Autumnalamide targeted proteins of the immunophilin family

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Previous works with autumnalamide reported that Store Operated Calcium (SOC) channels were blocked through mitochondrial modulation. In the present paper we studied the effect of autumnalamide on ionomycin Ca^{2+} fluxes. Thus, autumnalamide did not modify ionomycin-sensitive intracellular pools while the ionomycin-induced Ca^{2+} influx was blocked with similar potency whether the incubation was done before or after ionomycin-sensitive pools depletion. Nevertheless, autumnalamide was not able to inhibit ionomycin-induced Ca^{2+} influx once the membrane channels were activated. Moreover, the compound efficiently inhibited flufenamic acid (FFA) Ca^{2+} release induced in this organelle but not the next influx. Since in previous work the effect of autumnalamide was inhibited by cyclosporine A (CsA), structures that target this drug were studied. Therefore, the affinity of autumnalamide for cyclophilin D (Cyp D) was examined. The K_D obtained for Cyp D- autumnalamide was 1.51 ± 1.399 . Moreover, the K_D for Cyp A- autumnalamide was calculated. The peptide had a similar order of Cyp A binding affinity than CsA (8.08 ± 1.23 and 6.85 ± 1.1 μM respectively). After testing autumnalamide-binding capacity for Cyp A, the activity of this compound on Cyp A pathway was tested. Thus, the effect on interleukin (IL)-2 release on activated T-lymphocytes was checked. Autumnalamide was able to reduce IL-2 levels near to T cells in resting conditions. Next, the effect over calcineurin and NFATc1 was also evaluated. While CsA inhibits both calcineurin and NFATc1, autumnalamide did not produce any effect. From these results we can conclude that, autumnalamide targeted mitochondrion and prevent T-cells from IL-2 production through the modulation of SOC Ca^{2+} channels.

1. Introduction

Filamentous cyanobacteria of the genus *Phormidium* have a worldwide distribution and present a large chemical diversity with nearly 200 described species. The genus *Phormidium* forms mats on wet soil, mud, wetted rocks, macrophytes and in standing and running waters. Up to now, several species from different genus have been cultivated for the production of large quantities of biomass with wide range of applications, including animal and human nutrition, as biofertilizers in agriculture, cosmetics or with energy purpose to produce biodiesel (Markou and Georgakakis, 2011). Nevertheless, the use of active metabolites in the pharmaceutical sector is less studied. In the case of cyanobacteria, the production of toxic metabolites with therapeutic potential has

attracted the attention of pharmacy industry (Coates et al., 2013). Autumnalamide is a prenylated cyclic peptide obtained from the cyanobacteria *Phormidium autumnale* whose chemical structure has been recently elucidated (Fig. 1) (Audoin et al., 2014). This specie is one of the most common of the genus *Phormidium*, which colonizes stones in streams and rivers (Komárková, 2003). Initial studies on the biological activity of this natural compound has shown that autumnalamide is able to induce calcium influx blockade through store operated calcium channels (SOC channels) in SH-SY5Y neuroblastoma cells due to an effect at the mitochondrial level, disrupting the mitochondrial membrane potential (MMP) and inducing the opening of mitochondrial permeability transition pore (mPTP). Moreover, the effect of autumnalamide on SOC channels was abolished by pre-incubation with the immunosuppressant drug cyclosporine A (CsA), (Audoin et al., 2014). The mPTP complex is a multiproteic structure composed by different proteins. Among them, cyclophilin D (Cyp D) is an essential component with a molecular weight of 22 kDa (Elrod and Molkentin, 2013; Javadov and Kuznetsov, 2013). CsA is a cyclic 11-amino-acid peptide isolated from the fungus *Tolypocladium inflatum* and was described as

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a potent and selective mPTP inhibitor due to Cyp D binding in the matrix and on the inner surface of the mitochondrial membrane (Elrod and Molkentin, 2013; Hoppert et al., 2001). Besides Cyp D binding and mPTP opening inhibition, CsA has the ability to bind to all Cyclophilins (CyPs) present in the cell with different affinities due to a high degree of sequence conservation in their structure (Wang and Heitman, 2005; Fruman et al., 1994). The CyPs protein family structurally contain two main protein domains, a cyclophilin (Cyp)-like domain, which is the conserved domain across all family members and the specific domain depending on the family member (Lee and Kim, 2010). These proteins are present in all eukaryote and prokaryote cells like plants, fungi, bacteria, insects and human cells with a common peptidyl-prolyl isomerase activity. That is, CyPs catalyze the isomerisation of the peptide bonds from trans to cis form at the proline residues and facilitates protein folding among other functions (Wang and Heitman, 2005; Kofron et al., 1991; Davis et al., 2010). The location of these proteins is very broad and can be found in different organelles and also in the cytosol or in the extracellular medium (Lee and Kim, 2010; Kumari et al., 2013). Our initial studies on the biological activity of autumnalamide have shown that the compound induced the formation of mPTP. The mPTP can be induced by different stimulus over the proteins that form this structure like Cyp D (Audoin et al., 2014). Due to this and since the effect of autumnalamide on SOC channels was blocked by CsA, the main objective of this work was to go into detail about the knowledge of mechanism of action of autumnalamide.

2. Materials and methods

2.1. Chemicals and solutions

Carboxymethyl dextran (CM5) sensor chips, Hank's Balance Solution Surfactant P20 (HBS-EP) buffer (pH 7.4, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.15 M NaCl, 3 mM Ethylenediamine tetraacetic acid (EDTA), 0.005% polysorbate), amine coupling kit (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) and ethanolamine-HCl were supplied by BiacoreAB (Uppsala, Sweden). Percoll was obtained from Pharmacia (Uppsala, Sweden). Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). RPMI and Foetal calf serum (FBS) were bought from Gibco (Glasgow, UK). The Pan T cell Isolation Kit (human) and the monoclonal antibody to human CD3, clone BW264/56, FITC were purchased from Miltenyi Biotec (Germany). Human interleukin (IL)-2 ELISA kit was obtained from Invitrogen (Spain). Active human Cyclophilin D (Cyp D) and Active Human Cyclophilin A (Cyp A) full-length proteins were from Abcam (Cambridge, UK). Anti-nuclear factor of activated T cells (NFAT) c1 mouse monoclonal antibody [AT1C3] to NFATc1 and rabbit polyclonal anti-lamin B1 antibody were from Abcam (UK). FURA-2AM was obtained from Molecular Probes. Polyvinylidene fluoride (PVDF) membrane was from Millipore (Temecula, CA). Polyacrylamide gels and molecular weight marker Precision Plus Protein Standards Kaleidoscope was from BioRad (Barcelona, Spain). The Protease Inhibitor Complete Tablets and Phosphatase Inhibitor Cocktail Tablets were from Roche (Spain). Bovine serum albumin (BSA) and CsA (purity $\geq$ 98.5%), Flufenamic acid (FFA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and the rest of Chemicals and reagents were obtained from Sigma-Aldrich (Madrid, Spain). The strain of *Phormidium autumnale* was purchased from the Culture Collection of Algae and Protozoa (UK, CCAP1446/10). The strain was cultured and extracted as previously described (Audoin et al., 2014). Autumnalamide was isolated with purity higher than 95% as demonstrated by its ^1H NMR spectrum. The composition of saline solution (PBS) used for

Fig. 1. Autumnalamide chemical structure.

human T lymphocytes purification was in (mM): 137 ClNa, 8.2 Na_2HPO_4 , 1.5 KH_2PO_4 , 3.2 KCl and 2 EDTA. The composition of Umbreit saline solution was in (mM): 119 NaCl, 1.2 Mg (SO_4), 1.2 NaH_2PO_4 , 22.8 NaHCO_3 , 5.9 KCl, 1 CaCl_2 and 1 g/L Glucose. The pH was equilibrated between 7.2-7.3. Stock solutions of drugs were done in dimethylsulphoxide (DMSO).

2.2. Human neuroblastoma culture cells and human T lymphocytes isolation

Neuroblastoma cell line SH-SY5Y was purchase from American Type Culture Collection (ATCC) Number CRL-2266. The cells were plated in 25 cm^2 flasks at a cultivation ratio of 1:10. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) from ATCC and F12 Medium (Invitrogen) 1:1 supplemented with 10% fetal bovine serum (FBS) (Gibco) plus 100 UI/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. The neuroblastoma cells were expanded weekly using 0.05% trypsin/EDTA (1x) (Invitrogen).

Peripheral lymphocytes were isolated from human fresh heparinised blood from healthy volunteers as previously described (Alfonso et al., 2001). The blood was diluted in the same proportion with PBS plus EDTA 2 mM previously equilibrated at room temperature. 4 mL of diluted blood were placed over 3 mL of isotonic percoll (57.5%) carefully avoiding the mixture of the two phases. Once the tubes were prepared they were centrifuged at 3000 rpm, 25 min at room temperature. After centrifugation, different phases were obtained and only the fraction that containing the population of white blood cells was collected and washed three times by centrifugation with PBS-EDTA at 1500 rpm, 10 min, room temperature to remove remaining percoll. Lymphocyte purity was always higher than 80%. T lymphocytes were purified from this population with a human Pan T cell Isolation Kit. This is an indirect magnetic labeling system for the isolation of untouched T cells. T cell purity was always higher than 95%. Assessment of cell purity was performed by flow cytometry by using a monoclonal antibody to human CD3 labeled with FITC. Viability ($>95\%$) was determined by trypan blue exclusion. Pure T cells were maintained in RPMI 1640 plus 10% FBS and plated in 24 plastic tissue-culture dishes in humidified 5% CO_2 and 95% air atmosphere at 37 $^\circ\text{C}$.

2.3. Measurements of cytosolic free calcium

Cells were seeded onto 18 mm glass cover slips and used between 48 and 72 h after plating at a density of 120,000 cells/glass cover slip. For cytosolic Ca^{2+} measurements, cells were washed twice with saline solution (Umbreit) supplemented with 0.1% BSA. Umbreit composition was (mM): NaCl 119, $\text{Mg}(\text{SO}_4)$ 1.2, NaH_2PO_4 1.2, NaHCO_3 22.85, KCl 5.94, Glucose 0.1% and CaCl_2 1. In all assays the solutions were equilibrated with CO_2 before being used, adjusting the final pH between 7.2–7.4. The cells were loaded with the Ca^{2+} -sensitive fluorescent dye FURA-2 AM (0.5 μM) for 6.5 min at 37 °C and 300 rpm. Loaded cells were washed twice with saline solution and the cover slips were placed in a thermostatic chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diphot 200 microscope equipped with epifluorescence optics (Nikon 40X- immersion UV- Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution with the drug to the incubation chamber. Cytosolic Ca^{2+} levels as FURA-2 ratio was obtained from the images collected by fluorescent equipment, Ultra-high-speed wavelength switching illumination system (Lambda-DG4) for excitation and Lambda 10-2 for emission from Sutter Instruments Co., USA. The Light source was a xenon arc bulb and the different wavelengths used were chosen with filters. Cells were excited at 340 and 380 nm lights alternately and emission was collected at 510 nm.

2.4. Viability assay

Cells were incubated with different concentrations of autumnamide ranging from 0.001 to 10 μM for 24 and 48 h. After incubation, cells were washed twice with saline solution. MTT (500 $\mu\text{g mL}^{-1}$) dissolved in saline solution was added to the 96-well microplate for 1 h at 37 °C (300 rpm) in the dark. Finally the MTT incubation media was removed and cells were washed once before the addition of SDS at 5%. Colored formazan salt aggregates were measured at 595 nm in a spectrophotometer plate reader.

2.5. Binding experiments: surface activation, ligand immobilization and binding

A Biacore X SPR biosensor with Control Software and BIAevaluation software version 3.0 from Biacore (GE Healthcare, Uppsala, Sweden) was used to check the binding between molecules. Sensor surface activation and ligand immobilization were performed by using HBS-EP as running buffer at a flow rate of 5 $\mu\text{L min}^{-1}$ and 25 °C. CM5 sensor chip were used as surface where Cyp D or Cyp A was immobilized as ligand. The CM5 chip is a glass slide coated with a thin layer of gold with a matrix of carboxymethylated dextran covalently attached. The CM5 chip was activated using an amine coupling kit. Following manufacture instructions, a mixture (1:1, v/v) of EDC and NHS was applied for 2 min over the sensor chip. After activation, the ligand, 100 $\mu\text{g mL}^{-1}$ of Active human Cyp A or Cyp D full-length protein dissolved in sodium acetate 10 mM at pH 4.5 was added. In these conditions, a typical covalent binding curve was obtained. Then, the CM5 chip surface was washed with HBS buffer and no fall in the signal was observed, indicating the protein immobilization onto the sensor chip surface. Finally, ethanolamine-HCl was injected to deactivate the remaining active esters, remove non-covalently bound protein and avoid non-specific binding (Sanchez et al., 2015; Alfonso et al., 2014). Next, analytes were added to check the binding between them and Cyp D or Cyp A. Individual binding curves for each analyte were analyzed by determining the kinetic constant of analytes-Cyp A/D binding, namely, the observed rate constant (K_{obs}), the association rate constant (K_{ass}), the dissociation rate constant (K_{diss}), and the kinetic equilibrium dissociation constant (K_D). At equilibrium, by

definition, $K_{\text{diss}}/K_{\text{ass}} = K_D$. The pseudo-first-order association rate constants K_{obs} (s^{-1}) were determined by using the 1:1 Langmuir association model of BiaEvaluation software (Biacore, Uppsala, Sweden). Then a representation of K_{obs} against the corresponding concentration of compound was done. These plots follow a linear correlation coefficient. From the equation of this representation, K_{ass} , $\text{M}^{-1} \text{s}^{-1}$, gradient of the Plot, and K_{diss} , s^{-1} , intercept of the Plot was obtained. Within these two values, the kinetic equilibrium dissociation constant K_D for the analyte-Cyp A/D binding was obtained.

The duration of the sample injection was 2 min at 10 $\mu\text{L min}^{-1}$ flow rate. Next, dissociation of bounded molecules in HBS-EP buffer flow was studied. The bounded drugs were removed from the chip surface before the next injection by adding 1 M Glycine-HCl at pH 2.5 for 1 min. The association phase was used to quantify the compound-Cyp A/D interactions.

2.6. Interleukin 2 release

Human T lymphocytes at the concentration of 1×10^6 cells mL^{-1} were plated in 24 well plates and pre-treated for 2 h with autumnamide (10 μM). Then, cells were stimulated with Concanavalin A (Con A) at 50 $\mu\text{g mL}^{-1}$ for 48 h to induce IL-2 release. The amount of IL-2 released to the culture medium was evaluated using a Human IL-2 ELISA kit.

2.7. Calcineurin phosphatase activity assay

Calcineurin phosphatase activity was used as a measure of Cyp A activity by means of a calcineurin Phosphatase Assay Kit (Enzo Life Sciences, Inc., Farmingdale, NY). This is a complete colorimetric assay kit to measure calcineurin phosphatase activity. The Cyp-A-drug complex was allowed to form for 1 h at room temperature. 1 nM of Cyp A and different concentrations of drugs were employed dissolved in deionised water. After 1 h, recombinant calmodulin (0.25 μM) and recombinant calcineurin (40 units per well) were added to the complex formed between Cyp A and Autumnamide or CsA. The complex was incubated for 30 min. Next; RII phosphopeptide substrate was added at a final concentration of 0.15 mM allowing the development of the reaction for 1 h at room temperature. Then 100 μL of development reagent was added and incubated for 20 min at room temperature. Finally, the A_{620} of the 96-well plate samples was measured using Multi-Mode Microplate Reader Synergy™ 4 equipment from Biotek (Winooski, VT, USA).

2.8. Western blotting

Cells were incubated first with autumnamide or CsA for 2 h after which Con A (50 $\mu\text{g mL}^{-1}$) was added for 48 h. After incubation, T cells were centrifuged and washed twice with ice-cold saline solution (Umbreit) at 1500 rpm, 5 min and 4 °C. Then, the pellet was resuspended in 50 μL of hypotonic lysis buffer solution to extract cytosolic proteins for 15 min with the following composition mM: 20 Tris-HCl, 10 NaCl and 3 MgCl_2 , 10X phosphatase inhibitors and 7X protease inhibitor. Cells were pelleted at 3000 rpm, 10 min at 4 °C to remove the cytosolic protein fraction. The supernatant was collected and the pellet obtained was resuspended in 30 μL of nuclear lysis buffer composed by: 100 mM Tris, 2 mM NaVO_4 , 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1% Triton-X-100, 10% Glycerol, 0.1% SDS and 0.5% Deoxycholate Sodium containing 1 mM PMSF and 10X protease inhibitor cocktail for 30 min vortexing every 10 min. Samples were then centrifuged at 14000g at 4 °C for 30 min. The supernatants of interest were collected as protein nuclear fraction. The determination of protein concentration was done using Direct Detect (Millipore) using bovine serum albumin (BSA) as standard protein.

Fig. 2. Effects of autumnamide and ionomycin cytosolic Ca^{2+} profile in SH-SY5Y neuroblastoma cells. A. First arrow indicates the addition of $2 \mu M$ ionomycin. Second arrow shows addition of $10 \mu M$ of autumnamide. The third arrow indicates the addition of Ca^{2+} $1 mM$ to the bath solution B. Cytosolic Ca^{2+} profile in cells first pre-incubated with $10 \mu M$ autumnamide (first arrow) followed

Samples of cell lysates containing $10 \mu g$ of nuclear fraction of total protein were used for electrophoresis. Samples for electrophoresis were dissolved in a 15–20% polyacrylamide gel and blotted to PVDF membrane by reduced SDS-PAGE. To determine the protein size and also to monitor the progress of the electrophoresis run, Precision Plus Protein Standards Kaleidoscope molecular weight marker was used. Then, membranes were blocked with 0.5% BSA in washing buffer (PBS + 0.1% Tween) and incubated 10 min with primary anti-NFATc1 (1:1000). After three washes with washing buffer (PBS + 0.1% Tween 20), membranes were incubated with secondary anti-Mouse IgG conjugated with horseradish peroxidase. The immunoreactive bands were detected using the Supersignal West Pico or Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and the Diversity GeneSnap software (Syngene). NFATc1 signal was normalized using Lamin B1 (1:1000).

2.9. Statistical analysis

All experiments were carried out by duplicate a minimum of three times. Results were analyzed by using one-way analysis of variance ANOVA with Dunnett's post hoc analysis. p values < 0.05 were considered statistically significant. All results were expressed as the mean $\pm$ SEM of 3 or more experiments.

3. Results

Considering the results obtained in a previous work, autumnamide was able to completely inhibit SOC channels by its effect over mitochondrion (Audoin et al., 2014). Therefore, several additional calcium (Ca^{2+}) experiments were carried out in order to clarify the effect of autumnamide on Ca^{2+} fluxes different from SOC. To do this, the effect of autumnamide on ionomycin Ca^{2+} fluxes was studied. Ionomycin is a Ca^{2+} ionophore that induces intracellular pools depletion and allows Ca^{2+} entrance from the extracellular medium (Morgan and Jacob, 1994). Thus, autumnamide was added in a Ca^{2+} -free medium after ionomycin pools depletion, and a significant cytosolic Ca^{2+} influx reduction was observed after ion addition (Fig. 2A). In the same way, when the compound was incubated in a Ca^{2+} -free medium before ionomycin addition, no modification on intracellular stores was observed neither on ionomycin-sensitive pools depletion. Nevertheless, once Ca^{2+} ion was restored, a significant ion-influx inhibition was obtained comparing to ionomycin (Fig. 2B). Moreover, autumnamide did not inhibit the ion entrance once the influx is activated (Fig. 2C). Thus, autumnamide did not affect intracellular pools modulated by ionomycin; nevertheless cytosolic Ca^{2+} entrance was reduced a 30% when the incubation with autumnamide was done after or before ionomycin-sensitive pools depletion. Therefore, as it was reported, the compound inhibits SOC entrance.

In order to confirm if autumnamide inhibits other Ca^{2+} influx different from SOC, flufenamic acid (FFA) was employed. FFA is a non-steroidal drug that acts inducing Ca^{2+} release from the mitochondrion in a Ca^{2+} free medium (Jiang et al., 2012; Damsker et al., 2009; Zeng et al., 2012). Thus, when FFA was incubated in the absence of Ca^{2+} , a significant Ca^{2+} release from internal stores was induced. In addition, when Ca^{2+} was added to the medium an ion influx was observed (Fig. 3A). Nevertheless, when autumnamide was incubated before the FFA addition, the Ca^{2+} release from mitochondrion was significantly inhibited, besides after addition of Ca^{2+} the ion reached similar levels than FFA (Fig. 3A). Therefore, the

by ionomycin $2 \mu M$ (second arrow). The third arrow indicates $1 mM$ of Ca^{2+} addition to the bath solution. C. The first arrow shows the ionomycin addition. The second arrow shows the Ca^{2+} addition to the medium and the third the addition of $10 \mu M$ of autumnamide. The experiments were performed in duplicate. The results of each replica are mean of 20 cells. Mean $\pm$ SEM of three independent experiments.

Fig. 3. Effect of FCCP or autumnalnamide and FFA on cytosolic Ca²⁺ profile in SH-SY5Y neuroblastoma cells. A. First arrow indicates autumnalnamide addition and the second arrow the FFA addition. The third arrow indicates the addition of Ca²⁺. B. The FCCP addition to the medium (first arrow) prior to FFA addition (second arrow). The third arrow indicates the addition of the ion to the Ca²⁺-free bathed solution. The experiments were performed in duplicate. The results of each replica are mean of 20 cells. Mean $\pm$ SEM of three independent experiments.

compound does not affect mitochondrial Ca²⁺ influx, however the release of the ion from this organelle was largely reduced. In order to know the effect of other mitochondrial uncouplers on FFA, FCCP was employed (Ma et al., 2012). As shown in Fig. 3B, FCCP produced a small increase in Ca²⁺ levels; and the following addition of FFA did not induce a Ca²⁺ release. Thus, after the ion addition to the bath solution, a similar Ca²⁺ influx as FFA control was observed. Therefore, as in the case of autumnalnamide, the prior incubation with FCCP inhibited the Ca²⁺ release induced by FFA without affecting the next Ca²⁺ influx. Summarizing the results described above, both, FCCP and autumnalnamide were able to decrease the release of Ca²⁺ from mitochondrion induced by FFA; nevertheless the cytosolic Ca²⁺ entrance was not blocked. In addition, autumnalnamide inhibited cytosolic Ca²⁺ influx induced by the ionophore while ionomycin-sensitive intracellular pools were not modified. Therefore, autumnalnamide targeted mitochondrion without inducing Ca²⁺ release from this organelle but inhibiting SOC influx.

In order to explain the effect of autumnalnamide on Ca²⁺ handling and since in a previous work it was observed the effect of autumnalnamide inhibiting Ca²⁺ influx, which was reverted by CsA (Audoin et al., 2014), the structures that this drug targets were studied. With

this purpose, the highly sensitive biosensor Biacore X was used to determine the binding between mitochondrial Cyp D, used as ligand, attached to the sensor surface and using autumnalnamide as ligate. First, CsA was used as control of binding for the immobilized Cyp D. As Fig. 4A shows, when different concentrations of CsA (5, 10, 20 and 40 μ M) were added over the immobilized Cyp D, typical association curves profiles were obtained. In the presence of 5 μ M CsA the signal was 10.05 RU, while in the presence of 40 μ M of CsA the response reached 31.8 RU. Individual binding curves from Fig. 4A were analyzed as described in the material and methods section. K_{obs} for each concentration of CsA were obtained. When K_{obs} were represented against each CsA concentration, (Fig. 4B), a linear regression with a correlation coefficient of $r = 0.97985$ for Cyp D was obtained. From this representation, K_{ass} , $M^{-1} s^{-1}$, slope, K_{diss} , s^{-1} , and Y-intercept, were obtained. Within these two values, the kinetic equilibrium dissociation constant K_D (Y-intercept/slope) for the CsA-Cyp D association was obtained. The value of this constant was $4.5 \times 10^{-6} \pm 3.05 \times 10^{-6} M$ for the CsA-Cyp D binding assay (Fig. 4B). After setting up these conditions the binding of Cyp D and autumnalnamide was next studied. In this case, different concentrations of autumnalnamide (30, 50, 70 and 100 μ M) were dissolved in HBS-EP buffer and added onto the previously immobilized Cyp D. Thus, as Fig. 4C shows, association curves for the binding of autumnalnamide with Cyp D were obtained with responses ranging from 10 RU at the lowest autumnalnamide concentration to 220 RU in the presence of 100 μ M of the compound. As in the case of CsA, the interaction between autumnalnamide and Cyp D follows a pseudo-first order kinetic where K_{obs} was calculated. When each K_{obs} was represented against the corresponding concentration of autumnalnamide from Fig. 4C, linear regression plots with a correlation coefficient of $r = 0.98$, for the binding of autumnalnamide to Cyp D were obtained. From these representations the kinetic equilibrium constant for the binding of autumnalnamide to Cyp D was calculated. In the case of the Cyp D-autumnalnamide binding, a $K_D = 1.51 \pm 1.399 \mu M$ (Fig. 4D) was obtained. Due to Cyts share a common region domain and since autumnalnamide showed good binding affinity for Cyp D, the effect of this compound over Cyp A, another important immunophilin, was also evaluated. Straightaway, the same procedure was followed for Cyp A immobilization over a CM5 chip as described in Material and Methods. Thus, when different concentrations of CsA were added over the immobilized Cyp A, typical association curves were obtained. In the presence of 5 μ M CsA the signal was 17.54 RU, while in the presence of 40 μ M of CsA the response reached 55.45 RU (Fig. 5A). In this case, a K_D for CsA-Cyp A was $6.83 \times 10^{-6} \pm 1.1 \times 10^{-6} \mu M$ (Fig. 5B). In the same way, different concentrations of autumnalnamide were dissolved in HBS-EP buffer and added onto the immobilized Cyp A. Thus, as Fig. 5C shows, association curves for the binding of autumnalnamide with Cyp A were obtained with responses ranging from 34.11 RU at the lowest concentration to 102.5 RU at the highest concentration of autumnalnamide. From the association curves a K_D of $8.08 \pm 1.23 \mu M$ was obtained (Fig. 5D). So far the results obtained here indicated that autumnalnamide showed higher affinity for Cyp D than CsA and also a good affinity for Cyp A, although slightly smaller than that of CsA.

Hence, autumnalnamide showed Cyp A binding, and also acts by inhibiting SOC influx; both targets are related with the modulation of interleukins (ILs) (Sweeney et al., 2009; Djuric et al., 2000), we then tested the effect of autumnalnamide on IL-2 production in human T lymphocytes. In previous work the effect of autumnalnamide in SH-SY5Y cell viability was checked for 1 h, and the compound produced a 42% of decrease in cell viability at the concentration of 25 μ M (the highest concentration tested) (Audoin et al., 2014). In order to develop experiments with prolonged exposure times and in different cellular model, it was necessary to check cell toxicity of autumnalnamide. To do this, different concentrations

Fig. 4. CsA/autumnalnamide association curves and ligand binding analysis. A. Cyp D-CsA association: association curves after addition of different amounts of CsA to immobilized Cyp D. Different CsA concentrations were injected using HBS-EP as running buffer and a flow rate of $10 \mu\text{g mL}^{-1}$. The association curves were obtained after subtraction of their respective solvent control. B. Analysis of ligand binding: kinetic plot of apparent association rate constant K_{obs} (s^{-1}) obtained from plot A (calculated by BiaEvaluation software) versus CsA concentration. C. Cyp D-autumnalnamide association: association curves after addition of different amounts of autumnalnamide to immobilized Cyp D. Different autumnalnamide concentrations were injected using HBS-EP as running buffer and a flow rate of $10 \mu\text{g mL}^{-1}$. The association curves were obtained after subtraction of their respective solvent control. D. Analysis of ligand binding: kinetic plot of apparent association rate constant K_{obs} (s^{-1}) obtained from plot C (calculated by BiaEvaluation software) versus autumnalnamide concentration. Representative experiment of $n = 3$.

ranges of autumnalnamide were tested for 24 and 48 h in human T lymphocytes. As Fig. 6 shows, the concentrations tested for the two exposure times did not produced any changes on cell viability. Once again, the concentration of $10 \mu\text{M}$ was selected to develop the current experiments at 48 h.

To develop IL-2 experiments, a control of human T lymphocytes was treated with Con A alone, to induce IL-2 release, or in combination with autumnalnamide. After 48 h of incubation, IL-2 release in the culture medium was measured. As Fig. 7 shows, the stimulation of human T lymphocytes with Con A for 48 h efficiently induces IL-2 production comparing with non-stimulated control cells from $47.72 \pm 1.58 \text{ pg mL}^{-1}$ to $742.77 \pm 152.23 \text{ pg mL}^{-1}$ respectively ($p \leq 0.001$). In this conditions, when T lymphocytes were pre-incubated for 2 h with $10 \mu\text{M}$ autumnalnamide, before stimulation with ConA for 48 h, a reduction from $742.77 \pm 152.23 \text{ pg mL}^{-1}$ to $111.61 \pm 3.4 \text{ pg mL}^{-1}$ ($p \leq 0.01$). The well-known IL-2 inhibitor CsA was used as positive control of IL-2 inhibition. Thus, when cells were pre-incubated with CsA $0.2 \mu\text{M}$ for 2 h before Con A stimulation, IL-2 production was significantly reduced to control values $146.25 \pm 111.34 \text{ pg mL}^{-1}$ ($p \leq 0.01$). From these results we conclude that both compounds suppressed IL-2 release from human activated T lymphocytes with a similar efficacy.

In view of these results two additional experiments were performed to evaluate the mechanisms underlying the effects of autumnalnamide in IL-2 blockade. Some compounds, as in the case

of CsA, act docking calcineurin phosphatase activity over NFATc1 and the next IL-2 induction due to the binary complex formed with Cyp A (Randak et al., 1990). Therefore, in view of the binding of autumnalnamide to Cyp A, the ability of autumnalnamide to inhibit calcineurin phosphatase activity was next compared. In this case, a compound concentration close to the calculated K_D was used. As Fig. 8 shows, when Cyp A was incubated in the presence of $6 \mu\text{M}$ CsA a significant reduction in calcineurin phosphatase activity of $28.33 \pm 3.07\%$ ($p \leq 0.01$) was observed. In the same way, in the presence of $10 \mu\text{M}$ of autumnalnamide a significant $18.87 \pm 2.21\%$ ($p \leq 0.001$) reduction was observed over calcineurin phosphatase activity.

It is known that T lymphocyte stimulation with Con A induce a cytosolic Ca^{2+} increase (Smith-Garvin et al., 2009). As a consequence, Cyp A forms a complex with calcineurin that binds to and dephosphorylates cytosolic NFATc1. The dephosphorylation of the nuclear factor leads to the translocation to the nucleus. The increase in the nuclear expression of NFATc1 in a transcriptionally active form leads to the increase of IL-2 production (Liu, 2009; Xiong et al., 2013). Therefore, the nuclear expression of NFATc1 in activated human peripheral T lymphocytes in the presence of autumnalnamide was next checked and compared with the effect of CsA in the same condition. Representative western blot bands for these results are shown in Fig. 9A. As shown in Fig. 9B, the quantification of western blot band intensities in each condition showed

Fig. 5. CsA/atumnalamide association to immobilized Cyp A and ligand binding analysis. A. Cyp A-CsA association: association curves after addition of different amounts of CsA to immobilized Cyp A. Different CsA concentrations were injected using HBS-EP as running buffer and a flow rate of $10 \mu\text{g mL}^{-1}$. The association curves were obtained after subtraction of their respective solvent control. B. Analysis of ligand binding: kinetic plot of apparent association rate constant K_{obs} (s^{-1}) obtained from plot A (calculated by BiaEvaluation software) versus CsA concentration. C. Cyp A-autumnalamide association: association curves after addition of different amounts of autumnalamide to immobilized Cyp A. Different autumnalamide concentrations were injected using HBS-EP as running buffer and a flow rate of $10 \mu\text{g mL}^{-1}$. The association curves were obtained after subtraction of their respective solvent control. D. Analysis of ligand binding: kinetic plot of apparent association rate constant K_{obs} (s^{-1}) obtained from plot C (calculated by BiaEvaluation software) versus autumnalamide concentration. Representative experiment of $n=3$.

that when cells were stimulated with Con A for 48 h nuclear band intensity of NFATc1 exhibited a $67.21 \pm 0.12\%$ ($p < 0.01$) increase comparing with T cells in resting conditions. Pre-incubation of the cells with CsA before activation decreased NFATc1 band intensity close to control values, in agreement with previously reported results (Ishikawa et al., 2003). However, autumnalamide did not significantly reduce the increase in nuclear NFATc1 in activated human T lymphocytes that reach higher levels than Con A treated cells.

Altogether, the results presented here indicate that, autumnalamide does not modify ionomycin-sensitive intracellular Ca^{2+} pools, besides confirm that the compound inhibits the SOC channels. Moreover, it binds to mitochondrion Cyp D, presents binding affinity for Cyp A and inhibits calcineurin and human IL-2 production in activated T lymphocytes but, unlike CsA, it lacks of an inhibitory effect over NFATc1.

4. Discussion

The structure of the prenylated cyclic peptide named autumnalamide was recently identified and the same paper reported the only available data on the compound, indicating that autumnalamide alters mitochondrial membrane potential in neuroblastoma cells and also induces the mPTP opening (Audoin et al.,

2014). The opening of this structure can be induced by different substances that do not necessarily target the proteins that form the mPTP structure (Tornerio et al., 2002). There are many molecules from the own organism that act as inducers of mPTP, such as β -Amyloid (βA) protein, as well as molecules with natural origin like mastoparan from the venom of *Vespula lewisii*; honokiol from *Magnolia* spp. or molecules from synthetic sources like MT-21. Nevertheless, almost no molecule acts targeting directly Cyp D, both for blocking or inducing the formation of mPTP. In the case of βA protein, it induces the production of Reactive Oxygen Species (ROS), mastoparan forms channels in the mitochondria and MT-21 directly induces the release of cytochrome c from mitochondria. All these factors lead to the opening of mPTP (Watabe et al., 2000; Ding and Nam Ong, 2003; Du and Yan, 2010; Arora et al., 2012). Thus, with the exception of honokiol, none of these compounds induces the mPTP opening directly by binding to a specific component of the multiprotein complex, as we observed with autumnalamide in the present paper. On the other hand, Ca^{2+} experiments reveal that autumnalamide neither modify ionomycin-sensitive intracellular Ca^{2+} pools, nor the ionomycin Ca^{2+} entrance through SOC channels once the influx is activated. Nevertheless, the Ca^{2+} profiles obtained from the present study show that the FFA-induced Ca^{2+} release in SH-SY5Y is inhibited by pre-treatment with FCCP, which collapse the mitochondrial membrane potential that normally drives

Fig. 6. Effect of autumnamide over T lymphocytes cell viability. T lymphocytes cells were incubated for 24 and 48 h with autumnamide at the concentration range from 1 nM to 10 μM. Bar columns represent the different concentrations for each times. Effect on cell viability tested by MTT test. Data are mean ± SEM of 3 independent experiments. Data are mean ± SEM of 3 independent experiments.

Fig. 7. Effect of CsA or autumnamide on IL-2 production in human T lymphocytes stimulated with Con A. Human T lymphocytes were pre-treated for two hours with CsA (0.2 μM) or autumnamide (10 μM) and then with Con A (50 μg mL⁻¹) for 48 h. Mean ± SEM of three experiments. All values are shown in percentage to the Con A treated cells. **Significant differences between autumnamide or CsA treated cells with respect to cells treated with Con A ($p < 0.01$). # Significant differences between Con A treated cells and control ($p < 0.001$). Mean ± SEM of 3 independent experiments.

the Ca^{2+} into the mitochondrion (Collins et al., 2000). The effect of autumnamide over FFA may be achieved through either a protonophore-like effect, similar to FCCP, or the induction of the mPTP, as previously described (Audoin et al., 2014). Thus, in the case of FCCP in SH-SY5Y cells, the uncoupling of the mitochondrial transport chain produced in mitochondria, substantially inhibits Ca^{2+} release induced by FFA probably because both FCCP and FFA act by disrupting mitochondrial membrane potential, and one drug blocks the effect of the other (Chi et al., 2011). Moreover, CsA abolished the effect of autumnamide efficiently in comparison with FCCP on SOC Ca^{2+} influx (Audoin et al., 2014). In accordance with these previous results and due to the effect observed over mPTP by autumnamide, the binding of the prenylated cyclic peptide from the cyanobacterium *Phormidium autumnale* was tested on Cyp D, an essential component of the mPTP. Biosensor data analysis indicates that the compound binds with good affinity to mitochondrial Cyp D. Autumnamide almost shows 3 times more affinity for mitochondrial Cyp D than CsA. Moreover, due to the findings

Fig. 8. Effect of autumnamide or CsA on Ca^{2+} /calmodulin-dependent on Phosphatase Activity of Calcineurin. Phosphatase activity of calcineurin determined after pre-incubation with Cyp A or Cyp A + CsA (6 μM)/autumnamide (10 μM). All values are shown in percentage with respect to Cyp A control. **Significant differences between Cyp A control and Cyp A + CsA ($p \leq 0.01$). ***Significant differences between Cyp A control and Cyp A + autumnamide ($p \leq 0.001$). Data are mean ± SEM of 3 independent experiments.

Fig. 9. Effect of CsA or autumnamide over NFATc1 nuclear levels in human T lymphocytes activated with Con A. A. NFATc1 levels were studied as nuclear fraction after 48 h incubation with compounds. Human T lymphocytes were pre-treated for 2 h with CsA (0.2 μM) or autumnamide (10 μM) and afterward incubated with Con A (50 μg mL⁻¹) for 48 h. A. Representative image of one experiment. B. Mean of the ratio of the nuclear NFATc1/lamin B1 band intensity. All results are presented as the percentage of NFATc1 in the nucleus. ##Significant differences with respect to control cells ($p \leq 0.01$). **Significant differences with respect to Con A treated cells ($p < 0.01$). Data are mean ± SEM of three experiments.

observed by SPR and taking into account the results observed on mPTP besides the uncoupling effect (Audoin et al., 2014), this effect could be related with the interaction between autumnamide and mitochondrial Cyp D in a more specific way than in the case of FCCP or FFA that is produced mainly by the disruption of membrane potential. Cyps share a common domain; therefore compounds that

bind to one of this protein family usually show binding affinity for other CyPs as CsA display (Kumari et al., 2013). Thus, autumnalnamide also shows, as CsA, Cyp A binding affinity. This cytosolic protein plays a central pathway of many physiological and pathological processes in the cells such as inflammation, cardiovascular diseases, immunomodulation or sepsis among others (Nigro et al., 2013). In the case of CsA, once the complex between Cyp A- CsA is formed, calcineurin phosphatase activity is blocked and the translocation of NFAT to the nucleus is avoided, inhibiting the transcription activity over genes that encodes for IL-2 (Fruman et al., 1992). Autumnalnamide reduces calcineurin phosphatase activity, while NFAT was not reduced after the binding to Cyp A, nevertheless, autumnalnamide, as CsA, reduces IL-2 production in T lymphocytes. While CsA exerts its effect through the docking of calcineurin (Naesens et al., 2009), the inhibition of IL-2 produced by autumnalnamide probably occurs partially by a calcineurin-independent pathway since the nuclear entry of the NFATc1 is closely linked to its dephosphorylation and the nuclear levels of this transcription factor are not blocked after autumnalnamide treatment. Molecules that inhibit CRAC channels are also expected to reduce IL-2 production (Sweeney et al., 2009; Djuric et al., 2000). As FFA or BTP2, two compounds that modulate SOC channels, act by reducing the production of this IL in white blood cells, being Ca^{2+} an essential signal for its production (Kankaanranta et al., 1996; Cardenas and Heitman, 1995). In the case of autumnalnamide, the indirect blockade produced on SOC channels, the main Ca^{2+} entrance in T cells, due to the effects that produce in mitochondrion, could explain the effect over IL-2 inhibition, although it does not block NFAT translocation. Then, other transcription factors or mitogen-activated protein kinases (MAPK) could be related with the modulation of interleukins (ILs) by autumnalnamide where the restriction of cytosolic Ca^{2+} induced by the compound may be essential (Audoin et al., 2014; Cardenas and Heitman, 1995; Kar et al., 2011). On the other hand, autumnalnamide can be a useful tool to study molecules that targeted Cyp D, other applications could be attributed to autumnalnamide due to the binding to Cyp A. This effort mainly led to the development of compounds with immunosuppressive or anti-inflammatory effects.

As a conclusion, we have a drug that has good affinity for two important immunophilins engaged in many diseases and produces an effective blockade on IL-2 production and low toxicity. Due to the multi-functional properties of Cyp A and the functions where it is involved, such as protein folding, trafficking, assembly or cell signaling, autumnalnamide could open a new window to improve the treatment of many diseases where Cyp A is a key player (Nigro et al., 2013). With respect to Cyp D, the fact that mPTP is a process regulated by a wide type of stimulus, it makes this multiproteic complex an ideal pharmacological target implicated in multiple processes, where autumnalnamide directly binds to (Tornerio et al., 2002).

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The research leading to these results has received funding from the following FEDER cofounded-grants. From CDTI and Technological Funds, supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01, AGL2014-58210-R, and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016, and through Axencia Galega de Innovación, Spain, ITC-20133020 SINTOX. From CDTI under ISIP Programme, Spain, IDI-20130304APTAFOOD.

From the European Union's Seventh Framework Programme managed by REA – Research Executive Agency (FP7/2007-2013) under grant agreement 312184PHARMASEA. We wish to thank the Clínica Losada Arránz, especially Ms. Paula López Arránz for providing the human blood samples for T cells purification. Jon Andoni Sánchez is supported by a fellowship from Plan Galego de Investigación e Crecemento, Xunta de Galicia, Spain.

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