experiments. The mass spectrometry proteomics

data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011170 and 10.6019/PXD011170.

In vitro validation of brain glycogen phosphorylase as Arzanol partner by immunoblotting, DARTS and competitive elution

MS-based result has been validated by three orthogonal experiments exploiting the immunoblotting technique. After affinity purification, the obtained mixtures of proteins from the arzanol-bared and the control beads have been separated by 1D-SDS-PAGE (8% polyacrylamide) and then transferred on a nitrocellulose membrane (Macherey-Nagel). After blocking in Milk 5% (1h, RT), the membrane has been incubated overnight with the specific primary antibody for the bGP (1:1000 in 5% milk; Invitrogen, Thermo Scientific).
Drug Affinity Responsive Target Stability (DARTS) experiment has been performed incubating (1h, RT, 400 RPM) lysate from HeLa cells (200µg of proteins for each experimental condition) with different concentrations of Arzanol (0, 1 µM, 10 µM and 100 µM final concentrations in 1% DMSO). Then, samples have been treated or not with subtilisin (defect 1:500) in presence of 1mM $CaCl_2$ to prevent enzymatic auto-digestion. The partial proteolysis by subtilisin has been quenched after 1h (RT) adding PMSF (phenylmethylsulfonyl fluoride) 1mM final concentration. After 10 minutes, samples were prepared for 1D-SDS-PAGE (8% polyacrylamide) and the obtained gel has been used for the immunoblotting. Nitrocellulose membrane has been incubated with the primary antibody specific for bGP (1:1000 in 5% milk; Invitrogen, Thermo Scientific) and then normalized with primary antibody against GAPDH (1:1000 in 5% milk; Invitrogen, Thermo Scientific).
A competitive elution has been performed incubating both Arzanol-bared beads and a control matrix with a control lysate, an Arzanol-enriched lysate (100 µM final concentration) or an AMP-enriched lysate (1mM final concentration). Protein mixtures eluted from each resin have been separated by 1D-SDS-PAGE (8% PolyAcrylamide) and the immunoblotting against bGP (1:1000 in 5% milk; Invitrogen, Thermo Scientific) has been carried out.

Surface Plasmon Resonance Analysis

bGP was immobilized onto a CM5 sensor chip by using standard amine coupling procedures. 1X HBS-P was used as running buffer. The carboxymethyl dextran surface was activated with a 5 min injection of a 1:1 ratio of EDC and NHS (100 mM at 5 µL/min). bGP was diluted to 100 ng/mL in potassium acetate (10 mM, pH 4.5) and injected onto the activated chip surface (flow rate 5 µL/min) until reaching ~17 000 RU. Remaining active groups of the sensor chip were blocked with a 7 min injection of ethanolamine·HCl (1.0 M, pH 8.5) at 5 µL/min. For these biosensor experiments, arzanol or AMP (0.25–5 µM) was diluted in HBS-P containing 1% DMSO. Each concentration was tested at least three times. Since both dissociated to baseline within a reasonable time, no regeneration was required. The interaction experiments were carried out at a flow rate of 10 µL/min over a 3 min injection time. The dissociation time was set at 600 s. Rate constants for association (ka), dissociation (kd) and the dissociation constant (K_D) were obtained by globally fitting data from all the injection of different concentrations of each compound by using BIAevaluation software (GE Healthcare) with a simple 1:1 Langmuir binding model. The same experiment was carried out using the same concentrations of free AMP as bGP ligand on a control protein as lysozyme which has been immobilized onto a CM5 sensor chip with the same procedure reaching ~10 000 RU.

Molecular Docking Analysis

In order to identify the most probable binding complex between the human bGP and the Arzanol, we perfomed a molecular docking analysis starting from the crystallographic structure of the enzyme (pdbID: 5IKP)[3] and from the design and the refinement/minimization (with a universal force field, UFF, and a conjugate gradient algorithm until a ΔE lower than 0.001kJ/mol) of the ligand using Avogadro software (version 1.2)[4]. Molecular docking was performed using Autodock Vina (version 1.1.2)[5] on an Intel Core i7/Mac OS X 10.13 – based platform, considering as docking zone both the entire enzyme and the AMP binding site (with a grid of 70, 75 and 76 Å and of 29, 29 and 20 Å in the x, y, and z directions, respectively).The estimation of the predicted equilibrium dissociation constant ($K_{d,pred}$) of Arzanol or AMP (the geometry of which was taken from the crystallographic structure the enzyme reported above) in complex with human bGP was done using the NNscore 2.0 python script.[6] The final complex geometry was rendered by PyMol software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.) whereas the 2D representation was created using PoseView server.[7]

In vitro biological activity assay

An *in vitro* kinetic assay has been carried out evaluating glycogenolytic activity of bGP in presence of different concentrations of Arzanol and/or AMP.
Lysate from HeLa cells has been treated (see above for details) with an enzymatic mixtures (final concentrations in PBS pH 6.9 : 100 µg of proteins/30 µL; 5% glycerol; 2 mM EDTA, 0,25% glycogen, 0,8 mM NADP, 10 mM $Mg(C_2O_2H_3)_2$, 5 µM alpha-D-glucose 1,6-bisphosphate, 6 U/30 µL glucose-6-phosphate dehydrogenase, 6 U/30 µL phosphoglucomutase). The assay mix has been first divided into 3 aliquots to add AMP to the final concentrations of 1mM or 0.5mM, keeping one of the samples as AMP-free control. Each of the aliquots has been splitted again for the incubation with Arzanol (final concentrations: 5 µM or 50 µM in 1%DMSO), saving aliquots without the molecule as controls. Part of the lysate has been treated with PBS pH 6.9 not containing enzymatic mixtures to subtract the basal response of the lysate. All the samples have been incubated at 37°C for 15 minutes (400 RPM) and 30 µL of each mix has been loaded in triplicate in a 384-multiwell plate. Variations in the absorbance at 340 nm have been calculated reading the plate in a kinetic mode each 5 minutes for 200 minutes in total (37°C, medium interval shaking) on MultiskanGO Spectrophotometer (Thermo Scientific).

References

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Author Contributions

Dr. del Gaudio, Dr. Pollastri and Dr. Mozzicafreddo were involved in experimental investigations, data curation, formal analysis and data validation. Prof. Alberto Minassi and Raffaele Riccio were involved in project administration and funding acquisition. Prof Maria Chiara Monti was involved in data curation, formal analysis, data validation and she wrote the original draft.