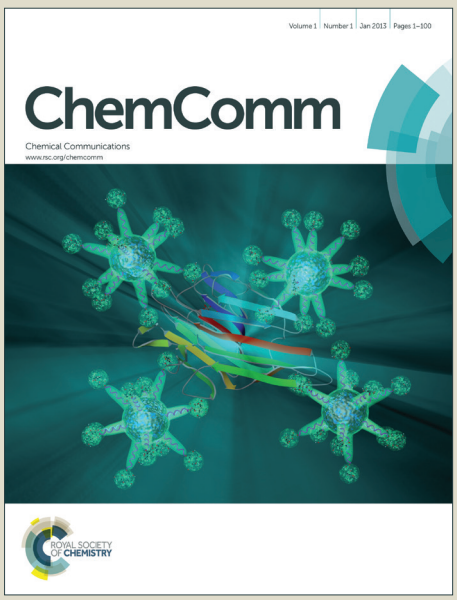
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Theonellasterone, a steroidal metabolite isolated from a *Theonella* sponge, protects Peroxiredoxin-1 from oxidative stress reactions.

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

L. Margarucci,[a] M. C. Monti,[a*] A. Tosco,[a] R. Esposito,[a] A. Zampella,[b] V. Sepe,[b] M. Mozzicafreddo,[c] R. Riccio[a] and A. Casapullo[a*]

Abstract: Peroxiredoxin-1, a key enzyme in cellular detoxification pathway, has been identified through a chemo-proteomic approach as the main partner of thonellasterone, a marine bioactive metabolite. A combination of chemical and biochemical assays disclosed its mechanism of action at a molecular level.

Since about 40% of current pharmaceuticals derives from biological sources [1,2], the biomedical potential of structural and chemical examination of natural organisms has been widely recognized [1-4]. In addition to their possible employ as drugs, natural compounds can also act as biological probes in the discovery of novel therapeutic targets and to clarify their role in complex biological processes. Among natural fonts, marine world is a huge source of original molecules for the treatment of human pathologies [5]. In particular, sponges of the genus *Theonella* have fascinated the scientific community for their remarkable assortment of bioactive secondary metabolites [6-9]. Among them, Theonellasterol and Theonellasterone (THS, Figure 1), 4-methylene-24-ethylsteroids containing a relatively rare double bond and a biosynthetically unusual methylene functionality, have been shown to modulate in HepG2 cells the trans-activation of Farnesoid-X-Receptor (FXR), an important pharmacological target in the treatment of cholestatic disorders [10-14]. Thus, on the basis of structural considerations, THS has been considered a good probe for chemical proteomics, a mass spectrometry-based affinity chromatography approach, to unveil its whole interactome and thus shed more lights on its mechanisms of action. The discovery of the multi-target profile of a small molecule or a drug is essential for a wide-ranging assessment of possible side effects and toxicities. Furthermore, a drug hitting several targets can be functional in a one-step therapy of several unrelated diseases, or in amplifying a specific therapeutic application [15].

Following our ongoing studies on natural products targetomics [16-19], we have applied chemical proteomics to the case of THS. In this approach, a small bioactive molecule is conveniently modified and acts as a bait, fishing out its specific interactors from a cell lysate or a tissue extract. Once eluted, the cellular partners are identified by means of MS and bio-informatic analysis. Later on, the biological relevance of the relevant interactions are assessed by biochemical assays [16-19].

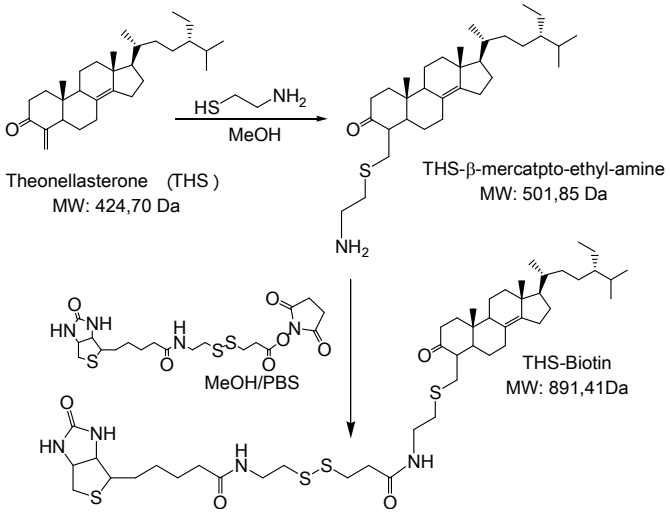


Figure 1: Reaction pathways of THS with β-mercapto-ethyl-amine and NHS activated S-S-biotin to obtain THS-Biotin adduct used in chemical proteomics experiments.

In a first phase, THS has been modified by a biotin-containing linker to take advantage, in the following affinity chromatography step, of the strong biotin-streptavidin interaction (Figure 1). THS was put in reaction with β-mercapto-ethyl-amine, which nucleophile -SH group attacked on the THS conjugated carbonyl by a Michael-like addition (Figure 1). This reaction gave a THS adduct, purified by RP-HPLC and characterized by MS and MS-MS (Figure S1), later treated with a *N*-hydroxysuccinimide activated S-S biotin linker, a disulphide-containing spacer arm. This treatment gave rise to an amide bond, providing the THS-biotin derivative reported in Figure 1 with approximately 80% Yield. The reaction was monitored by RP-HPLC-MS and the product (MW of 891.41 Da, Figure S2) was then purified by RP-HPLC-UV. The use of a disulphide bridge-containing spacer arm is a common practice in chemical proteomics. This linker provides a labile site, selectively cleavable using mild

reducing agents such as glutathione or dithiothreitol (DTT), useful to reduce the amount of aspecific binding during the affinity chromatography phase [20].

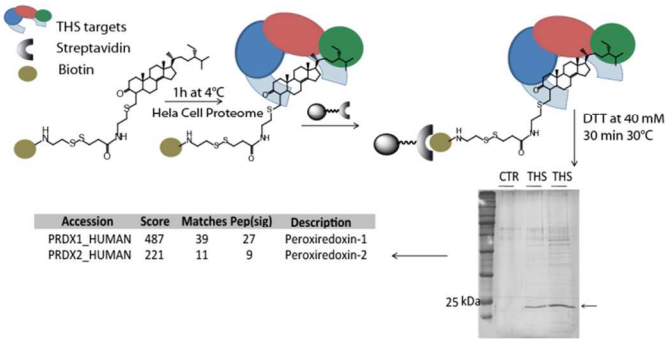


Figure 2:THS chemical proteomic workflow starts with affinity purification step followed by THS-partners separation by 12% SDS-PAGE and ends with targets identification by nano-LC-MSMS analysis coupled with bio-informatics.

HeLa cells protein extracts were then separately incubated with the THS-Biotin adduct (100 nmol) and the biotin linker modified by β-mercapto-ethyl-amine (100 nmol), as opportune control (Figure S3), to promote the interaction between the compound and its potential partner(s) in solution. Then, the THS-Biotin, and control adducts, together with their interactors, were isolated from the solution using a sample of streptavidin-bearing matrix beads. Subsequently, the tightly bound proteins were released after cleavage of the disulphide bridge. The protein mixtures eluted from the THS-Biotin and control experiments were resolved by 12% SDS-PAGE (Figure 2). As a result, a main band at around 25 kDa was only visible in the THS experiments (indicated by arrow in figure 2) and was subjected, after excision, to an *in situ* digestion protocol [21]. The tryptic peptide mixture was analyzed through nano-flow RP-HPLC/MSMS and proteins identification was performed by submitting the peak lists to the Mascot database. The proteins identified with the highest score belong to Peroxiredoxins family, namely Peroxiredoxin-1 and -2 (PRX-1 and PRX-2).

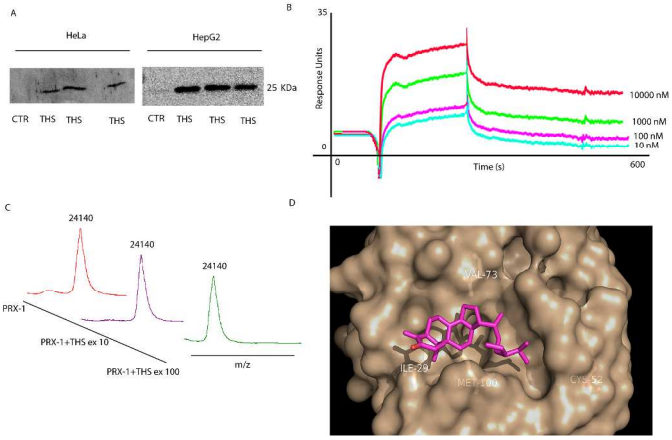


Figure 3: Panel A shows immune-blotting analysis of THS fishing for partners on HeLa cell lysates using antibody against PRX-1. Panel B shows SPR sensorgrams obtained on PRX-1 modified sensor chip with different concentration (0.01-10 μM) of free THS. Panel C shows MALDI spectra for PRX-1 incubated with THS at 10 μM and 100 μM. Panel D shows THS best orientation on PRX-1 surface obtained by molecular docking analysis.

The binding of THS-Biotin to PRX-1, chosen as main probe due to its highest identification score, was also assessed by immune-blotting analysis, in which PRX-1 was detected only in the THS-Biotin lane, in respect to the control experiment (Figure 3A). Then, surface plasmon resonance was employed as a further validation of the chemical proteomics results and to measure the binding affinity between THS and PRX-1, using PRX-6 as negative control. Both proteins were immobilized on a CM-5 sensor chip prior the injection of THS at various concentrations (0.01– 10 μM, Figure 3B). Sensorgrams analysis allowed us to calculate the dissociation constant (K_D) of PRX-1-THS complex as 330±200 nM, while no binding was observed for PRX-6 (see also Figure S4). Furthermore, due to a free highly reactive Cys residue into the PRX-1 active site [22] and the THS α,β-unsatured carbonyl function, a MALDI-MS analysis of the native protein in presence and in absence of different concentrations of THS was performed to disclose a plausible covalent binding between the two counterparts. As reported in Figure 3C, none covalent binding was detected in each tested condition, suggesting a main role of non-covalent recognition event in the PRX-1-THS complex formation. Later, a *in silico* docking analysis was carried out to get a more detailed picture of the THS/PRX-1 complex formation, analyzing the most feasible binding mode of THS on the whole PRX-1 surface (see methods section in SI) [23-25]. THS was found to produce a stable PRX-1 complex with a K_D of 8.74 μM. More in details (Figure 3D), we reported the most favourable pose of THS on PRX-1 surface, established exclusively by virtue of a higher number of hydrophobic interactions (through Ile 29, Val 73, Met 100). Consistent with the experimental results, the most favourable THS pose was buried in a protein region apart from the active site containing the Cys 52 reactive centre.

PRX-1 belongs to the 2-Cys PRX enzymes, in which the peroxidative cysteine (Cys 52 for PRX-1) is selectively oxidized by H_2O_2 to a cysteine-sulfenic derivative, which in turn reacts with the thiol moiety of Cys173 in the COOH-terminal region of a different subunit to form an inter-subunits disulfide bridge [21]. During peroxidase catalysis, the thiol group of the peroxidative cysteine is occasionally over-oxidized to sulfinic acid, resulting in the inactivation of its peroxidase activity [22,26]. In this scenario, our aim was to test the effect of THS on PRX-1 oxidation, induced by H_2O_2 both on HeLa cell lysates and on HeLa living cells. Immuno-blotting analyses were carried out using an antibody against PRX-1 in its oxidized form (named anti PRX1-SO_3). First, HeLa cell lysates were treated by 5 mM DTT to promote PRX-1 in its reduced form; then, samples were incubated or not with THS at 250 μM, while H_2O_2 at 5 and 50 mM was added for 15 min at r.t. As shown in Figure 4A, the treatment with THS clearly protects PRX-1 from cysteine over-oxidation, since the band corresponding to oxidized PRX-1 was strongly reduced. Later, we tested the THS effects on cell proliferation for 24 h and, as reported in Figure 4B, THS did not affect HeLa cells viability up to 100 μM, as measured by MTT assays. Thus, HeLa cells were treated with THS at 100 μM for 12 h and then oxidized by H_2O_2 at 100 μM. The obtained lysates were immune-blotted and probed with anti-PRX1-SO_3. THS was found able to protect PRX-1 to cysteine over-oxidation mediated by H_2O_2 even in a living cell system (Figure 4C).

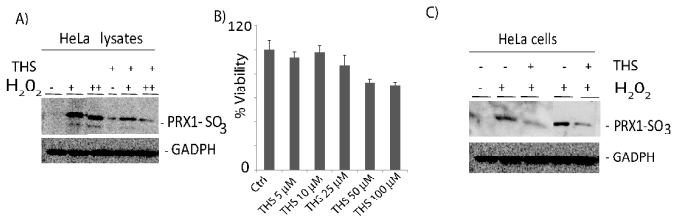


Figure 4: Panel A shows immune blotting analysis using anti-PRX-1SO_3 on 60 ug of HeLa cell lysates pretreated with DTT at 5 mM and then incubated or not with THS at 250 µM and with H_2O_2 at 5 mM (+) or 50 mM (++). Panel B shows MTT assays on HeLa cells treated for 24 h with THS at different concentrations and panel C shows HeLa cells incubated or not with THS at 100 µM for 12h and then treated with H_2O_2 at 100 µM for 5 min before lysis. GADPH has been used to normalize.

Conclusions

The discovery of small molecules that bind a specific target and modulate the function of proteins is an important and challenging step in chemical biology research.

Here, chemical proteomics discloses PRX-1 as the main target of THS, a bioactive 2-oxo-4-methylene-24-ethylsteroid isolated from a *Theonella* sponge [11], a promising agent in the treatment of metabolic disorders able to negatively modulate FXR transactivation [14]. PRXs are a family of ubiquitous peroxidases involved in removing H_2O_2 and organic hydroperoxides [26]. It is widely reported that PRXs can defend cells from oxidative stress reactions by oxidizing itself [27]. It has been proposed that PRXs in mammalian cells act as a dam against oxidative stress, and the ratio active/inactive enzyme might play a role to mediate signaling cascades in several cellular processes [28, 29]. In the past years, alterations in the protein levels of PRXs were found in various kinds of cancers; PRX-1 and PRX-2 have been found to be elevated in several human cancer cells and tissues such as oral, esophageal, pancreatic, follicular thyroid, breast and lung cancers [30]. The elevated PRX-1 and PRX-2 levels enhance the aggressive survival phenotype of cancer cells, and confer an increased resistance to the chemotherapy and radio-therapy [30]. On this basis, they may be considered potential targets for anti-cancer drugs [31, 32]. In this research, THS has been demonstrated to target PRX-1 and protect its cysteine over-oxidation induced by H_2O_2 both *in vitro* and in living cells. Future experiments will be planned to explore the capacity of THS to modulate PRXs-mediated cellular protection effects against other oxidative stressors, thus acting as anti-cancer drug.

Notes and references

[a] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano, Salerno, Italy;

[b] Department of Pharmacy, University of Napoli "Federico II", Via Montesanto, 80131, Naples, Italy;

[c] School of Biosciences and Veterinary Medicine, University of Camerino, via Gentile III da Varano, 62032 Camerino, Italy;

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

1. J.W.H. Li and J.C. Vederas, *Science*, 2009, **325**, 161.
2. E.E. Carlson, *ACS Chem. Biol.*, 2010, **5**, 639.
3. S. Terracciano, M. Rodriguez, M. Aquino, M.C. Monti, A. Casapullo, R. Riccio and L. Gomez-Paloma, *Curr.Med. Chem.*, 2006, **13**, 1947.
4. L. Gomez-Paloma, M.C. Monti, S. Terracciano, A. Casapullo and R.Riccio, *Curr. Org. Chem*., 2005, **9**, 1419.
5. W.R. Sawadogo, M. Schumacher, M.H. Teiten, C. Cerella, M. Dicato and M. Diederich, *Molecules*, 2013, **18**, 3641.
6. J.K. Guo, C.Y. Chiang, M.C. Lu, W.B. Chang and J.H. Su, *Mar. Drugs,* 2012, **10**, 1536.
7. A. Vilasi, M.C. Monti, A. Tosco, S. De Marino, L. Margarucci, R. Riccio and A. Casapullo, *Mar. Drugs,* 2013, **11**, 1288.
8. M. Bucci, A. Cantalupo, V. Vellecco, E. Panza, M.C. Monti, A. Zampella, A. Ianaro and G. Cirino, *PLoSOne*, 2013, **8**, e57801.
9. L. Margarucci, M.C. Monti, A. Mencarelli, C. Cassiano, S. Fiorucci, R. Riccio, A. Zampella and A.Casapullo, *Mol. Biosyst*., 2012 , **8**, 1412.
10. E. Kho, D.K. Imagawa, M. Rohmer, Y. Kashman and C. Djerassi, *J. Org. Chem.,* 1981, **46**,1836.
11. S. De Marino, R. Ummarino, M.V. D'Auria, M.G. Chini, G. Bifulco, B. Renga, C. D'Amore, S. Fiorucci, C. Debitus and A. Zampella, *J. Med. Chem.*, 2011, **28**, 3065.
12. B. Renga, A. Mencarelli, C. D'Amore, S. Cipriani, M.V. D'Auria, V. Sepe, M.G. Chini, M.C. Monti, G. Bifulco, A. Zampella and S. Fiorucci, *PLoSOne*, 2012, **7**, e30443.
13. S. De Marino, R. Ummarino, M.V. D'Auria, M.G. Chini, G. Bifulco, C. D'Amore, B. Renga, A. Mencarelli, S. Petek, S. Fiorucci and A. Zampella, *Steroids*, 2012, **77**, 484.
14. V. Sepe, R. Ummarino, M.V. D'Auria, O. Taglialatela-Scafati, S. De Marino, C. D'Amore, B. Renga, M.G. Chini, G. Bifulco, Y. Nakao, N. Fusetani, S. Fiorucci and Zampella, *Mar. Drugs*, 2012 **10**, 2448.
15. U. Rix and G. Superti-Furga, *Nat. Chem. Biol*., 2009, **5**, 616.
16. L. Margarucci, M. C. Monti, A. Tosco, R. Riccio and A. Casapullo, *Angew. Chem., Int. Ed.*, 2010, **49**, 3960.
17. L. Margarucci, M. C. Monti, C. Cassiano, M.Mozzicafreddo,M. Angeletti, R. Riccio, A. Tosco and A. Casapullo, *Chem. Commun.,* 2013, **49**, 5844.
18. C. Cassiano, R. Esposito, A. Tosco, A. Zampella,M. V.D'Auria, R. Riccio,A. Casapullo and M. C. Monti, *Chem. Commun*., 2014, **50**, 406.
19. C. Cassiano, L. Margarucci, R. Esposito, R. Riccio, A. Tosco, A. Casapullo and M. C. Monti, *Chem. Comm.*, 2014, **50**, 6043.
20. N. Kanoh, H.Takayama, K. Honda, T. Moriya, T. Teruya, S. Simizu, H. Osada and Y. Iwabuchi, *BioconjugateChem.*, 2010, **21**, 182.
21. A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen and M. Mann, *Nat. Protoc.,* 2006, **6**, 2856.
22. S.G. Rhee , H.A. Woo , I.S. Kiland S.H. Bae, *J. Biol. Chem.*, 2012, **287**, 4403.
23. O. Trott and A. J. Olson, *J. Comp. Chem.*, 2010, **31**, 455.
24. T.J. Jonsson, L.C. Johnson and W.T. Lowther, *J. Biol. Chem*., 2009, **284**, 33305.
25. M.D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek and G. R. Hutchison, *J. Cheminform*., 2012, **4**, 1.
26. J.W. Park, G. Piszczek, S. G. Rhee and P. B. Chock, *Biochemistry*, 2011, **50**, 3204.
27. W.T. Lowther and A.C. Haynes, *Antioxid Redox Sign.,* 2011, **15**, 99.
28. C. X. Liu, H.C. Zhou, Q.Q. Yin, Y.L. Wu and G. Q. Chen, *Exper. Cell Res*., 2013, **319**, 170.
29. Z.A. Wood, E. Schroder, J. R. Harris and L.B. Poole, *Trends Biochem. Sci.*, 2003, **28**, 32.
30. S.W. Kang, S.G. Rhee, T.S. Chang, W. Jeong and M.H. Choi, *Trends Mol. Med*., 2005, **11**, 571.
31. B. Zhang, Y. Wang and Y. Su, *Cancer Lett*., 2009, **28**, 6154.
32. S. Fourquet, M.E. Huang, B. D'Autreaux and M.B. Toledano, *Antioxid. Redox Sign.*, 2008, **10**, 1565.