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Original Paper

Spongionella Secondary Metabolites Regulate Store Operated Calcium Entry Modulating Mitochondrial Functioning in SH-SY5Y Neuroblastoma Cells

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Key Words
Calcium • SOC Channels • Mitochondria • Cyclophilin D • Cyclosporine A • *Spongionella* sp.

Abstract
Background/Aims: The effect of four secondary metabolites isolated from sponge *Spongionella*, gracilins H, A, L and tetrahydroaplysulphurin-1 on Calcium ion (Ca^{2+}) fluxes were studied in SH-SY5Y neuroblastoma cells. ***Methods and Results:*** These compounds did not modify cytosolic baseline Ca^{2+}-levels. Nevertheless, when cytosolic Ca^{2+}-influx through store operated calcium channels (SOC channels) was stimulated with Thapsigargin (Tg), a strong inhibition was observed in the presence of gracilin A, gracilin L and tetrahydroaplysulphurin-1. Since these compounds were able to protect mitochondria from oxidative stress, the role of this organelle in the Ca^{2+}-influx inhibition was tested. In this sense, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and Cyclosporine A (CsA) were used. Surprisingly, both the inhibitory effect over Tg-sensitive stores and Ca^{2+} influx through SOC channels produced by FCCP were abolished with different potencies by *Spongionella* compounds in a similar way than CsA. CsA is able to avoid Mitochondrial Permeability Transition Pore (mPTP) opening. As well as CsA, Spongionella compounds reverted mPTP opening induced by FCCP. In the case of CsA the mPTP blockade is due to the direct binding to Cyclophilin D (Cyp D), a mitochondrial matrix protein. This association was also observed between gracilin L and tetrahydroaplysulphurin-1 and Cyp D. Therefore, Spong*ionella* compounds modulate mitochondrial activity by preventing mPTP opening by binding to CypD. ***Conclusions:*** These effects make *Spongionella* compounds as new family of compounds with promising activity in human diseases where mitochondrial alterations are implicated.

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Introduction

Ca^{2+} homeostasis is strictly regulated in animal cells. Small changes in intracellular Ca^{2+} levels can produce a broad range of intracellular effects in the cellular machinery [1]. In resting conditions, the extracellular Ca^{2+} level is 1 mM while in the cytosol is 100 nM aprox, depending on the cellular model. These differences between the extracellular medium and the cytosol can be reached because of the low Ca^{2+}-permeability of the plasma membrane and by the narrow relationship between pumps, channels, exchangers and binding proteins located in this membrane. The adjustments of cytosolic Ca^{2+} levels are regulated by the entry from the extracellular media through voltage-gated calcium channels and through the Store Operated Calcium Channels (SOC channels) and by different intracellular binding proteins and organelles like mitochondria or the endoplasmic reticulum (ER) [2]. Of those organelles, mitochondria are indispensable on cellular Ca^{2+} regulation through SOC channels. In this way, when Ca^{2+} levels are increased in the cytosol, due to SOC channels opening, mitochondria are able to capture the ion to maintain cytosolic normal levels. Otherwise, Ca^{2+} is released from this organelle when cytosolic levels are decreased. Thus, mitochondrion has three transport systems that regulate the fluxes of Ca^{2+}. On one hand, the Ca^{2+}uniporter, which is driven by the negative charge of the mitochondrial membrane potential (MMP) and introduces Ca^{2+} to the mitochondrion, and on the other hand Na^{+}/Ca^{2+} and Ca^{2+}/H^{+} exchangers responsible for Ca^{2+} efflux to cytosol [3, 4]. In addition, there is an alternative mechanism in the mitochondria to regulate Ca^{2+} concentrations, when Ca^{2+} levels exceed the buffering capacity of the inner membrane exchangers, known as mitochondrial permeability transition pore (mPTP) [3]. Moreover, the immunophilin Cyclophilin D (Cyp D) is a very important structure located in the mitochondrial matrix at physiological conditions [5, 6]. Under pathophysiological situations, when a stimulus produces cellular stress in the cell, such as reactive oxygen species (ROS) or any MMP alteration or elevated cytosolic Ca^{2+} levels steadily, Cyp D translocates from the physiological location to the inner mitochondrial membrane. In this location, Cyp D is part of the multiproteic complex of the mPTP. The complex, besides the Cyp D, is composed by other structures such as hexokinase, voltage-dependent anion channel (VDAC) or the adenine nucleotide translocator (ANT) [3]. The whole structure is identified by a sudden increase in the inner mitochondrial membrane permeability. The formation of the mPTP produces the loss of ion homeostasis and proton motive force required for ATP production [7]. This multiproteic complex can be pharmacologically modulated. Cyclosporine A (CsA) is a cyclic peptide of fungal origin and it is one of the most relevant drugs for its affinity for different immunophilins located in different cellular regions [8]. This immunosuppressant drug also has effect on mitochondrial immunophilin Cyp D by decreasing the formation of mPTP [9]. And mPTP deregulation is related with an increase of cell death. Due to this, the compounds that bind to Cyp D structure, could constitute an interesting tool for the treatment of many diseases where mitochondria are implicated, such as ischemic insults, muscular dystrophies, multiple sclerosis, amyotrophic lateral sclerosis and Alzheimer's disease (AD) while others [10].

Sponges are aquatic metazoan with a large distribution due to the their ability to adapt to different environments [11]. These marine organisms produce as self-protection a wide type of bioactive compounds with very interesting mechanisms of action and therefore attractive as potential drugs [12]. These marine bioactive compounds include molecules with many pharmacological effects, i.e. anti-inflammatory and immunosuppressive, anticoagulant, antibacterial, antifungal or anticancer [13]. In this sense, bisnorditerpene gracilin H, norditerpene gracilin A, 12-hydroxy derivative gracilin L and the diterpenoid 3´-norspongionlactone tetrahydroaplysulphurin-1 are secondary metabolites isolated from the marine sponge *Spongionella* with unusual structural characteristics and unknown mechanism of action [14]. In previous works these *Spongionella* compounds were described as neuroprotective modulating mitochondrial functioning and preserving neurons against oxidative damage [15]. The neuroprotection against oxidation conditions suggested that these metabolites could be interesting lead candidates in drug development for

neurodegenerative diseases [16]. The aim of this work was to further study the effect of these bioactive compounds in cytosolic Ca^{2+} fluxes and the role of mitochondria through the mPTP complex in this effect.

Material and Methods

Source of Natural compounds

The library of compounds was provided by the Marine Biodiscovery Centre (Department of Chemistry, University of Aberdeen), from which four secondary metabolites of *Spongionella* sp., gracilin H, A, L and tetrahydroaplysulphurin-1 were chosen to develop the experiments. Compounds were purified from their sponge sources, which were freeze-dried, and extracted with MeOH and $MeOH/CH_2Cl_2$ to obtain a crude extract. The crude extract of each organism was dissolved in H_2O and passed through Diaion HP20 resin and re-concentrated under vacuum conditions to obtain a salt-free extract. This extract was subjected to multiple steps of liquid/liquid fractionation and SiO_2, Sephadex LH-20 and RP-C18 chromatography to obtain the pure compounds. The structure elucidation of these compounds was based on their High-resolution electrospray ionisation mass spectrometry analysis as well as direct comparison with the previously reported Nuclear Magnetic Resonance (NMR) spectral data [14].

Chemicals

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), sodium acetate, glycine-HCl and amine coupling kit (1-ethyl-3- (3-dimetylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and ethanolamine-HCl were supplied by BiacoreAB (Uppsala, Sweden). Bovine Serum Albumin (BSA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and Cyclosporine A (CsA) and other chemicals were reagent grade and purchased from Sigma Chemical Co. (Madrid, Spain). FURA-2 acetoxymethyl (AM) ester was obtained from Molecular Probes (Leiden, The Netherlands). Thapsigargin (Tg) and ionomycin were from Alexis Corporation (Läufelfingen, Switzerland). MitoProbe™ Transition Pore Assay Kit was purchase from Invitrogen. Active human Cyclophilin D (Cyp D) full-length protein was from Abcam (Cambridge, UK).

Cell culture

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% foetal bovine serum (FBS) (Gibco) plus 100 UI/mL penicillin and 100 µg/mL streptomycin. The neuroblastoma cells were expanded weekly using 0.05% trypsin/EDTA (1x) (Invitrogen).

Measurements of cytosolic free calcium

Cells were seeded onto 18-mm glass cover slips and used between 48-72 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, Mg (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.

mPTP opening measurements

The MitoProbe™ Transition Pore Assay Kit was used to measure the mPTP opening. Calcein-AM dye and $CoCl_2$ as quencher are included in the kit. Cells were loaded for 1 h with 10 nM of calcein-AM at 37ºC and 300 rpm in the presence of 1 mM $CoCl_2$ to quench cellular calcein fluorescence except the fluorescence into the mitochondrial matrix. The solution employed for cell load was saline solution (Umbreit) plus BSA 0.1%. Loaded cells were washed with saline solution and kept on ice until use. Fluorescent measures were done in a confocal laser-scanning microscope Nikon D- Eclipse C1 by using the 488 nm lasers for excitation and 515 nm emission filters. The images were collected using 40 x oil immersion objective (Nikon). Clusters of mitochondria were selected as regions of interest. Sequential digital images were acquired every 30 seconds (s) during 15 min and the fluorescence was represented as % of variation respect to control cells.

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 µL min^{-1} and 25°C. CM5 sensor chips were used as surface where CypD 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 µg/mL of active human Cyp D protein dissolved in sodium acetate 10 mM at pH 4.5 or 6 was added to be immobilized over a CM5 sensor chip. Finally ethanolamine-HCl was injected to deactivate the remaining active esters. Next, analytes were added to check the binding between them and Cyp D. Once analytes were tested and interaction was observed, individual binding curves were analyzed by determining the kinetic constants of analytes-Cyp 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, $K_{diss}/K_{ass} = K_D$. The pseudo-first-order association rate constants K_{obs} (s^{-1}) were determined for each compound concentration 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 each compound was done. These plots follow a linear correlation coefficient. From the equation of these representations, K_{ass}, M^{-1} 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 each analyte-Cyp D binding was obtained.

The duration of the sample injection was 2 min at 10µL 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 D interactions.

Statistical analysis

In each experiment, 30 cells were used. 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. A probable level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM of three or more experiments.

Results and Discussion

Spongionella compounds were reported to be active against hydrogen peroxidation in primary cortical neurons through an effect closely linked to mitochondrial dysfunction [15]. Ca^{2+}dyshomeostasis is a key factor in the development of pathophysiological disorders such as neurodegenerative, autoimmune or inflammatory diseases, among others [9, 17, 18]. Mitochondrion plays an important role in the intracellular Ca^{2+} homeostasis. In this paper we study the effect of four secondary metabolites obtained from the sponge *Spongionella*, gracilins H, A, L and tetrahydroaplysulphurin-1 (Fig. 1) over cytosolic Ca^{2+} levels in the human SH-SY5Y neuroblastoma cell line. First, the direct effect of these compounds was checked. As shown in Fig. 2A-D, at 1 µM none of these compounds exhibited any effect

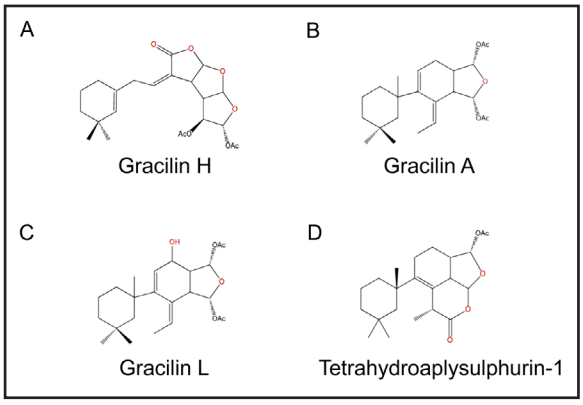

Fig. 1. *Spongionella* compound structures. Gracilin H (A), gracilin A (B), gracilin L (C) and tetrahydroaplysulphurin-1 (D).

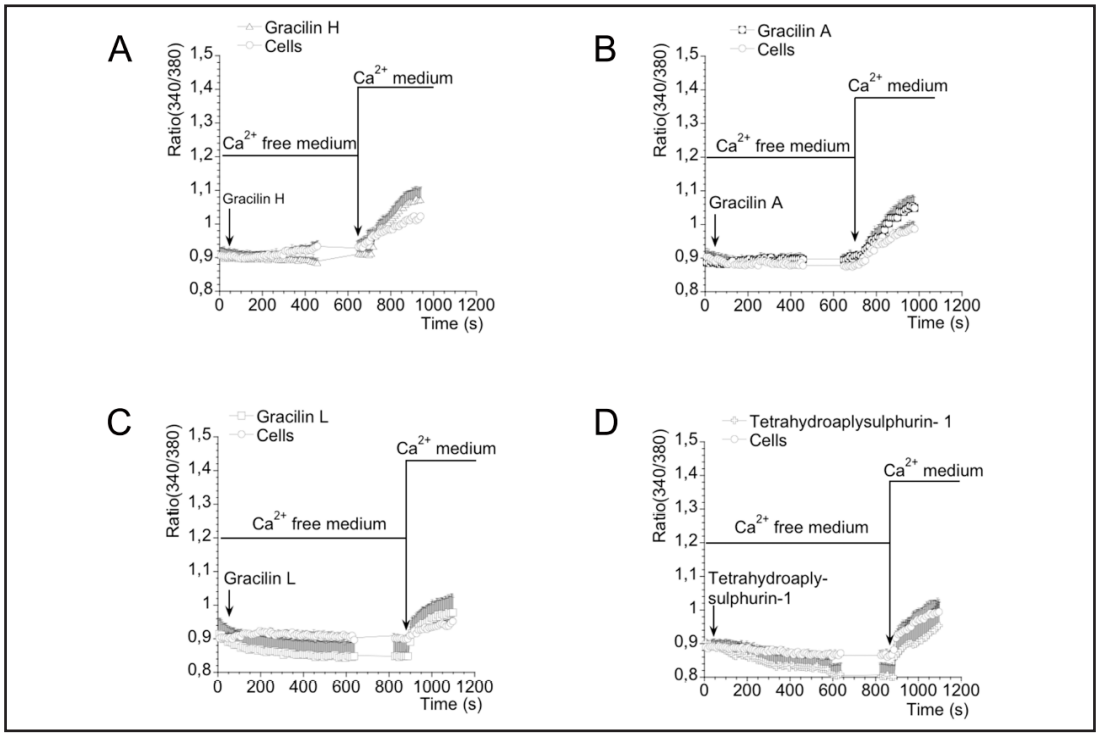

Fig. 2. Effect of *Spongionella* compounds on cytosolic Ca^{2+} profile of SH-SY5Y neuroblastoma cells. Cytosolic Ca^{2+} profiles of cells treated with 1 µM of gracilin H (A), 1 µM of gracilin A (B), 1 µM of gracilin L (C) or 1 µM of tetrahydroaplysulphurin-1 (D) in a Ca^{2+}-free medium. The arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. Mean ± SEM of three experiments.

on the basal levels of cytosolic Ca^{2+}, not even when 1 mM of Ca^{2+} was restored to the bath solution. Lower and higher concentrations of compounds were tested, and no effects were observed (data not shown). Then, we checked the effect of these compounds over SOC entry, using Tg to activate Ca^{2+}-influx through SOC channels. Tg is a sesquiterpene lactone that inhibits the Ca^{2+}-ATPase from endoplasmic reticulum (ER) and induces Ca^{2+}-pools depletion. According to the store-operated model, ER depletion induces the subsequent external Ca^{2+}-influx through SOC channels in the plasma membrane [19, 20]. The effect of compounds was tested before the depletion of the Tg-dependent stores. As shown in Fig. 3A, gracilin H produces a significant decrease on Tg-sensitive stores depletion, however when 1 mM Ca^{2+} is restored to the medium Ca^{2+}-influx was not modified. On the contrary, gracilin A and L did not modify pools depletion, but after the addition of 1 mM Ca^{2+}, a significant and sustained reduction on Tg-induced Ca^{2+}-influx, 34% ± 0.028% and 45.5% ±

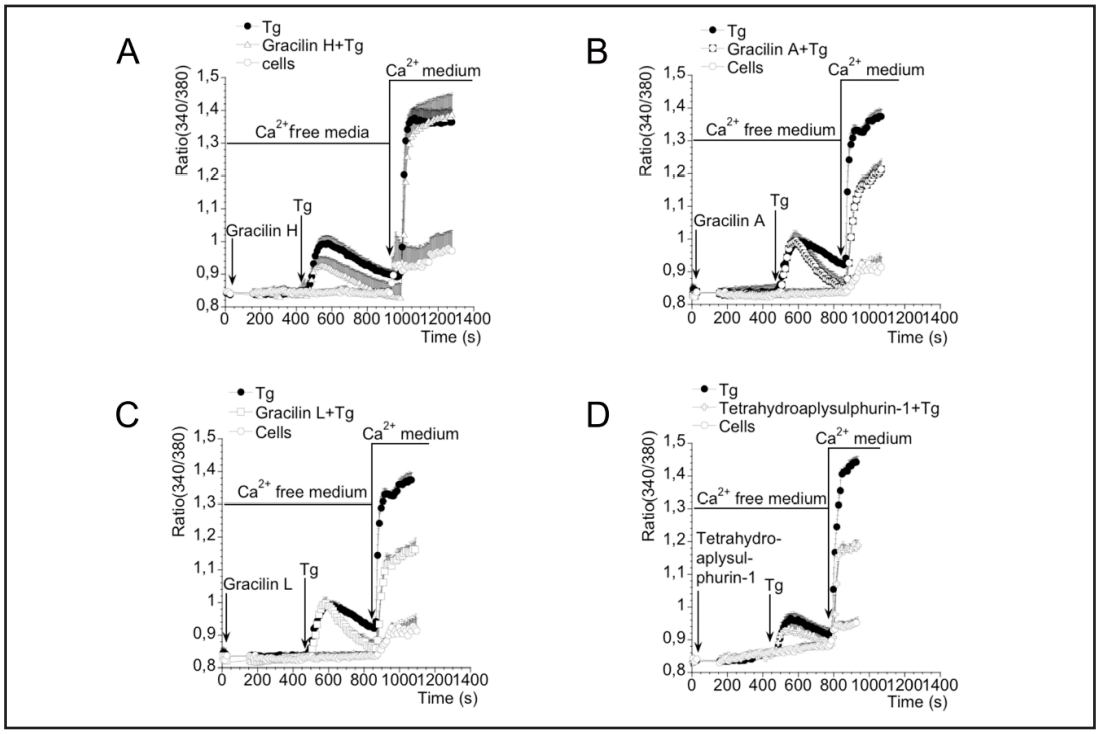

Fig. 3. Effect of *Spongionella* compounds and Tg on cytosolic Ca^{2+} profile of SH-SY5Y neuroblastoma cells. Cytosolic Ca^{2+} profiles of cells first preincubated with 1 μM gracilin H (A), 1 μM gracilin A (B), 1 μM gracilin L (C) or 1 μM tetrahydroaplysulphurin-1 (D) and then with 2 μM Tg. First arrow indicates the addition of *Spongionella* compounds and second arrow indicates Tg addition. Ca^{2+} (1 mM) was restored to the extracellular medium in the third arrow. Mean ± SEM of three experiments.

0.022% respectively, was observed (Fig. 3B and 3C). Similarly, tetrahydroaplysulphurin-1 did not elicit any modification on the depletion of Tg-sensitive pools, but a 40% 0.017% reduction of Ca^{2+}-influx was observed after addition of 1 mM Ca^{2+} (Fig. 3D). Lower and higher concentrations of each compound were tested, and while lower concentrations did not show any effect, higher concentrations did not produce a larger effect than that reported with 1 μM (data not shown). Therefore *Spongionella* compounds significantly inhibited SOC entry and modulated Tg-dependent stores, although these effects surprisingly seemed not be related. To rule out if the inhibitory effect on Ca^{2+}-influx of these compounds was separate from ER effect, compounds were incubated after Tg-stores depletion. In these conditions, while gracilin H did not significantly modify cytosolic Ca^{2+} entrance, Fig. 4A, a significantly decrease in Ca^{2+}-influx was observed when gracilin A, L and tetrahydroaplysulphurin-1 were added, 23.2% 0.028%, 21.9% ± 0.011% and 19.6% ± 0.017% reduction respectively (Fig. 4B, 4C and 4D). Thus, with the exception of gracilin H, *Spongionella* compounds inhibited SOC entry after Tg-sensitive stores depletion, although a stronger inhibition of SOC entry was observed when the incubation was done before Tg-sensitive stores depletion. In this way, only gracilin H was able to reduce Tg-sensitive stores without affecting SOC influx, and the rest of compounds did not produce any direct effect on the ER; therefore the interaction with other cellular structure engaged in the modulation of SOC channels should be suspected.

Mitochondria act as Ca^{2+}-buffer to protect SOC entry against feedback inhibition and ensure the physiological lasting maintenance of SOC influx signaling [21-23]. Therefore, since *Spongionella* compounds had some effect over these organelles [15, 16], it was important to clarify to which extent the inhibitory effect on SOC fluxes of these compounds was influenced by mitochondrion. To do this FCCP and CsA were used. FCCP is a well-documented mitochondrial uncoupler able to depress Ca^{2+}-entry through SOC channels and also to induce ROS production. This effect is due to MMP alteration that leads into the

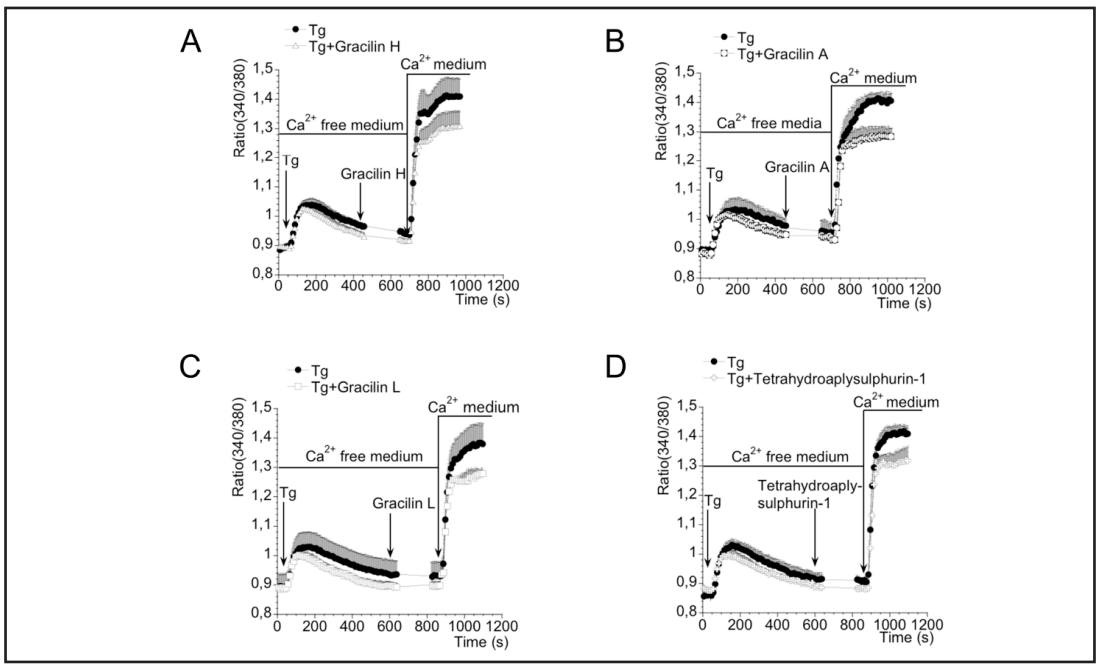

Fig. 4. Effect of *Spongionella* compounds after Tg-stores depletion on cytosolic Ca^{2+} levels of SH-SY5Y neuroblastoma cells. Cytosolic Ca^{2+} profiles of cells first incubated with 2 μM Tg (first arrow) and then with 1 μM of gracilin H (A), gracilin A (B), gracilin L (C) or tetrahydroaplysulphurin-1 (D) (second arrow). The third arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. Mean ± SEM of three experiments.

opening of the mPTP in some cellular models [22, 24-28]. Therefore the effect of FCCP over SOC channels in human SH-SY5Y neuroblastoma cell line was checked. As Fig. 5A shows, FCCP incubation produced a small increase in FURA-2 ratio due to the release of Ca^{2+} from mitochondrion. This effect was already described in other cellular models [29]. Once Tg was added, a significant reduction in Ca^{2+}-stores depletion was observed. Thus, when the ion was replaced in the medium, a sustained reduction of 65.2% ± 0.0075% in Ca^{2+} was shown as compared with Tg control. CsA is an immunosuppressant drug able to keep MMP and to avoid mPTP opening due to the direct binding to Cyp D working in opposite way to FCCP [30, 31]. Thus, the effect of CsA over SOC influx was also studied. As Fig. 5B shows, CsA produces a significant decrease in Tg-sensitive stores depletion, a 28.75% ± 0.017% on SOC influx compared with the Tg control. It is noted that CsA inhibits inositol triphosphate receptors from ER, and in consequence the ER emptying induced by Tg is smaller and the subsequent Ca^{2+} entrance through SOC channels is decreased [30, 32]. Therefore, in the same way than *Spongionella* compounds, both CsA and FCCP decrease the Ca^{2+}-influx induced by Tg. Since these drugs have opposite effects on mitochondria, the combined effect of both compounds was checked. As Fig. 6A shows, when CsA was added before FCCP and then Tg, no effect on cytosolic Ca^{2+} profile was observed. Similarly (Fig. 6B), the incubation with FCCP before CsA followed by Tg addition showed no effect on either pools depletion or SOC entry. Therefore, mitochondria have an important role in Tg-pools depletion and in the resulting SOC entry related with mPTP opening in human SH-SY5Y neuroblastoma cell the same than in other neuronal models [33]. In this context the involvement of mitochondria on the effect of *Spongionella* compounds on cytosolic Ca^{2+} levels was checked. Compounds were incubated before FCCP and followed by Tg addition. As Fig. 7A shows, the effect of FCCP on Tg-sensitive stores was inhibited by gracilin H. Once Ca^{2+} was restored to the medium, the reduction of Ca^{2+}-influx produced by FCCP was also inhibited. Fig. 7B shows that the incubation with gracilin A before FCCP addition inhibits the effect on Tg-sensitive Ca^{2+}stores depletion and

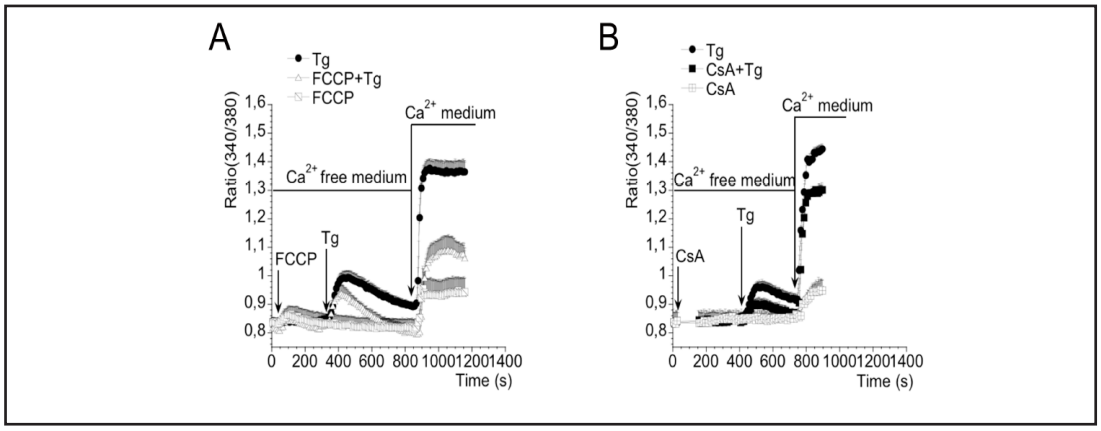

Fig. 5. Effect of FCCP or CsA and Tg on cytosolic Ca^{2+} profile of SH-SY5Y neuroblastoma cells. (A) Cytosolic Ca^{2+} profiles of cells first preincubated with 10 μM FCCP and then incubated with 2 μM Tg. First arrow indicates the addition of FCCP and second arrow indicates the addition of Tg. The third arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. (B) Cytosolic Ca^{2+} profiles of cells first preincubated with 0.2 μM of CsA and then with 2 μM of Tg. First arrow indicates the addition of CsA. Second arrow indicates the addition of Tg. The third arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. Mean ± SEM of three experiments.

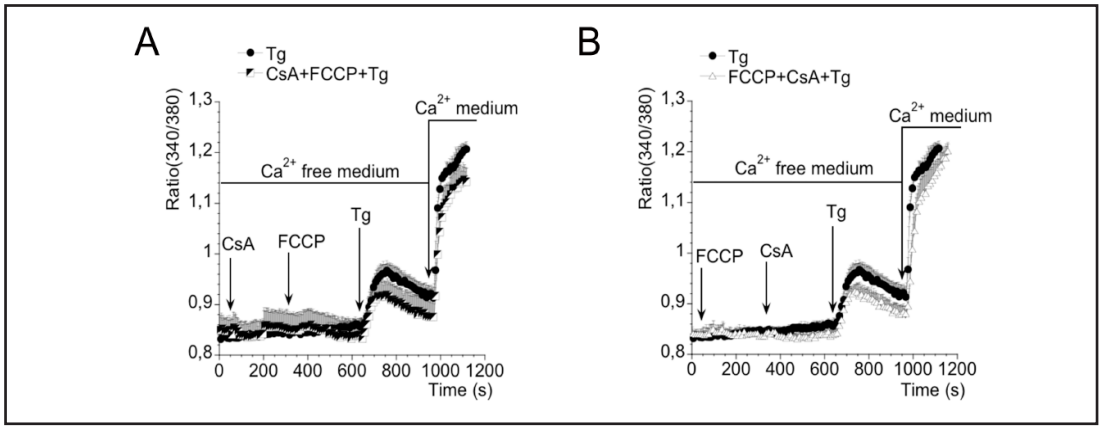

Fig. 6. Effects of coincubation of CsA and FCCP plus Tg on cytosolic Ca^{2+} profile of SH-SY5Y neuroblastoma cells. (A) Cytosolic Ca^{2+} profiles of cells first preincubated with 0.2 μM CsA, then with 10 μM FCCP and finally with 2 μM Tg. First arrow indicates the addition of CsA, second indicates the addition of FCCP and third arrow indicates the addition of Tg. The fourth arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. (B) Cytosolic Ca^{2+} profile in cells first preincubated with 10 μM FCCP followed by 0.2 μM CsA and then with 2 μM Tg. First arrow indicates the addition of FCCP, second indicates the addition of CsA and third arrow indicates the addition of Tg. The fourth arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. Mean ± SEM of three experiments.

when 1 mM of Ca^{2+} was added to the bath solution, the effect of FCCP over SOC entry was partially inhibited since there is an increase of 24.4% ± 0.015% in Ca^{2+}-influx in comparison with the FCCP effect. In the case of gracilin L, Fig. 7C, Tg-pools depletion reached similar levels than Tg control, and the compound fully inhibited FCCP effect over Ca^{2+}-influx. The same effect was observed with tetrahydroaplysulphurin-1, Fig. 7D. This compound reversed the effects over Tg-stores induced by FCCP and also on Ca^{2+}-influx. In summary, the same than CsA, all *Spongionella* compounds were able to inhibit FCCP effects over Tg-sensitive Ca^{2+}stores and SOC entry. Similarly, when FCCP was added before *Spongionella* compounds the same effect was observed (data not shown). Therefore, *Spongionella* compounds have similar effects than CsA and FCCP on cytosolic Ca^{2+} levels. Thus, all compounds with the

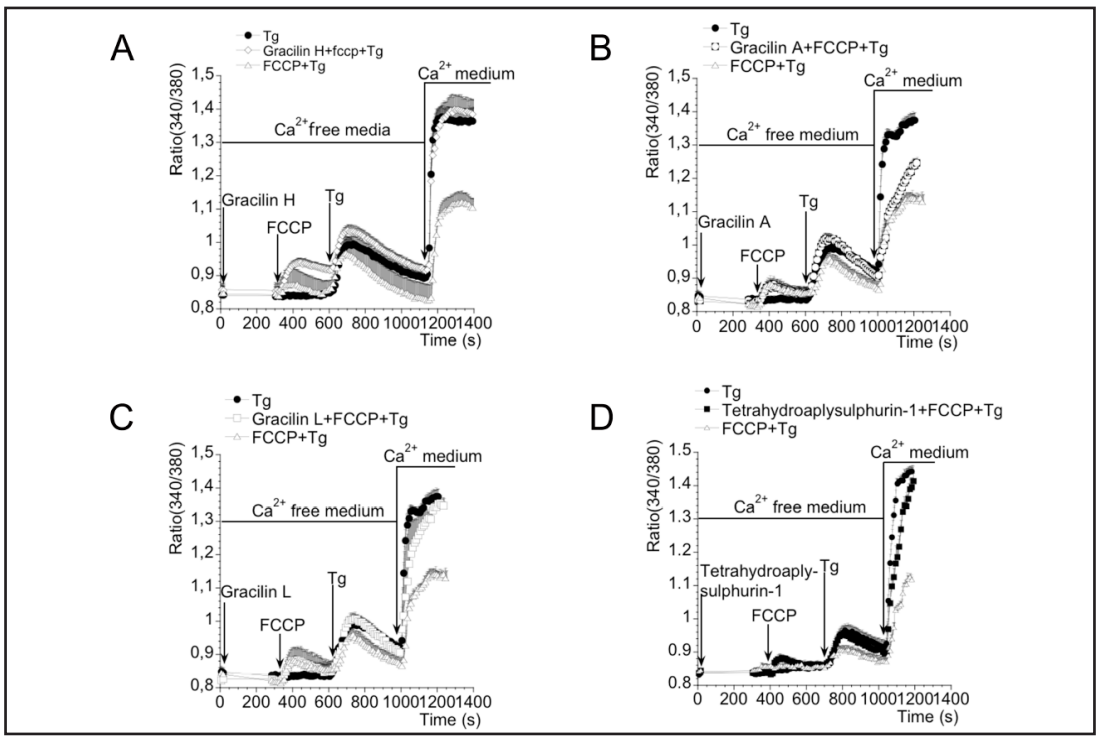

Fig. 7. Effect of *Spongionella* compounds, FCCP and Tg on cytosolic Ca^{2+} profile of SH-SY5Y neuroblastoma cells. Cytosolic Ca^{2+} profile of cells first preincubated with 1 μM gracilin H (A), gracilin A (B), gracilin L (C) and tetrahydroaplysulphurin-1 (D), then with 10 μM FCCP and finally with 2 μM Tg (third arrow). The fourth arrow indicates the addition of 1 mM of Ca^{2+} to the bath solution. Mean ± SEM of three experiments.

exception of gracilin A were able to maintain SOC entry with similar effectively than CsA. Regarding the inhibition of SOC entry, gracilin H, gracilin L and tetrahydroaplysulphurin-1 were able to fully reverse the effect induced by the protonophoric uncoupler.

CsA was described to inhibit FCCP effect due to the binding of Cyp D, avoiding the formation of the mPTP and maintaining the normal SOC entry [22, 34]. To clarify if *Spongionella* compounds have this effect and directly modulate mPTP, we used calcein-AM and $CoCl_2$ as quencher. In healthy cells, the dye, but not the quencher, enters the mitochondria that remains brightly fluorescent until mPTP activation triggers the fluorescence quenching. Thus, when mPTP is formed mitochondrial calcein fluorescence decreases. As Fig. 8A shows, a 30% ± 6.30% decrease in calcein fluorescence was observed in the presence of FCCP, while no modification in calcein fluorescence was observed when CsA was added. In cells incubated with CsA before FCCP, no changes in fluorescence were registered, Fig. 8A. That is, mPTP was formed in the presence of FCCP whereas with CsA this activation was avoided. The same effects were observed by using *Spongionella* secondary metabolites. The compounds did not induce any effect in calcein fluorescence but the formation of mPTP in the presence of FCCP was also avoided (Fig. 8B-E). Thus, as for CsA, *Spongionella* compounds inhibited the mPTP opening induced by FCCP. Moreover, in previous papers *Spongionella* compounds were described to preserve neurons against oxidative damage and to block apoptosis markers production, caspase-3, in mouse cortical neurons [15]. These results are in accordance with the effects observed by these compounds over mPTP. Probably, due to the early inhibition of mPTP, the apoptotic signaling pathways are blocked, since this pore is a key step in the intrinsic apoptotic pathway [35].

On the other hand, CsA was able to reduce SOC entry activated by Tg but also CsA was able to revert the inhibitory effect of FCCP in this Ca^{2+}-influx [32, 36]. In addition, in mouse neuronal 2a transgenic AD cell model, CsA was able to inhibit the SOC entry reduction due to the inhibition of mPTP formation [22]. In addition, it is well documented that CsA binds to

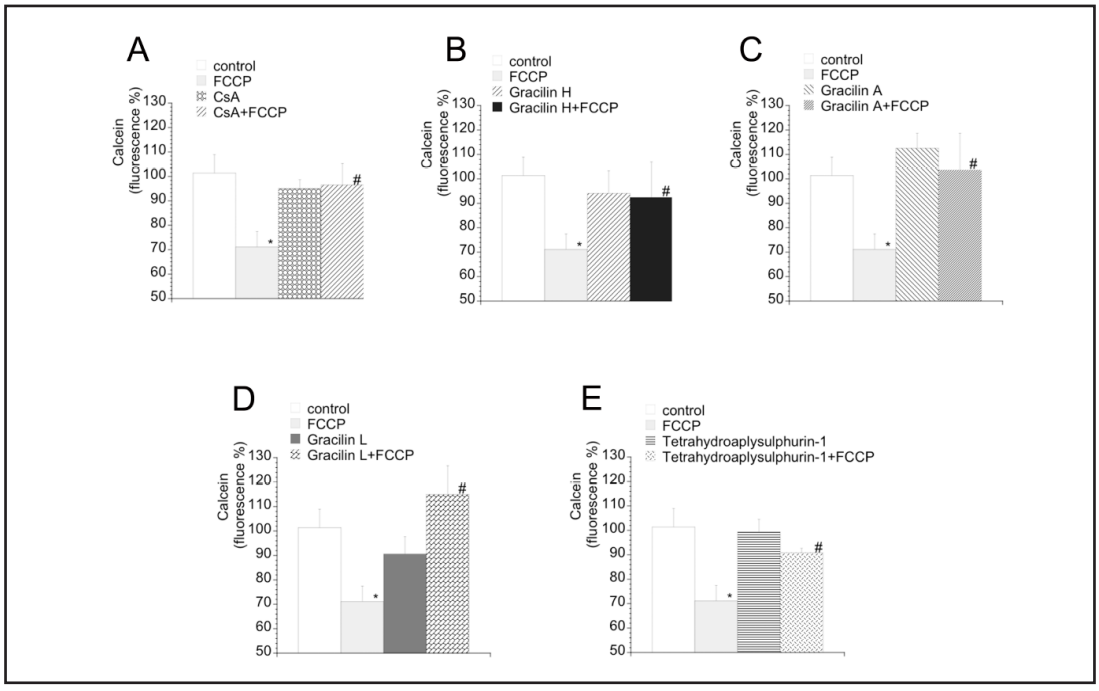

Fig. 8. Effect of FCCP, CsA and *Spongionella* compounds on mPTP opening in SH-SY5Y neuroblastoma cells. (A) Changes in calcein fluorescence in the presence of 10 μM FCCP, 0.2 μM CsA, and 0.2 μM CsA plus 10 μM of FCCP. (B) Changes in calcein fluorescence in the presence of 10 μM FCCP, 1 μM gracilin H and 1 μM gracilin H plus 10 μM FCCP. (C) Changes in calcein fluorescence in the presence of 10 μM FCCP, 1 μM gracilin A and 1 μM gracilin A plus 10 μM FCCP. (D) Changes in calcein fluorescence in the presence of 10 μM FCCP, 1 μM gracilin L, and 1 μM gracilin L plus 10 μM of FCCP. (E) Changes in calcein fluorescence in the presence of 10 μM FCCP, 1 μM tetrahydroaplysulphurin-1, and 1 μM tetrahydroaplysulphurin-1 plus 10 μM of FCCP. Mean ± SEM of three experiments. * significant differences between FCCP treated cells and untreated cells (control). # significant differences between FCCP treated cells and cells coincubated with *Spongionella* compounds and FCCP.

Cyp D and in consequence inhibits the formation of mPTP opening [22, 37]. Therefore, since *Spongionella* compounds shown the same effect than CsA, Cyp D was checked as cellular target for them. The binding between Cyp D and *Spongionella* compounds was studied by using a Biacore X SPR biosensor. Cyp D was used as ligand attached to the sensor surface and *Spongionella* compounds or CsA (positive control) were used as ligate. As Fig. 9A shows, after the activation of the amine groups by EDC-NHS addition, 100 μg mL^{-1} of human Cyp D dissolved in sodium acetate pH (4.5) were added to the sensor chip. Under these conditions, a typical covalent binding curve was obtained. Then, the CM5 chip surface was washed with HBS-EP buffer flow and no fall in the signal was observed, indicating Cyp D was strongly immobilized onto the sensor chip surface. Next, different concentrations of CsA were added at 25°C, by using HBS-EP as running buffer at flow rate of 10 μL min^{-1}. In this case, typical association dose-dependent curves were observed. As Fig. 9B shows, in the presence of 5 μM of CsA the signal was 10.6 RU, while in the presence of 40 μM of CsA the response reached 110.8 RU. The individual binding curves were analyzed, as described in material and methods, and K_{obs} for each CsA concentration was obtained. The representation of K_{obs} *versus* CsA concentration fitted a linear regression with a correlation coefficient of r=0.979, Fig. 9C. From this representation the value of K_D for CsA-Cyp D binding was obtained, 4.5 ± 3.05 μM CsA. After setting up these conditions with CsA we studied the binding of Cyp D-*Spongionella* compounds. In this case, different concentrations were dissolved in buffer and added onto the immobilized Cyp D. While gracilin H and gracilin A did not show binding affinity to this protein, association curves for gracilin L and tetrahydroaplysulphurin-1 were

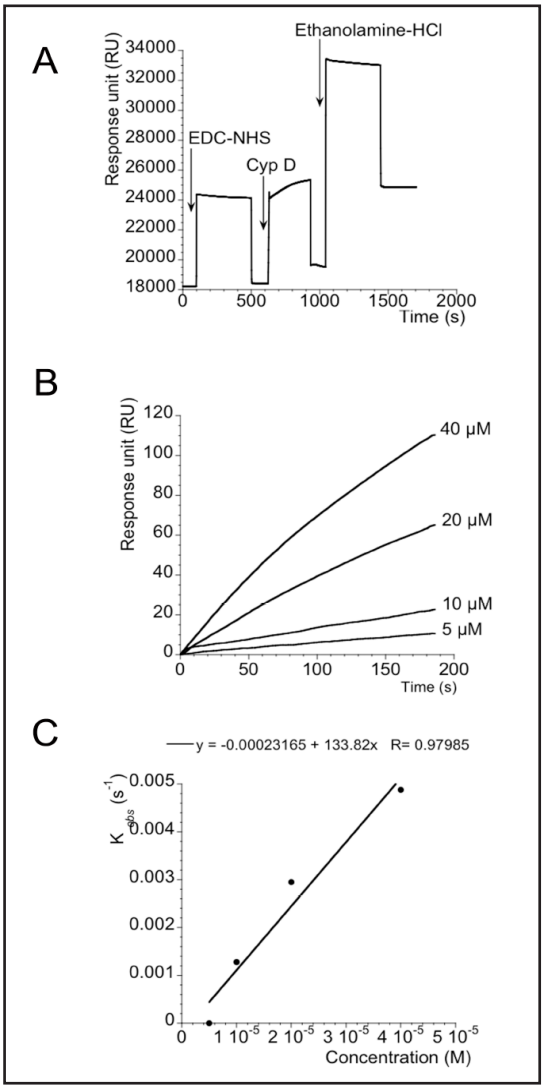

Fig. 9. CM5 dextran chip activation, Cyp D immobilization, CsA association curves and ligand binding analysis. (A) Activation: first arrow shows the addition of EDC/NHS to activate CM5 dextran surface. Immobilization: the second arrow shows the addition of 100 µg mL^{-1} Cyp D. Blocking: the third arrow indicates the blocking of remaining activated sites with ethanolamine/HCl. (B) 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 µg mL^{-1}. The association curves were obtained after subtraction of their respective solvent control. (C) Analysis of ligand binding: kinetic plot of apparent association rate constant K_{obs} (s^{-1}) obtained from plot in B (calculated by BiaEvaluation software) *versus* Cyp D concentration. Representative experiment of n=3.

obtained. As Fig. 10 A and C shows, after 200 s, responses from 4 to 16 RU and 2 to 16 RU respectively were observed. K_{obs} for each curves was obtained and represented against the corresponding concentration of compound obtaining a linear regression with a correlation coefficient of r = 0.9961 and r = 0.9959 respectively (Fig. 10B and D). From these representations the kinetic equilibrium constants for Cyp D-gracilin L/tetrahydroaplysulphurin-1 binding were obtained, being K_D=17.9 ± 5 µM for gracilin L and K_D=30.6 ± 1 µM for tetrahydroaplysulphurin-1. Therefore as happens with CsA, gracilin L and tetrahydroaplysulphurin-1 bind to Cyp D while gracilin H and A, despite inhibiting mPTP opening, did not exhibit a direct binding to Cyp D. In order to improve the affinity of Cyp D by *Sponginella* compounds we investigated other pH immobilization values close to the isoelectric point (pI) of Cyp D. When Cyp D was dissolved in pH 6 sodium acetate and then immobilized onto the sensor surface, K_D for CsA-Cyp D binding was highly decreased, 3.05 ± 0.1 nM CsA, however in these conditions no associations were observed when *Spongionella* compounds were checked. Our data proved that gracilin L and tetrahydroaplysulphurin-1, the same as CsA, directly bind to mitochondrial Cyp D, a critical part of the structure of mPTP and as consequence its formation is inhibited. Thus, mPTP regulation produced by these two compounds avoid the impaired mitochondrial buffering capacity, which efficiently allows the Ca^{2+} uptake to maintain SOC entry from feedback inhibition produced by FCCP in SH-SY5Y cells. Multiple mechanisms may explain the modulation of mPTP [3]. However, in the case of gracilin H and gracilin A the interaction with other unidentified structure would be related with the reversion of mPTP induced by FCCP. Gracilin L and tetrahydroaplysulphurin-1 target Cyp D and avoid FCCP-alterations at mitochondrial level, maintaining the mitochondrial normal functioning and consequently a normal SOC entry, as in the case of CsA. However, based only in their structure is difficult to conclude this effect over Cyp D. In comparison with gracilin A, the presence of an hydroxyl group in gracilin L could play an important role in the interaction with Cyp D. Furthermore,

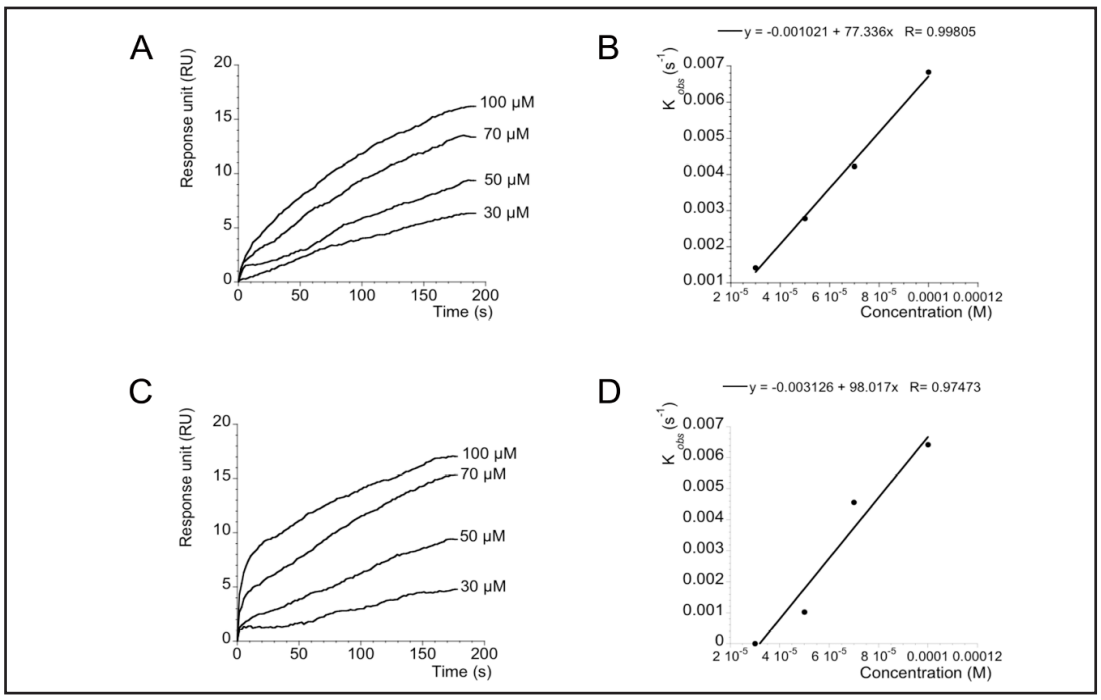

Fig. 10. Gracilin L and Tetrahydroaplysulphurin-1 association and ligand binding analysis. Association curves after addition of different amounts of gracilin L (A) and tetrahydroaplysulphurin-1 (C) to immobilized Cyp D. Different gracilin L and tetrahydroaplysulphurin-1 concentrations were injected using HBS-EP as running buffer and flow rate of 10µg mL^{-1}. The association curves were obtained after subtraction of their respective solvent control. (B and D) kinetic plots of apparent association rate constant K_{obs} (s^{-1}) obtained from figures A and C respectively (calculated by the BiaEvaluation software) *versus* Gracilin L and tetrahydroaplysulphurin-1 concentration. Representative experiment of n=3.

CsA also present hydroxyl groups on its structure. In comparison with gracilin H, the different disposition of aliphatic rings, and the presence of only one acetate substituent group in tetrahydroaplysulphurin-1, could facilitate the interaction with Cyp D. These results suggest the existence of a new compound family with similar activity than CsA on Cyp D. Therefore, these drugs are useful to prevent the mPTP opening and the mitochondrial damage, as in case of oxidative stress. In this sense, *Spongionella* derived compounds have shown high activity to protect mitochondrial function *in vitro* and they have been described as promising compounds for diseases where ROS have a central role, as in AD. Thus, *in vivo* assays revealed that some of *Spongionella* compounds were able to reduced Amyloid-β peptide (Aβ) and hyperphosphorylated tau levels [15, 16]. There is a strong relationship between mitochondrial Aβ and Cyp D interaction and the accumulation and production of mitochondrial ROS as a result of this interaction that leads in the Cyp D recruitment and the increased susceptibility to induce mPTP opening [9].

Conclusions

In a similar fashion than CsA, the *Spongionella* compounds gracilin L and tetrahydroaplysulphurin-1 directly interact with Cyp D, an important structure of mPTP, considered as a potential drug target for neurodegeneration [38]. On the other hand, the unclear origin of AD leads to study several drug targets. Thus, the new bioactivities of *Spongionella* compounds, as SOC entry regulators and mPTP inhibitors provide more evidences as promising active compounds with therapeutic applications to neurodegenerative diseases not only in the AD. In these sense, the low molecular weight of these compounds, comparing to CsA, could overcome some of the problems that present this drug such as the

impossibility to cross blood-brain barrier, the low cell permeability and the wide toxic effects [39]. In addition, it is well know that cyclophilins are key players for other human diseases such as cancer, cardiovascular and immunology alterations, and therefore the association of *Spongionella* compounds with Cyp D opens the possibility to further study the affinity to other cyclophilins as it happens with CsA [40].

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Disclosure Statement

The authors declare no conflict of interest.

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