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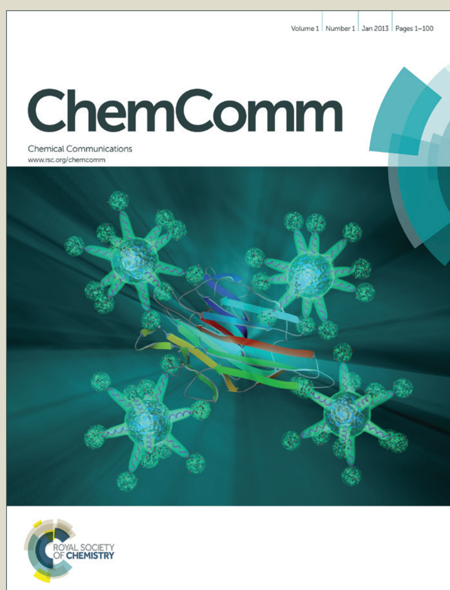

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In cell Scalaradial Interactome Profiling Using a Bio-Orthogonal Clickable Probe

C. Cassiano,[a] L. Margarucci,[a] R. Esposito,[a] R. Riccio,[a] A. Tosco,[a] A.Casapullo,[a] * M.C. Monti[a] *

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Abstract: A bio-orthogonal click-chemistry procedure was developed to allow the *in cell* interactome profiling of scalaradial, an anti-inflammatory marine natural product. The results were validated through the application of the classical *in vitro* chemical proteomics and several bio-physical methods and peroxiredoxins, 14-3-3 and proteasome were recognized as main scalaradial targets.

Low molecular weight drugs usually target more than a single protein in cell living systems, as pointed out in several recent publications for almost 80% of existing drugs.[1,2] The recognition of all the binding partners of new potential leads is a necessary step into the drug development process,[3] in order to deeply understand the drug-related multi-pharmacological profile and/or toxic side-effects. The most common approaches are based on mass-spectrometry (MS) chemical proteomics using a matrix-immobilized compound as a bait for *in vitro* fishing out specific partners from cell lysates (**Fig. 1A**).[4-7] Nonetheless, cell lysis experimental procedures, usually employing sonication and/or detergents, could affect proteins native conformation leading to accidental loss of some physiological targets of the sample molecule[8] and, furthermore, the compound anchored on the solid matrix could be forced into a not likely conformation leading to an altered identification of its interactors.[2] More recently, new approaches based on bio-orthogonal click-chemistry[9-11] have been developed to discover the interactors of small-drugs in living cells under physiologically relevant settings. In this paper, we employed the copper-(I)-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition, a straightforward reaction leading to a stable 1,2,3-triazole adduct through the reaction of azides and terminal alkynes,[12] to guide the *in cell* identification of the protein targets of Scalaradial (SLD), a natural scalarane sesterterpenoid isolated from the sponge *Cacospongia mollior*.[13] SLD has been reported to inhibit PLA_2 activity,[14-15] EGF-stimulated Akt phosphorylation and NF-κB activation, causing cell death by inducing apoptosis.[16-18] Thus, we selected this interesting natural product for its relevant anti-inflammatory profile as a probe for a comparative analysis between *in vitro* and *in vivo* chemical proteomics procedures, aiming to a rational validation of the two techniques along with the discovery of new SLD targets responsible for its remarkable pharmacological activity. A typical *in cell* chemical proteomics procedure starts with the chemical decoration of the compound of interest with an azide or a terminal alkyne, as suitable functional groups for bio-orthogonal reactions. Then, a living cell sample is incubated with the tagged molecule to promote its internalization and the interactions with the target proteins. The cell lysate, obtained upon mild treatment, is then exposed to the appropriate reagents mixture and the click chemistry reaction between the azide and alkyne functional groups allows the connection of the drug to a solid support. This adduct is then isolated, washed and the pulled-back proteins are submitted to SDS-PAGE and MS-based analysis for their characterization (**Fig.1B**).

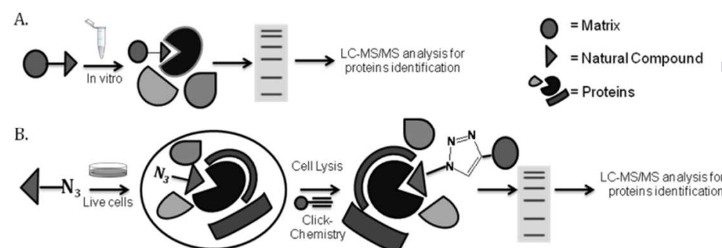

Figure 1: *In vitro* (A) and *in cell* (B) chemical proteomics workflow followed by nano-ESI-LC-MSMS targets identifications.

Our experimental approach has been organized in two basic steps: a) Set-up and optimization of the click-chemistry procedure on SLD; b) *in vitro* and *in cell* SLD chemical proteomic analyses, validated through bio-physical methods. First, the suitable SLD-derivative was prepared by coupling SLD with the 11-azido-3,6,9-trioxaundecan-1-amine (TRX-Az, **Fig. 2**). The TRX-Az linker was preferred to an alkyne one to avoid the potential *in vivo* alkyne reduction, likely due to the cellular reductase enzymes action,[19] and also for its good solubility in aqueous buffers and ability to cross the cellular membranes.[20] The product formation was monitored by RP-HPLC and MS/MS (**Fig. S1**). On the basis of the MS and MS/MS spectra and our previously published data,[14-15, 21] a reaction mechanism was proposed, based on the formation of a Schiff base intermediate by reaction of the TRX-Az terminal amino group and one of the SLD aldehyde carbonyls, followed by a cyclization step whose product evolved to a pyrrole species (**Fig. 2**). The SLD-Az derivative was then purified by RP-HPLC and lyophilized, giving approximately

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95% yield. The following step was the optimization of the in solution Cu(I)-catalyzed cyclization of the SLD-Az with the 4-pentyn-1-amine (PINA linker, **Fig. 2**), using Na-L-(+)-Ascorbate as reducing agent, for the *in situ* generation of Cu(I) from Cu(II)SO_4, and Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) as stabilizer. RP-HPLC-MS and MSMS analyses (**Fig. S2**) gave a product ion at *m/z* 694.45, corresponding to the presumable product.The better reaction conditions were found at 100 μM $CuSO_4$, 5 mM Na-L-(+)-Ascorbate and 100 μM TBTA, under nitrogen flux, using SLD-AZ and PINA in a 1:2 ratio (**Fig. 2**).

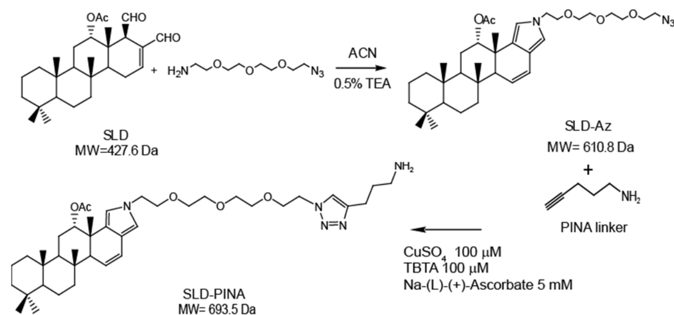

Figure 2:SLD reaction with TRX-Az generated SLD-Az which has been modified by PINA linker giving SLD-PINA adduct. Each reaction has been monitored by LC-MS-MS analysis.

The SLD interactome profile was then carried out through an *in vitro* affinity purification methodology both on a sample of bee venom PLA_2 as known interactor (**Fig. S3**) and on HeLa cell lysates, in order to produce a reference experiment for the development of the *in cell* procedure. SLD was linked to the 2-(2-amino-ethyl-disulfanyl)-ethyl-amine linker (TIOS), a spacer arm often used in chemical proteomics.[22] The SLD-bearing spacer arm was purified by RP-HPLC-UV and immobilized on the CDI agarose solid support by coupling the linker terminal amino group with the activated carbonyl on the matrix, with a final >90% immobilization yield after 16 h of reaction (**Fig. 3** and **Fig.S3**).

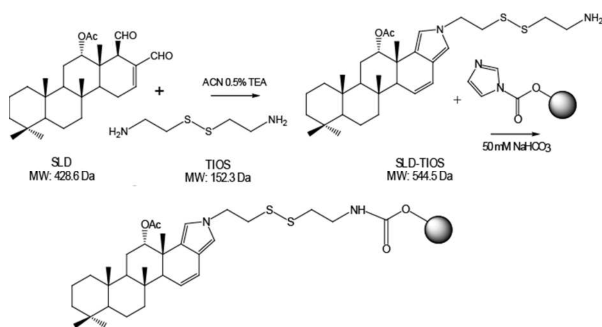

Figure 3:SLD reaction with TIOS and SLD-TIOS immobilization on CDI agarose solid matrix. Each reaction was monitored by LC-MS-MS analysis.

Following the *in vitro* chemical proteomics protocol reported in SI,[23] several proteins belonging to the 14-3-3 and peroxiredoxin families, reported in **Fig.S4**, were recognized as SLD main partners. These results were confirmed by immune-blotting analysis, in which a relevant enrichment of 14-3-3ε and PRX1, chosen as main probes due to their high identification scores, was evident in the SLD lane when compared to the control experiment (**Fig. 4A**). In a following test, the protein affinities between original free SLD and PRX1 or

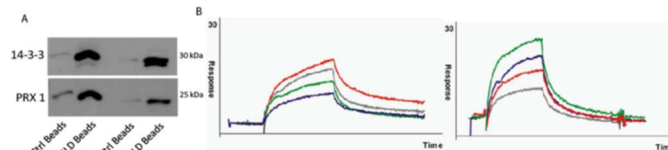

Figure 4: Panel A: Western blot analysis on the protein eluates from control and SLD-beads incubated with HeLa cell lysates (two independent experiments) using 14-3-3ε and PRX1 as probes. Panel B: Sensorgrams obtained from the injections of SLD (0.01–25 μM from bottom to up) on the immobilized 14-3-3 ε (left) and PRX-1 (right).

14-3-3ε were measured by SPR, giving calculated dissociation constants of 2.24±2.07 and 0.99±0.18 μM for PRX1 and 14-3-3ε, respectively, evidence of a strong interaction between the counterparts **(Fig.4B).** Finally, the *in cell* click chemistry-based pull down assay was built-up and the SLD interaction profile was then compared, to give evidence of its soundness, with the one previously recovered by the *in vitro* procedure. In a first instance, the optimized click-chemistry conditions were tested on a HeLa cell lysate sample to assess the recovery of the SLD main interactors and improve, if necessary, the reaction conditions to use with the living cell system. The SLD-AZ adduct (1 μM and 10 μM) was incubated for 1 h at 4°C with the cell lysate and then mixed with CDI-Agarose matrix bearing PINA linker in presence of 100 μM $CuSO_4$, 5 mM Na-L-(+)-Ascorbate and 100 μM TBTA under nitrogen. After several washes to remove the unbound proteins, the SLD partners were eluted, separated by 12% SDS-PAGE and revealed by immunoblotting using anti-14-3-3ε and anti-PRX1 antibodies. As reported in **Fig.5A**, 10 μM of SLD-Az gave a selective fishing of 14-3-3ε and PRX1 in respect of the control and more efficient than 1 μM of SLD-Az. Then, the experiment conditions were improved using 10 μM of SLD-Az in presence of 250 μM $CuSO_4$, 2.5 mM Na-L-(+)-Ascorbate and 500 μM TBTA under nitrogen (**Fig. 5B**). Before starting with the *in cell* experiments, we tested if the SLD biological properties may have been hampered by the chemical modifications, testing their action on bee venom PLA_2 inhibition, on cell proliferation and apoptosis. As reported in **Fig. S5**, both molecules showed the same effect on PLA_2 inhibition, and did not affect HeLa cells viability at the tested doses, as measured by MTT assays, while both induced apoptosis at higher doses, as already described for SLD.[24]

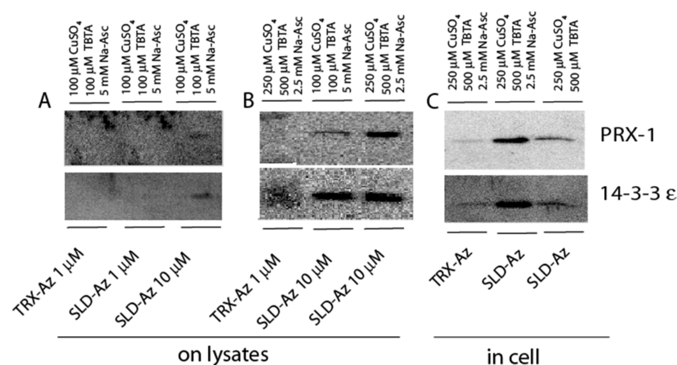

Figure 5: Western blot analysis on proteins eluted from control and SLD-beads incubated with HeLa cell lysates (*in vitro*) or on living HeLa cells (*in cell*) using 14-3-3ε and PRX1 as probes. Panel A and B are relative to SLD-based pulldown using different quantities of metabolite and different click chemistry conditions as reported. Panel C shows 14-3-3ε and PRX 1 enrichment operated by SLD-Az in living HeLa cells fished out by CDI-Agarose beads modified with PINA linker.

Thus, living HeLa cells were treated with 1 μM of SLD-Az or with 1 μM of TRX-Az as control, for 6 hours to allow the drug internalization and the binding to its cellular targets. Cells were then washed with fresh media to remove the amount of non-internalized

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drug and lysed by gentle treatment with a suitable lysis buffer. Lysates were then treated with the catalytic mixture (250 µM $CuSO_4$, 2.5 mM Na-L-(+)-Ascorbate and 500 µM TBTA) and the PINA-bearing CDI-Agarose beads for 1 h at 4°C to promote the Huisgen 1,3-cycloaddition and then to pull-down the SLD-AZ interacting proteins. The same reaction conditions were also applied without the presence of Na-L-(+)-Ascorbate as additional control (**Fig. 5C**). The pulled-down samples were then separated by SDS-PAGE followed by both immunoblotting analysis and Comassie staining. As reported in **Fig.5C**, immunoblotting revealed that both PRX-1 and 14-3-3ε were clearly enriched by SLD-Az. Moreover, the gel bands excised out by both SLD-Az and control lanes were subjected to an *in situ* digestion protocoland the peptide mixtures were then analyzed through nano-flow LC-MS/MS, followed by Mascot database search for protein identification. The SLD-Az interactors list was refined by removing the hits shared with the control experiments and proteins with a Mascot score lower than 75, thus obtaining a list of approximately 90 proteins (**Fig. S6**). These proteins were clustered using the STRING software to get large protein complexes more visible (**Fig. 6**). In spite of the large number of potential hits, often due to a high background content possibly derived to the nonspecific binding of abundant proteins,[25] our attention was focused on a large protein sub-network accounting for the SLD reported pharmacological profile,[14-18] in which PRXs with several 14-3-3 isoforms and the proteasome machineries complex are strictly connected. The recovery of PRX-1 and 14-3-3, common hits of the *in vitro* proteomic analysis, represented a validation for the *in cell* procedure, and in addition the proteasome was found as a formerly undetected SLD target. The last evidence was confirmed by Western blotting analysis, using monoclonal antibodies against the 26S proteasome subunit α-7 and Psmc3, as reported in **Fig.6**.

In conclusion, we have successfully optimized an *in cell* pull-down LC-MS/MS methodology based on the click-chemistry copper-catalyzed variant of the Huisgen1,3-dipolar cycloaddition for the interactome profiling of the anti-inflammatory marine metabolite SLD. The simultaneous definition of its *in vitro* profiling helped as reference point for the validation of the *in cell* procedure. We obtained a list of cellular potential targets and, among them, PRXs, 14-3-3 isoforms and proteasome were selected as relevant SLD partners on the basis of their involvement in the inflammatory process. These targets, then validated by SPR and western blotting analyses, are functionally connected to the PI3K and NF-κB pathways already known to be modulated by SLD.[14-18] Our findings could have important outcomes in future applications of the incell fishing for partners methodology, and for a deep evaluation of SLD bio-pharmacological significance.

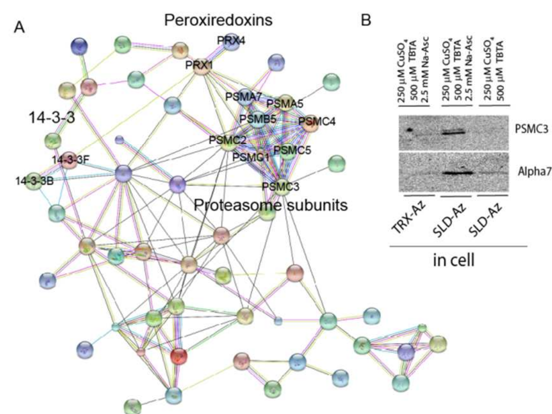

Figure 6: Panel A: Network of *in cell* SLD target proteins obtained by STRING software using 91 identified proteins filtered to remove disconnected ones. Panel B: Western blot analysis on proteins eluates from controls and SLD-beads incubated with HeLa cells using PSMC3 and Alpha7 proteasome subunits as probes.

Notes and references

[a] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano, Salerno, Italy email: mcmonti@unisa.it, casapullo@unisa.it tel: 0039-089-969243 fax: 0039-089-966202.
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