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***N*-Formyl-7-amino-11-cycloamphilectene, a marine sponge metabolite, binds to tubulin and modulates microtubule depolymerization[†]**

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Abstract

The importance of protein–small molecule interaction in drug discovery, medicinal chemistry and biology has driven the development of new analytical methods to disclose the whole interactome of bioactive compounds. To accelerate targets discovery of *N*-formyl-7-amino-11-cycloamphilectene (CALE), a marine bioactive diterpene isolated from the Vanuatu sponge *Axinella* sp., a chemoproteomic-based approach has been successfully developed. CALE is a potent anti-inflammatory agent, modulating NO and prostaglandin E2 overproduction by dual inhibition of the enhanced inducible NO synthase expression and cyclo-oxygenase-2 activity, without any evidence of cytotoxic effects. In this paper, several isoforms of tubulin have been identified as CALE off-targets by chemical proteomics combined with bio-physical orthogonal approaches. In the following biological analysis of its cellular effect, CALE was found to protect microtubules against the colcemid depolymerizing effect.

Introduction

For centuries, secondary metabolites biosynthesized in the natural kingdom evolved for optimal interactions with bio-macromolecules, modifying their molecular scaffold to fit better with cellular targets. Nowadays, the interactions between small molecules and proteins are an important task for the advancement of basic science and drug development, and their study can be approached from different perspectives.¹

[†]Electronic supplementary information (ESI) available: HPLC run of CALE–NH₂ bearing linker, MS and MSMS analysis of the covalent adduct between CALE–NH₂ and dithiobis-succinimidylpropionate, HPLC runs of CALE–NH₂ bearing linker immobilization on amine beads, electrophoretic profile of proteins eluted from CALE and control beads, proteins identified from control experiments, proteins sequence coverage of the selected CALE interactors, Mascot report and peak lists for Hsp90β and tubulin, analysis of CALE and Hsp90β affinity by surface plasmon resonance and cell viability assay. See DOI: 10.1039/c3mb70315k

The marine environment is a particularly rich source of pharmacologically relevant molecules produced by a wealthy source of plants, animals and micro-organisms.^{2–5} In this scenario, the identification of their cellular interactome is crucial to unambiguously define their mechanism of action and thus to understand their pharmacological effects.^{6–8}

The importance of protein–small molecule interactions in drug discovery and biology has driven the development of new methods to identify all the possible cellular targets for a bioactive molecule, in order to determine its interaction network. Indeed, recent reports indicate that multi-target engagement occurs in up to 80% of current drugs.⁹

Among the new emerging analytical technologies, affinity-based methods integrated with modern proteomics are the most common used approaches to move toward target discovery.^{10–13} In MS-based chemical proteomics, a small bioactive molecule needs to be linked to a matrix through a spacer arm. This structural modification has to be considered an essential chemical artifice to pull-down its specific interactors from a cell lysate or a tissue extract. Once eluted, cellular partners are then identified by high resolution MS and bioinformatics analysis.^{12,13} Later on, the biological activity of the selected compound with its cellular interactor(s) needs to be investigated by biological assays.

In this paper, we describe the application of MS-based chemical proteomics to determine the biological partner(s) of *N*-formyl-7-amino-11-cycloamphilectene (CALE, Fig. 1), a marine diterpene isolated from the Vanuatu sponge *Axinella* sp.¹²

Chemically, CALE belongs to the class of diterpenes based on amphilectene and cycloamphilectene skeletons, usually found in sponges of the order Halichondrida,^{14,15} whose structures are characterized by poorly functionalized tri- or tetra-cyclic carbon frameworks, often containing one or more isonitrile, isocyanate or isothiocyanate functions. From a pharmacological point of view, CALE is a potent anti-inflammatory agent, modulating NO and prostaglandin E2 overproduction by dual inhibition of the enhanced inducible NO synthase expression and cyclo-oxygenase-2 activity.¹⁶ Indeed, this metabolite inhibited the production of NO and prostaglandin E2 in murine peritoneal macrophages stimulated by zymosan, without any evidence of cytotoxic effects. Nevertheless, it was practically ineffective when inducible NO synthase was already expressed. Immune-blotting analysis of mouse peritoneal macrophage lysates showed that inducible NO synthase protein expression was reduced by the presence of CALE during zymosan treatment.¹⁶

Thus, captivated by the high potential of this molecule as an anti-inflammatory agent and following our studies on targets discovery of marine natural products by chemoproteomics,^{17–21} we selected CALE as a candidate for the application of the above mentioned approach to identify its interactors.

Results and discussion

CALE-bearing matrix preparation

To achieve a quantitative immobilization of CALE on agarose beads, the *N*-formyl group was removed by acidic hydrolysis to give a more reactive primary amine (CALE–NH₂, Fig. 2A and Fig. S1, ESI[†]). CALE–NH₂ was then treated with dithiobis-succinimidylpropionate,

an activated disulphide-containing spacer arm, to form a covalent adduct (Fig. 2B and Fig. S2, ESI[†]), whose yield was monitored by RP-HPLC-MS (MW = 576.4 Da) and purification performed by RP-HPLC-UV. The CAle-bearing spacer arm was then immobilized on an agarose solid support, through coupling with the primary amine on the matrix (Fig. 2C), with a final 80% immobilization yield (see Fig. S3, ESI[†]). All residual amino groups on the resin were quenched with acetic anhydride.

The use of a spacer arm between the small molecule and the solid support is a common practice in chemical proteomics to prevent steric hindrance that could hamper the protein accessibility. The spacer arm can also incorporate a cleavable site, as a disulphide bridge, which is stable in many biological applications and that can be selectively cleaved with mild reducing conditions to decrease the amount of unspecific binders fished out by solid supports.²²

The identification of CAle interactors by affinity chromatography and mass spectrometry

HeLa cells protein extracts were separately incubated with the CAle-modified and control beads to promote the interaction between the immobilized compound and its potential partner(s) in solution. The amount of un-specifically interacting proteins was reduced by several washing steps of the matrix beads, while the tightly bound proteins were released after disulphide bond cleavage with DTT. The protein mixtures eluted from CAle and the control experiments were resolved by 10% SDS-PAGE (Fig. S4, ESI[†]), and the entire gel lanes were divided into a few pieces and subjected to an *in situ* digestion protocol.²³ The peptides originating from each gel slice were analyzed through nano-flow RP-HPLC MS/MS and protein identification was performed by submitting the peak lists to the Mascot database. The list of the CAle interacting proteins was refined by removing the hits shared with the control experiments (see Table S1, ESI[†]). The combination of four independent experiments gave a high confident list of putative CAle interactors (Table 1). We were particularly intrigued by the proteins identified with the highest score (Table 1), and among them Hsp90 β and α/β tubulin (see Table S2 and Fig. S5–S8, ESI[†]).

In vitro validation of CAle partners

The interactions of CAle with Hsp90 β and tubulin were then confirmed by immune-blotting analysis, as clearly reported in Fig. 3A. Moreover, an *in vitro* pull-down experiment was performed to prove the direct interaction between CAle and its protein targets, by incubating samples of control and CAle-modified beads with both the recombinant Hsp90 β and tubulin. CAle was found to significantly enrich tubulin compared to the control, while no differences were found for Hsp90 β between CAle and the control beads (Fig. 3B). Thus, tubulin was confirmed as the CAle direct primary target, while HSP90 β could be considered as the secondary partner, probably fished out by tubulin, which is a HSP90 β client protein, as reported by *in vitro* and *in cell* studies.^{24,25}

[†]Electronic supplementary information (ESI) available: HPLC run of CAle–NH₂ bearing linker, MS and MSMS analysis of the covalent adduct between CAle–NH₂ and dithiobis-succinimidylpropionate, HPLC runs of CAle–NH₂ bearing linker immobilization on amine beads, electrophoretic profile of proteins eluted from CAle and control beads, proteins identified from control experiments, proteins sequence coverage of the selected CAle interactors, Mascot report and peak lists for Hsp90 β and tubulin, analysis of CAle and Hsp90 β affinity by surface plasmon resonance and cell viability assay. See DOI: 10.1039/c3mb70315k

Then, surface plasmon resonance was employed to measure the binding affinity of CALe with tubulin, using Hsp90 β as the negative control. Both proteins were immobilized on a CM-5 sensor chip prior to the injection of CALe at various concentrations (0.5–25 μ M, Fig. 3C). Sensorgrams analyses, using a 1 : 1 Langmuir algorithm, allowed us to calculate the dissociation constant (K_D) of the tubulin–CALe complex as 9.5 ($\pm$ 2.2) μ M, while no binding was observed for Hsp90 β (see also Fig. S9, ESI[†]).

An evaluation of the CALe effects on HeLa cells

In the first instance, CALe was submitted to an *in vitro* test and was unable to show either an inhibitory or stimulatory effect on tubulin assembly, as well as significant binding to the colchicine site (data not shown). Despite that, we moved to the evaluation of the potential effect of CALe on the microtubule cytoskeleton, performing an immuno-fluorescence analysis on HeLa cells. The effects of CALe on the cellular viability was measured, and no significant cytotoxicity was observed (Fig. S10, ESI[†]). Since 4 h incubation with 100 μ M CALe did not interfere with tubulin polymerization (Fig. 4A and B), we looked at the CALe effect on the tubulin assembly and disassembly in the presence of *N*-desacetyl-*N*-methylcolchicine (colcemid), a well-known inhibitor of microtubule polymerization.²⁶ As reported in Fig. 4C, the microtubule cytoskeleton of HeLa cells is severely damaged after 2 h of treatment with 500 nM colcemid. When preincubated with CALe, a time-dependent protective effect of the marine compound on the microtubule integrity was revealed during the first 90 min after colcemid incubation, (Fig. 4E and F) even if this effect was lost after 120 min (Fig. 4D).

Conclusions

Target discovery in the field of bioactive natural compounds is a key strategy for understanding their biological effects. Here, we have analyzed the cellular interactome of the anti-inflammatory marine diterpene *N*-formyl-7-amino-11-cycloamphilectene with a chemical proteomics approach, identifying tubulin as its main target in HeLa cells. Furthermore, the interaction profile of the marine compound has been deepened by *in vitro* pulldown and surface plasmon resonance, revealing direct binding with tubulin.

The following biological assays gave evidence of a protecting effect of *N*-formyl-7-amino-11-cycloamphilectene on microtubule depolymerization, suggesting a potential alteration of the micro-tubule network slowing the colcemid-mediated microtubules disruption. This in turn is in accordance with the lack of cytotoxicity at all tested concentrations.

Thus, tubulin can be considered to be an *N*-formyl-7-amino-11-cycloamphilectene off-target, even if this interaction is not strictly linked to the reported anti-inflammatory activity of the natural product (Fig. S10, ESI[†]).¹⁶

This study broadens the knowledge on the biological profile of *N*-formyl-7-amino-11-cycloamphilectene and, since only few compounds are presently known as protecting agents against microtubule loss (for instance the neuroprotective peptide NAP),²⁷ this marine compound could be considered an interesting chemical tool to prevent microtubule damage.

Materials and methods

CALE chemical modification with dithiobis-succinimidylpropionate

To hydrolyze the amide bond, 10 mg of CALE were dissolved in 10 mL of a solution containing dioxane (50%), H₂O (25%) and HCl 1 M (25%). The reaction mixture was kept under shaking for 36 h at 100 °C, then lyophilized to remove the solvent, dissolved in ACN and subjected to RP-HPLC separation using an Agilent 1100 Series chromatographer equipped with a Phenomenex C18 column (250 × 4.6 mm). The gradient (solution A: 0.1% TFA, solution B: 0.07% TFA, 5% H₂O 95% ACN) started at 15% and ended at 95% B after 45 min at a flow rate of 1 mL min⁻¹. The peak was analysed by mass spectrometry on an LTQ-XL (Thermo Fisher) mass spectrometer.

10 mg (34.8 µmol) of cycloamphilectene amine (CALE-NH₂) were incubated with dithiobis-succinimidylpropionate (69.5 µmol) in NaHCO₃ (10 mM) containing 30% of ACN for 16 h at 30 °C. After RP-HPLC fractionation, mass spectrometry analysis was carried out on the major product of the reaction using an LTQ-XL (Thermo Fisher) mass spectrometer. The adduct was purified using an Agilent 1100 Series chromatographer equipped with a Phenomenex C18 column (250 × 4.6 mm). The gradient (solution A: 0.1% TFA, solution B: 0.07% TFA, 5% H₂O 95% ACN) started at 15% and ended at 95% B after 45 min at a flow rate of 1 mL min⁻¹.

The generation of a functional CALE affinity matrix

5.2 µmol of CALE-NH₂ bearing linker were dissolved in NaHCO₃ (50 mM) with 30% CH₃CN at pH 8.5 and added to 0.6 mL (dry volume) of the CarboxyLink beads and incubated for 2 h at 37 °C under continuous shaking. The amount of immobilized CALE-NH₂ was calculated integrating the peaks of the free CALE-NH₂ species after HPLC injections of supernatants at *t* = 0 h and *t* = 2 h, using an Agilent 1100 Series chromatographer. The HPLC runs were carried out onto a Phenomenex C18 column (250 × 2.0 mm) at a flow rate of 200 µL min⁻¹. The gradient (solution A: 0.1% TFA, solution B: 0.07% TFA, 5% H₂O 95% ACN) started at 15% and ended at 95% B after 45 min. The residual free amine groups on the CarboxyLink beads were blocked with 300 mM acetic anhydride dissolved in DMF at 10% TEA. The same experimental conditions were applied to the preparation of the control matrix. Briefly, 0.6 mL of CarboxyLink beads were incubated with 5.2 µmol of dithiobis-succinimidylpropionate in NaHCO₃ (10 mM) containing 30% of ACN followed by incubation with 0.6 mL of 1 M Tris-HCl to remove the succinimide. The residual free amine groups on the CarboxyLink beads were blocked as reported previously. The matrices were stored at 4 °C.

CALE interactome identification by affinity chromatography and mass spectrometry

HeLa cells were grown in DMEM medium (Sigma) supplemented with 10% (v/v) foetal bovine serum (Sigma), 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Sigma), at 37 °C in a 5% CO₂ atmosphere. For the cell lysates, cells were collected by centrifugation, washed twice with PBS 1× (10 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl pH 7.4) and resuspended in lysis buffer (PBS 1×, 0.1% Igepal) containing protease inhibitor cocktail (Sigma). After dounce homogenization, cellular debris was removed by centrifugation at

900×g for 10 min at 4 °C. The supernatant was carefully collected and the pellet resuspended in lysis buffer (500 µL) to increase the yield of the lysis. The second supernatant, obtained by centrifugation, was added to the first one followed by a Bradford assay to assess the protein concentration.

50 µL (dry volume) of CALe-NH₂ modified beads and the same amount of the control matrix were separately incubated with 1 mL of cell lysate (1 mg mL⁻¹) under continuous shaking for 16 h at 4 °C. The beads were collected by centrifugation (1000×g, 5 min, 4 °C) and subjected to three washes with PBS 1×.

The proteins bound to CALe were eluted by treating the beads with 30 mM DTT solution in PBS 1× (20 µL) at 30 °C for 30 min. The supernatant containing the eluted proteins was diluted two times with SDS-PAGE sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 0.001% bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol) and subjected to SDS-PAGE separation at 10% of acrylamide. After gel electrophoresis, the gels were subjected to colloidal coomassie (Biorad G-250) staining. All the enriched bands were cut out, reduced and alkylated (10 mM DTT and 54 mM iodoacetamide) and finally subjected to trypsin digestion (0.5 µg, Sigma Aldrich) for 16 h at 37 °C. A first supernatant for each band was collected and the peptides were extracted from the gel pieces by incubation with 100 µL of pure AcN. Finally, a second supernatant was collected and both were combined. Each peptide mixture was dried out in a vacuum and dissolved in 15 µL of 10% formic acid. 5 µL of the above mentioned peptide mixture, were injected onto a nano Acquity LC system (Waters Corp. Manchester, United Kingdom). The peptides were separated using a 1.7 µm BEH C-18 column (Waters Corp. Manchester, United Kingdom) at a flow rate of 400 nL min⁻¹. Peptide elution was achieved with a linear gradient from 10 to 60% B (solution A: 0.1% formic acid, solution B: 0.1% formic acid, 100% ACN) in 55 min. The MS and MS/MS data were acquired using a Q-TOF Premier mass spectrometer (Waters Corp., Micromass, Manchester, United Kingdom). The five most intense ions were automatically chosen by the MassLynx software and fragmented. After the mass spectrometric measurements, the data were automatically processed by ProteinLynx software to generate peak lists for protein identifications. Database searches were carried out with a MASCOT server. The SwissProt human database (release 2010_11 of 02 Nov. 10, 522019 sequences, 184241293 residues) was searched, allowing 2 missed cleavages, carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (ST) as variable modifications. The peptide tolerance was set to 80 ppm and the MS/MS tolerance to 0.8 Da. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD000106 and DOI: 10.6019/PXD000106.

Western blotting analysis on protein eluates

To further confirm the MS identification of Hsp90β and tubulin as CALe binders, the eluates from both the control and cycloamphilectene beads were analyzed by Western blotting. Briefly, 10 mL of each sample were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was first treated with a blocking solution (25 mM Tris pH 8, 125 mM NaCl, 0.1% Tween-20, 5% nonfat dried milk) for 1 h and then

incubated separately with a primary monoclonal antibody raised against Hsp90 β (1 : 2000 dilution; BD Biosciences, 556321) and β tubulin (1 : 2000 dilution; BD Biosciences). After incubation with peroxidase conjugated secondary antibodies an ECL and LAS 4000 (GE Healthcare, Waukesha, WI, USA) digital imaging system were used for detection.

***In vitro* validation of CALe interactors**

Human recombinant Hsp90 β (1 μ g) and tubulin from bovine brain (1 μ g) were separately incubated with CALe-NH₂ bearing beads (20 mL) in a PBS buffer for 1 h at 4 °C under continuous shaking. Several PBS washing steps were performed and the bound proteins were eluted by beads with 30 mM DTT solution in PBS 1 $\times$ (20 μ L) at 30 °C for 30 min. As a negative control, the same amount of each protein was incubated with control beads. All samples were analyzed by SDS-PAGE (10%) and the proteins were subjected to silver staining.

Binding affinity evaluation by surface plasmon resonance

HSP90 β and tubulin were separately immobilized onto a CM-5 sensor chip using standard amine coupling procedures. PBS 1 $\times$ was used as a running buffer. The sensor chip surface was activated with a 7 min injection of a 1 : 1 ratio of 100 mM EDC and 100 mM NHS at 10 μ L min⁻¹. 100 μ L of Hsp90 β and β tubulin, at a final concentration of 30 ng μ L⁻¹ in 10 mM sodium acetate at pH 4.5, were injected onto the activated chip surface at a flow rate of 5 μ L min⁻¹. The protein concentration was selected to obtain an optimal response (10 000–13 000 RU). The remaining active groups were blocked with a 7 min injection of 1.0 M ethanolamine-HCl, pH 8.5, at 10 μ L min⁻¹. Solutions of CALe (0.5–25 μ M), used in the biosensor experiments, were diluted in PBS 1 $\times$ containing 1% DMSO. Each concentration was tested at least three times. Since the dissociation curves reached the baseline within a reasonable time frame, no regeneration was required. All experiments were carried out at a flow rate of 10 μ L min⁻¹, employing a 3 min injection time. The dissociation time was set at 300 s. The rate constants for association (k_a) dissociation (k_d) and the dissociation constant (K_D) were obtained by globally fitting the data from all of the injections of different concentrations of each compound, using the BIAevaluation software, using the simple 1 : 1 Langmuir binding model.

Immunofluorescence analysis of tubulin polymerization

In order to analyse the effect of the CALe treatment on tubulin polymerization, we performed immunofluorescent analyses of the tubulin cytoskeleton. 4×10^4 HeLa cells per well were seeded on cover slips in 24-well plastic plates. After 24 h, cells were treated or not with 100 μ M CALe (4 h, 37 °C) and then with colcemid (Gibco® KaryoMAX) 500 nM for different time periods (30', 1 h, 1 h, 30', 2 h, 37 °C). The cover slips were treated as described elsewhere²⁸ and incubated with an antibody against α -tubulin (1 : 100; Santa Cruz 32293), diluted in blocking buffer over night at 4 °C. Labelling was visualized with the fluorescently conjugated secondary antibody DyLight 488 anti-mouse (Jackson). A secondary antibody was diluted 1 : 1000 in PBS. The images were collected on a Zeiss LSM 510 confocal microscope using LSM software. The images shown are representative of multiple fields and triplicate cover slips per experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
The chemical structure of *N*-formyl-7-amino-11-cycloamphilectene (CALE).

Fig. 2.

The reaction scheme of the CAle affinity matrix preparation. (A) The hydrolytic transformation of CAle to its amine derivative (CAle-NH₂). (B) The reaction scheme of the CAle-NH₂ chemical modification with dithiobis-succinimidylpropionate. (C) CAle-NH₂ immobilization onto the CarboxyLink beads.

Fig. 3.

(A) The Western blot analysis of CAlE-modified and control beads (CTRL) showed the metabolite ability to pull down Hsp90 β and tubulin in HeLa cell lysate. (B) The silver stained SDS-PAGE gel of "in vitro" pulldown, using recombinant Hsp90 (~90 kDa) and tubulin (~50 kDa). (C) Sensograms obtained from injections of CAlE (0.5–25 μ M) on immobilized tubulin.

Fig. 4.

HeLa cells were pre-treated or not with 100 μM CAle (4 h) and then incubated with 500 nM colcemid for different times and subsequently immuno-labeled for α-tubulin. The figure shows that the CAle treatment has no effect on tubulin polymerization after 4 hours (B), compared to control cells (A), while colcemid induces an evident microtubule depolymerization after 2 hours of treatment both on the control (C) and on pre-treated cells (D). If compared after 30 min of colcemid, the pre-treated cells (F) appear protected with respect to the control ones (E).

Table 1

A list of the CALe partners identified in four independent chemical proteomics experiments. The results are reported in terms of Mascot score (SCR) and number of identified peptides (in the brackets)

Proteins	SCR (n° pep)	SCR (n° pep)	SCR (n° pep)	SCR (n° pep)
Heat shock protein 90 β (HS90B_HUMAN)	1208(52)	474(20)	478(20)	512(20)
Tubulin beta chain (TBB5_HUMAN)	1289(40)	381(20)	132(10)	311(14)
Tubulin alpha-4A chain (TBA4A_HUMAN)	795(25)	415(23)	208(8)	237(10)
Tubulin alpha-1B chain (TBA1B_HUMAN)	466(20)	435(23)	198(8)	248(11)
Importin subunit beta-1 (IMB1_HUMAN)	252(10)	191(10)	195(6)	191(6)
ATP synthase subunit beta (ATPB_HUMAN)	365(17)	110(4)	41(4)	185(6)
Transportin-1 (TNPO1_HUMAN)	66(3)	401(16)	79(4)	89(3)
4F2 cell-surface antigen heavy (4F2_HUMAN)	111(9)	22(1)	118(4)	42(3)
Putative heat shock HSP 90- β (H90B2_HUMAN)	174(7)	76(2)	90(3)	131(4)
Calnexin (CALX_HUMAN)	67(3)	79(3)	104(5)	68(3)
Heat shock protein 75 kDa (TRAP1_HUMAN)	117(2)	120(2)	99(1)	183(3)