Chemical Biology

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Chemical Proteomics Discloses Petrosapongiolide M, an Antiinflammatory Marine Sesterterpene, as a Proteasome Inhibitor**

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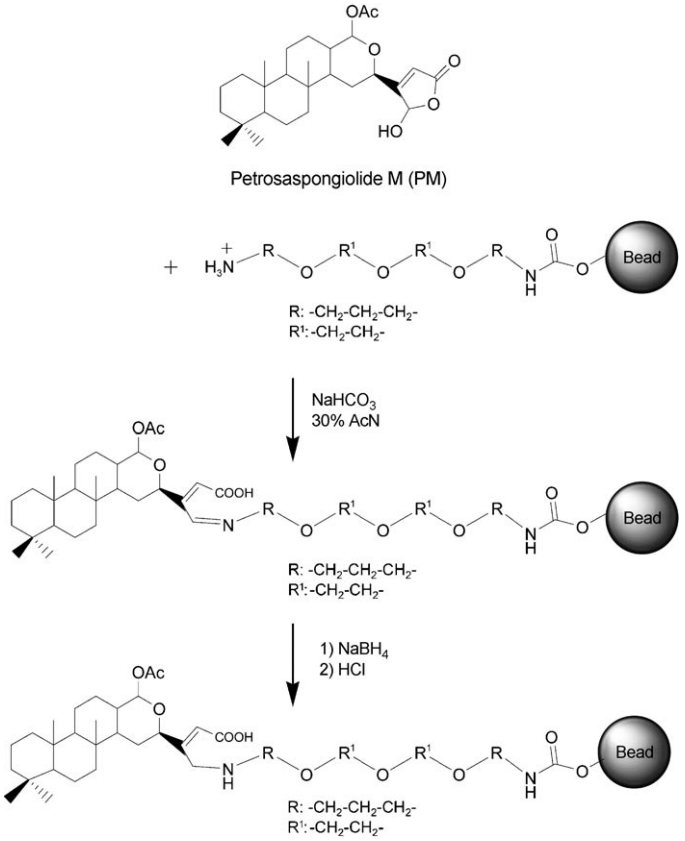
In memory of Luigi Gomez-Paloma

The identification of the macromolecular targets of small bioactive molecules is crucial in the field of chemical biology for understanding their mechanisms of action and developing new drug candidates.[1–8] Continuing our studies on marine natural products with antiinflammatory properties, we were intrigued by the peculiar pharmacological profile of petrosaspongiolide M (PM, Scheme 1), a γ-hydroxybutenolide marine terpenoid.[9–11] Its antiinflammatory activity, which was originally attributed to a strong inhibition of phospholipase A_2, has been studied extensively and clarified.[9–12] In addition, an in-depth pharmacological investigation revealed that PM also interferes with the action of NF-κB, strongly decreasing NF-κB–DNA binding.[13,14] (NF-κB, a protein complex that controls the transcription of DNA.) Nonetheless, whether PM exerts its pharmacological effects through binding and/or modifying specific proteins remains unknown.[14] More generally, the specific targets of naturally occurring small molecules modulating the pathway of NF-κB are for the most part not known.[15]

Since the NF-κB pathway plays a crucial role in the development of the inflammation response, it is desirable to identify the primary PM partners suitable as potential therapeutic targets. To reach this goal, a chemical proteomic strategy, using affinity chromatography coupled with mass spectrometry, was applied to macrophage cell proteomes.[15,16] From an experimental point of view, our work can be divided into the following steps: 1) chemical immobilization of PM on a solid support; 2) incubation of PM-modified beads with cell lysates; 3) MS identification of PM interactors; 4) evaluation of PM bioactivity in vitro and in live cells.

PM was covalently linked to the solid beads by taking advantage of its electrophilic functional group, the masked aldehyde of the γ-hydroxybutenolide ring. We selected an agarose matrix activated by 1,1′-carbonyldiimidazole, which was modified by 4,7,10-trioxa-1,13-tridecanediamine (PEG) as a spacer to avoid steric interference between the targets and the ligand-bearing matrix. The reaction between the spacer-modified resin and PM—the nucleophilic attack of the free amino group of PEG onto the C25 aldehyde moiety of PM—gave rise to an imine, which was subsequently reduced by treatment with $NaBH_4$ (Scheme 1 and Figure S1 in the Supporting Information). The experimental conditions (buffer, pH, incubation time, and temperature) were optimized to prepare beads with a final PM concentration of 18 μmol per mL resin; the coupling process was monitored by RP-HPLC analysis (see Figure S2 in the Supporting Information) and was completed by acetylating the residual free amino groups of the spacer.



Scheme 1. Chemical structure of petrosaspongiolide M (PM) and preparation of PM-modified beads.

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We treated the human-macrophage-derived THP-1 cell line with bacterial lipopolysaccharide (LPS) to induce an inflammatory response. The crude cell extracts of LPS-induced THP-1 were loaded on the PM-modified beads described above. Cell extracts were also incubated with a PM-free matrix as a control experiment to distinguish between specifically bound components and background contaminants. After extensive washing, the anchored proteins were released from the resin by elution with SDS-gel loading buffer (Figure 1).[17] The protein mixtures were then resolved by

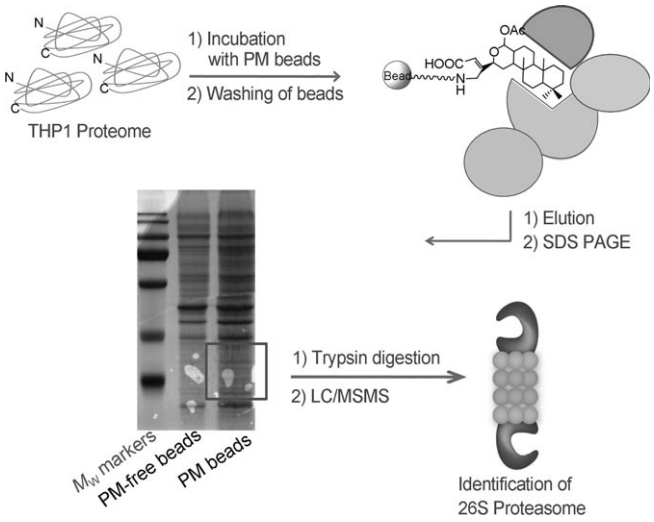

Figure 1. Representation of the chemical proteomic experiment applied to the discovery of PM targets. The proteins retained in the PM-affinity step were eluted from the support, subjected to SDS PAGE separation, digested in situ, and identified by mass spectrometry.

SDS-PAGE; the gel line was cut in 13 pieces, digested with trypsin, and analyzed by mass spectrometry through nanoflow reversed-phase HPLC MS/MS (Figure 1). Doubly and triply charged peptide species were fragmented, and all the MS/MS spectra were evaluated by a Mascot database search.[17]

To establish which proteins were specifically captured by PM, we compared the list of the proteins identified with those in the control experiment. As reported in the Venn diagram (Figure 2), 325 proteins were observed in both experiments while only 29 were exclusively identified in the binding experiment. These proteins were considered as potential PM interactors (Table 1).

Several proteins reported in Table 1 are highly expressed in THP-1 cells,[18] and their presence, typical in this kind of analysis, is explained mostly by their abundance rather than

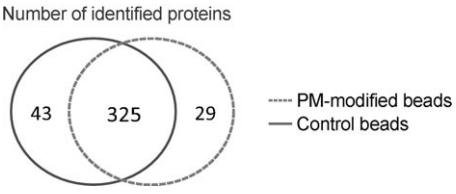

Figure 2. Venn diagram showing the number of proteins identified in either or both binding experiments.

true affinity as ligands.[19] We were particularly intrigued by the presence of several components of the proteasome enzymatic machinery, since the ubiquitin–proteasome system (UPS) is responsible for the degradation of most intracellular proteins, including those that control cell cycle progression, apoptosis, signal transduction, and the NF-κB transcriptional pathway.[20,21] As these findings are strictly connected with the previously reported pharmacological evidence,[14] we performed an in-depth investigation on the biological role of PM in the interaction with the proteasome machinery, through assays in vitro and in live cells.

The activity of PM on the 20S proteasome, the catalytic subunit of the entire 26S proteasome, was tested in vitro by detecting its ability to modulate the proteasome-mediated proteolysis of three fluorogenic peptide substrates, each specific for catalytic subunits of the enzyme.[21,22] We found that PM directly inhibits the proteasomal activity, in particular its caspase- and chymotrypsin-like activities (see Figure S3 in the Supporting Information). In a subsequent experiment, we assessed the ability of PM to inhibit proteasome functions inside the cell. In this experiment THP-1 macrophages were incubated with PM in a range of concentrations below the cytotoxic level (0.1–1 μM; for the MTT test see Figure S4 in the Supporting Information), and then the proteasome activity of cell lysates was measured. PM shows a significant inhibition of the caspase- and the chymotrypsin-like activity with IC_{50} values of 0.85 (±0.15) μM and 0.64 (±0.15) μM, respectively (Figure 3 A). Interestingly, PM stimulates the trypsin-like activity of the proteasome at 0.1 and 0.5 μM. This is not surprising, since this effect has been already reported for several inhibitors of caspase-like sites which

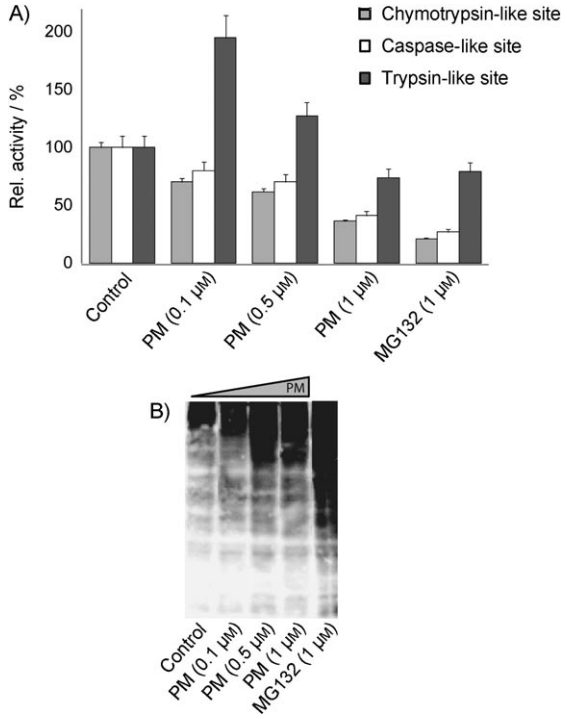

Figure 3. Effect of PM inhibition on 26S proteasome in cells. A) Effect of PM (0.1–1 μM) and MG132 (1 μM) on 26S proteasome activities in cells. B) Western blot analysis of polyUP accumulation after treatment with PM (0.1–1 μM) and MG132 (1 μM).

Table 1: Proteins identified as PM interactors (clustered by function).

Swiss-Prot code	Identified proteins[a]	M_w [Da]	Sequence coverage	No. of queries	Unique spectra	SCR[b]
Proteasome machinery[c]						
PSME2_HUMAN	proteasome activator complex subunit 2	28723	55%	20	10	471
PSME1_HUMAN	proteasome activator complex subunit 1	26700	10%	3	2	161
PSA2_HUMAN	proteasome subunit alpha type-2	25899	8%	5	2	149
PSA4_HUMAN	proteasome subunit alpha type-4	29484	28%	6	5	101
PSA5_HUMAN	proteasome subunit alpha type-5	26411	31%	7	5	119
PSB1_HUMAN	proteasome subunit beta type-1	26489	5%	2	1	47
PSB4_HUMAN	proteasome subunit beta type-4	29204	28%	6	4	79
PSA7_HUMAN	proteasome subunit alpha type-7	27887	23%	5	4	89
PRS6B HUMAN	26S protease regulatory subunit 6B	47366	12%	6	2	48
Cytoskeletal proteins						
ARP3 HUMAN	actin-related protein 3	47371	11%	9	4	178
ACTN1 HUMAN	alpha-actinin-1	103058	9%	5	4	237
Ras-related proteins						
RAC2-HUMAN	Ras-related C3 botulinum toxin substrate	21429	22%	9	5	120
RAB14_HUMAN	Ras-related protein Rab-14	23897	23%	5	3	119
RAB1B_HUMAN	Ras-related protein Rab-8B	22171	17%	6	3	177
RAB5C HUMAN	Ras-related protein Rab-5C	23483	40%	8	6	100
Kinases						
NDKA_HUMAN	nucleoside diphosphate kinase A	17149	11%	3	2	42
NDKB_HUMAN	nucleoside diphosphate kinase B	17298	10%	4	3	38
Oxidative stress proteins						
PRDX6_HUMAN	peroxiredoxin-2	25035	14%	10	2	219
PRDX3_HUMAN	thioredoxin-dep peroxide reductase	27693	14%	5	3	73
Ribosomal proteins						
R13AX_HUMAN	putative 60S ribosomal protein L13a-like	12135	10%	2	2	129
EF1A_HUMAN	elongation factor 1-alpha	50141	21%	8	4	111
Others						
ENPL_HUMAN	endoplasmin	92469	8%	10	7	237
SYG_HUMAN	Glycyl-tRNA synthetase	83140	14%	6	4	81
IFIT1_HUMAN	interferon-induced protein	55360	22%	12	7	286
PDIA6_HUMAN	protein disulfide-isomerase A6	48121	13%	7	4	266
1433E_HUMAN	14-3-3 protein epsilon	29174	25%	10	5	251
DECR_HUMAN	2,4-dienoyl-CoA reductase, mitocondrial	36068	46%	21	11	380
VIME_HUMAN	vimentin	53652	15%	5	5	74

[a] For details on the peak lists see the Supporting Information. [b] Mascot score (SCR) derived from the addition of individual peptide scores, where SCR > 36 is significant. [c] Mascot results are reported in the Supporting Information.

allosterically stimulates the trypsin-like activity.[23] Furthermore, it is well known that inhibition of the chymotrypsin-like site is the main event determining the inhibition degree of protein degradation.[22] In the same test the reference compound MG132, a well-known proteasome inhibitor,[24] was found to be slightly more effective than PM on inhibiting chymotrypsin and caspase activity, and similar behavior was observed for the modulation of the trypsin-like activity.

Additionally, we tested the effect of PM-mediated proteasome inhibition on the accumulation of the polyubiquitinated proteins (polyUP) by immunoblot analysis.[25] After treatment of THP-1 macrophage cells with PM, lysates were analyzed by western blotting using polyubiquitin antibodies. The immunoblot profile shown in Figure 3B shows a relevant degree of polyUP accumulation in the cells treated with PM which increases in a concentration-dependent manner. A major effect is evident in the case of MG132, in agreement with its higher potency of action measured in the previous tests.

Since the UPS abnormal activation or failure underlies the pathogenesis of many human diseases (i.e. cancer, inflammation, cardiovascular diseases, neurodegenerative disorders),[26,27] its modulating agents are considered attractive molecules for pharmacological intervention. In particular, two proteasome inhibitors have entered clinical trials for the treatment of cancer (Bortezomib)[24] and stroke patients (MLN-519),[28] of which the first is already on the market. On this basis, the relevant blocking of the proteasome activity in the living cells demonstrated by PM suggests this molecule as a potential new lead compound. Detailed investigations on the molecular mechanism of this inhibition are needed with the aim of the rational design of a new class of proteasome inhibitors.

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