A pipeline for natural small molecule inhibitors of endoplasmic reticulum stress

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Abstract: The homeostasis of eukaryotic cells is inseparable of that of the endoplasmic reticulum (ER). The main function of this organelle is the synthesis and folding of a significant portion of cellular proteins, while also being the major calcium reservoir of the cell. Upon unresolved ER stress, a set of stress response signaling pathways that are collectively labeled as the unfolded protein response (UPR) is activated. Prolonged or intense activation of this molecular machinery may be deleterious. It is known that compromised ER homeostasis, and consequent UPR activation, characterize the pathogenesis of neurodegenerative disease.

In an effort to discover new small molecules capable of countering ER stress, we subjected a panel of over 100 natural molecules to a battery of assays designed to evaluate several hallmarks of ER stress. The effect of the compounds on calcium homeostasis, key gene and protein expression, and levels of protein aggregation were evaluated in fibroblasts, and subsequently in neuronal cells. This framework resulted in the identification of several bioactive molecules capable of countering ER stress and deleterious events associated to it, among which delphinidin stands out as the most promising candidate against neurodegeneration.

Declarations of interest: none

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27 **Chemical compounds studied in this article:** Calcium ionophore A23187 (PubChem
28 CID: 24277964); thapsigargin (PubChem CID: 126969181); delphinidin (PubChem
29 CID: 68245); fisetin (PubChem CID: 5281614); myristic acid (PubChem CID: 11005);
30 sennoside B (PubChem CID: 91440); quercetin-3-*O*- β -D-glucoside (PubChem CID:
31 44259136).

32

33 **1. Introduction**

34

35 The endoplasmic reticulum (ER) plays a substantial role in the upkeep of the
36 proteostasis of any eukaryotic cell. Upon unresolved ER stress, several stress response
37 signaling pathways are activated in this organelle. These pathways are collectively
38 termed as the unfolded protein response (UPR) and aim to restore reticular homeostasis,
39 but may be deleterious upon sustained or intense activation. Shortly, the UPR relies on
40 three major signaling branches, each initiated by its respective sensor: the protein kinase
41 RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) or
42 the activating transcription factor 6 (ATF6) [1, 2].

43 Disturbances in the secretory pathway of proteins and consequent stress upon the
44 ER are hallmarks of several disease etiologies, including those of neurodegenerative
45 nature. Presently, neurodegenerative diseases have a high prevalence around the world,
46 and, as the population ages, their incidence is expected to increase [1, 3, 4]. Since
47 pharmacological intervention by modulating ER stress has shown promising results
48 against neurodegeneration in *in vitro* and *in vivo* studies [5-8], we consider the
49 development of ER-based strategies against neurodegenerative diseases a necessary step
50 to overcome the burden that these diseases represent to society.

51 Natural products have been used as medicinal agents from immemorial times and
 52 remain the best source of new drugs and drugs leads to modern medicine [9]. This
 53 chemical space has been gifted with immense structural diversity and has been
 54 evolutionarily tailored to provide drug-like molecules, displaying multiple chemotypes
 55 and pharmacophores [9, 10]. Furthermore, it is known that the time when
 56 pharmaceutical companies decided to reduce natural product research projects correlates
 57 with decreased numbers of new drugs entering the market [10]. A literature survey on
 58 nature-derived ER modulators [2] shows the importance of the natural product chemical
 59 space for this end, including molecules as distinct as basiliolides [11], fisetin [12-14],
 60 berberine [15-17], withaferin A [18, 19], agelasine B [20], cephalostatin 1 [21, 22], and
 61 hydroxytyrosol [23]. Notably, most of these molecules are associated to ER stress
 62 induction, while hydroxytyrosol and berberine are reported to ameliorate ER stress.

63 Herein we propose a pipeline for the identification of potential ER stress inhibitors
 64 among a library of natural products.

65

66 **2. Materials and Methods**

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68 **2.1. Chemical library and reagents**

69

70 The compounds 5,7,8-trihydroxyflavone, 5-deoxykaempferol, acacetin, apigenin,
 71 apigetrin, β -escin, chrysoeriol, chrysophanol, coumarin, cyanidin, delphinidin,
 72 diosmetin, diosmin, ellagic acid, eriocitrin, eriodictyol, eriodictyol-7-*O*-glucoside,
 73 eupatorin, ferulic acid, fisetin, flavanone, galangin, gallic acid, genkwanin, gentisic
 74 acid, guaiazulene, herniarin, hesperidin, homoeriodictyol, homoorientin, homovanillic

75 acid, hyoscyamine, isorhamnetin, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-
76 rutinoside, isorhoifolin, juglone, kaempferide, kaempferol, kaempferol-3-*O*-rutinoside,
77 kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperidoside, linarin, liquiritigenin,
78 lutein, luteolin, luteolin tetramethylether, luteolin-3',7-di-*O*-glucoside, luteolin-4'-*O*-
79 glucoside, luteolin-7-*O*-glucoside, malvidin, mangiferin, maritimein, myricetin,
80 myricitrin, myrtillin, naringenin-7-*O*-glucoside, narirutin, oleuropein, orientin,
81 pelargonidin, pelargonin, phloroglucinol, pyrogallol, quercetin-3-*O*-(6-
82 acetylglucoside), quercetagenin, quercetin-3,3',4',7-tetramethylether, quercetin-3,4'-
83 dimethylether, quercetin-3-methylether, quercetin-3-*O*-glucuronide, rhoifolin, robinin,
84 saponarin, scopolamine, sennoside A, sennoside B, sulfuretin, tectochrysin, tiliroside,
85 verbascoside and vitexin were purchased from Extrasynthese (Genay, France). The
86 molecules (-)-norepinephrine, (+/-)-dihydrokaempferol, 1,4-naphthoquinone, 18 α -
87 glycyrrhetic acid, 3,4-dihydrobenzoic acid, 3,4-dimethoxycinnamic acid, 3-
88 hydroxybenzoic acid, 4-hydroxybenzoic acid, 5-methoxypsoralen, ajmalicine,
89 berberine, betanin, betulin, boldine, catechol, cholesta-3,5-diene, chrysin, cynarin,
90 daidzein, emodin, galanthamine, genistein, guaiaverin, hesperetin, hydroquinone,
91 lupeol, myristic acid, naringenin, naringin, *p*-coumaric acid, phloridzin, pinocembrin,
92 plumbagin, quercetin, quercetin-3-*O*- β -D-glucoside, quercitrin, rosmarinic acid, rutin
93 and silibinin were acquired from Sigma-Aldrich (St. Louis, MO, USA). 5,8-Dihydroxy-
94 1,4-naphthoquinone and caffeine were from Fluka (Buchs, Switzerland), vanillin was
95 supplied by Vaz Pereira (Santarém, Portugal), vicenin-2 was purchased from Honeywell
96 (Charlotte, NC, USA). Chlorogenic acid was from PhytoLab (Vestenbergsgreuth,
97 Germany). Cinnamic acid was obtained through Biopurify (Chengdug, China).
98 Swertiamarin was from ChemFaces (Wuhan, China).

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum, penicillin/streptomycin solution (penicillin 5000 units/mL and streptomycin 5000 µg/mL), trypsin-EDTA (0.25%), Qubit® RNA IQ assay kit and Qubit® RNA HS assay kit, the SuperScript™ IV VILO™ MasterMix, the Qubit® Protein Assay Kit, the anti-BiP mouse monoclonal antibody and the anti-mouse secondary antibody were obtained from Invitrogen (Grand Island, NE, USA). Dimethyl sulfoxide (DMSO) was acquired from Fisher Chemical (Loughborough, UK). Isopropanol was obtained from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), calcium ionophore A23187, thapsigargin, RNazol®, chloroform, isopropanol, diethyl pyrocarbonate (DEPC), KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal, Aβ₂₅₋₃₅, 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI), protease inhibitor cocktail, phosphatase inhibitor cocktail, acrylamide/bis-acrylamide 30% solution, bromophenol blue, glycerol, sodium dodecylsulfate (SDS), glycine, Trizma® base, Trizma® hydrochloride, sodium deoxycholate, sodium chloride, potassium phosphate monobasic, potassium chloride, sodium phosphate dibasic, sodium bicarbonate, D-glucose and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA), while formaldehyde was from Bio-Optica (Milan, Italy). The fluorescent probe Fura-2/AM, as well as the anti-GAPDH rabbit monoclonal antibody and the anti-rabbit secondary antibody, were obtained from Abcam (Cambridge, UK). Thioflavin T was from Alfa Aesar (Kandel, Germany). The WesternBright® ECL HRP was supplied by Advansta (Menlo Park, CA, USA). Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Packs were acquired from Bio-Rad (Hercules, CA, USA).

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126 **2.2. Chemometric analysis**

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128 Since it is the most detailed and up to date database of annotated natural products
129 [24], we started from the COCONUT database (<https://coconut.naturalproducts.net>),
130 and created a data frame comprising all the molecules under study, where each row
131 corresponded to a different molecule and columns to features. Among all the original
132 features, we have decided to keep only 48, as the remaining ones did not contain
133 relevant information for this work (SMILE notation, fragments, among others). **Table**
134 **S1** contains a list of all variables kept and their description. All data was cleaned and
135 explored using Python 3.9. For preprocessing data, we have used Simple Imputer,
136 Standard Scaler, Ordinal Encoder and Label Encoder from Scikit-learn [25].
137 Dimensionality reduction was conducted using Scikit-learn [25] library (Principal
138 Component Analysis [PCA], Multidimensional Scaling [MDS], t-distributed Stochastic
139 Neighbor Embedding [TSNE] and Uniform Manifold Approximation and Projection
140 (UMAP) used the UMAP library [26]. For t-SNE we have used number of neighbors of
141 5, 13 and 45 and minimum distance of 0.01, 0.1 and 0.5. In the case of UMAP we have
142 iterated through perplexity of 5, 20, 50 and 100 and number of iterations of 300, 900,
143 1500 and 2000. Unless otherwise specified, specifications of each algorithm were set
144 for default. Graphics in **Figures 1, 7, 8** and **S3** were created using Seaborn or
145 Matplotlib and the remaining ones in GraphPad Prism 8.

146

147 **2.3. Cell culture conditions**

148

MRC-5 fibroblasts (European Collection of Authenticated Cell Cultures, Porton Down Salisbury, UK) were cultured in MEM and SH-SY5Y (American Type Culture Collection, LGC Standards S.L.U., Spain) cells were cultured in DMEM/F-12. Both mediums contained 10% FBS and 1% penicillin/streptomycin and both cell lines were maintained at 37 °C with 5% CO₂.

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155 **2.4. Viability assays**

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For viability assessment, MRC-5 fibroblasts were seeded at a density of 2×10^4 cells/well. After 24 h the cells were incubated with the compounds of interest. 24 h later, the wells are aspirated, the medium was replaced by MTT at 0.5 mg/mL and incubated for 2 h. At the end of this period, the solution was discarded and the formazan crystals in the wells dissolved in 200 µL of a 3:1 DMSO:isopropanol solution. The absorbance at 560 nm was read in a Thermo Scientific™ Multiskan™ GO microplate reader.

Results are presented as the percentage of control and correspond to the mean $\pm$ SEM of, at least, three independent experiments, each of them performed in triplicate.

166

167 **2.5. Cytosolic calcium level determination**

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MRC-5 fibroblasts were plated on black bottomed-96-well plates at the density of 2×10^4 cells/well. After 24 h, the fluorescent probe Fura-2/AM was added at 5 µM and incubated for 1 h. Then they were incubated with the selected nontoxic compounds in HBSS. The calcium ionophore A23187 was added at a final concentration of 5 µM after 2 h. Finally, 2 h later the fluorescence was read at 340/505 and 380/505 on a Cytation™

174 3 (BioTek (Vermont, MA, USA)) multifunctional microplate reader. Data analysis was
175 performed considering the ratio $F_{340/505}/F_{380/505}$. Results are presented as fold decrease vs
176 positive control and represent the mean $\pm$ SEM of, at least, three independent
177 experiments, each of them performed in triplicate.

178

179 **2.6. RNA extraction and quantification, cDNA synthesis and RT-qPCR** 180 **reaction**

181

182 Cells were seeded at 1.6×10^5 cells/well in 12-well plates, left at 37 °C for 24 h
183 and then incubated with the compounds that displayed positive results in previous
184 assays. After 2 h, thapsigargin (Tg) was used as a positive control and added at a final
185 concentration of 3 μ M, and a period of 16 h of incubation ensued. The cells were lysed
186 by aspirating the culture medium and adding 500 μ L of PureZOL RNA isolation
187 reagent, pipetting up and down multiple times. The lysate was kept at room temperature
188 for 5 min and then RNA extraction ensued.

189 The extraction was performed by phase separation, by adding 100 μ L of
190 chloroform, shaking, incubating for 5 min and centrifuging at 12000 g for 15 min at 4
191 °C. The aqueous phase was collected and 250 μ L of isopropyl alcohol were added,
192 following 5 min of incubation and new centrifugation at 12000 g for 10 min, at 4°C.
193 The supernatant was then discarded, and the pellet was washed with 75% ethanol,
194 vortexed, centrifuged at 7500 g for 5 min at 4 °C, air dried and resuspended in DEPC-
195 treated water. The RNA in the sample was quantified resorting to the Qubit® RNA HS
196 assay kit. Sample integrity was then evaluated with the Qubit® RNA IQ assay kit, and,
197 if in appropriate conditions, 1 μ g was converted to cDNA using the SuperScript™ IV
198 VILO™ MasterMix.

RT-qPCR reaction was performed in the following thermal cycling conditions: 3 min at 95 °C, 40 cycles of 95 °C for 3 s (denaturation), gene-specific temperature for 20 s (annealing temperatures listed on **Table 1**) and 20 s at 72 °C (extension). The employed mastermix was KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal. The reaction was conducted on a qTOWER3 G (Analytik Jena AG, Germany), and the data were analyzed on the software supplied along with the equipment (qPCRsoft 4.0). Primers for target genes were designed on Primer BLAST (NCBI, Bethesda, MD, USA) and synthesized by Thermo Fisher (Waltham, MA, USA). The respective nucleotide sequences are presented on **Table 1**. Product specificity was checked with melting curves. *GAPDH* was selected as reference genes for normalization of expression. All the RT-qPCR reactions were performed in duplicate, and the experiments were repeated, at least, four times. Results are presented as mean ± SEM.

211

Table 1. Data concerning analyzed genes and designed primer sequences.

Gene	Accession number	Primers	Annealing Temperature (°C)	Amplicon length (bp)
<i>GAPDH</i> (GAPDH)	NM_002046.6	F: AGGTCGGAGTCAACGGATTT R: TGGAATTTGCCATGGGTGGA	60	157
<i>HSPA5</i> (GRP78)	NM_005347.4	F: ACTCCTGAAGGGGAACGTCT R: TTTCAACCACTTGAACGGC	59.5	161
<i>DDIT3</i> (CHOP)	NM_001195053.1	F: AAGTCTAAGGCACTGAGCGT R: TTGAACACTCTCTCTCAGGT	59	93
<i>HSP90B1</i> (GRP94)	NM_003299.2	F: GCTCTATGTGCGCCGTGTAT R: ATCTGAGTCCACCACACCTT	60.5	91
<i>ATF4</i> (ATF4)	NM_001675.4	F: ACAACAGCAAGGAGGATGCC R: CCAACGTGGTCAGAAGGTCA	60	135
<i>EDEM1</i> (EDEM1)	NM_014674.2	F: GCGGGGACCCTTCAATCT R: CGGCTTTCTGGAACCTCGGAT	60	117

213

214

215 **2.7. Quantification and imaging of protein aggregates**

216

217 On black bottomed-96-well plates, MRC-5 and SH-SY5Y cells were seeded at the
218 densities of 2×10^4 and 3×10^4 cells/well, respectively. In the following day, they were
219 incubated with the selected molecules in the presence of A β_{25-35} at 10 μ M. After an
220 incubation period of 24 h, cells were washed with HBSS and incubated for 30 min with
221 thioflavin T at 5 μ M (prepared in HBSS). At this point, fluorescence was read at
222 450/482 nm on a CytationTM 3 (BioTek (Vermont, MA, USA)) multifunctional
223 microplate reader. Results represent the fold decrease on fluorescence compared to the
224 positive control. We present the mean $\pm$ SEM of, at least, three independent assays,
225 individually performed in triplicate.

226 To image protein aggregates, cells were seeded on clear bottomed-96-well plates
227 at the aforementioned density and treated with the molecules of interest for 24 h in the
228 presence of A β_{25-35} at 10 μ M. Cells were fixed on 4% formaldehyde at room
229 temperature for 15 min and incubated with 10 μ M thioflavin T for 60 min. The counter
230 staining was performed with DAPI at 0.25 μ g/mL, for 30 min. Finally, three washing
231 steps with HBSS for 5 min were carried out and cells were imaged under an Eclipse
232 Ts2R-FL (Nikon) fluorescence microscope equipped with a Retiga R1 camera. A FITC
233 filter was employed to observe thioflavin T and DAPI staining was imaged using a
234 DAPI filter. Images were analyzed resorting to the CellProfiler software version 4.2.1
235 (Broad Institute of MIT and Harvard, Cambridge, MA).

236

237 **2.8. Western blotting analysis**

238

239 MRC-5 and SH-SY5Y cells were seeded in 6-well plates at the densities of $3.2 \times$
240 10^5 and 4.8×10^5 cells/well. After 24 h, they were incubated with compounds selected

241 as promising in previous experiments. After 2 h, Tg at 3 μ M was added, and the plates
242 were left in the incubator for another 22 h. Ended this time frame, cell samples were
243 lysed in RIPA buffer containing 1% protease inhibitor cocktail and 1% phosphatase
244 inhibitor cocktail for 15 min at 4 °C, and subsequently centrifuged at 14000 g for 15
245 min to remove cell debris. The supernatant was then collected, and the protein content
246 was determined resorting to the Qubit[®] Protein Assay Kit.

247 The following step was to submit 30 μ g of each sample, previously denatured at
248 76 °C for 10 min, to 10% SDS-PAGE electrophoresis, in a gel containing 10 %
249 acrylamide/bisacrylamide. The proteins were then transferred to a nitrocellulose
250 membrane on a Trans-Blot[®] Turbo (Bio-Rad; Hercules, CA, USA). The membrane was
251 blocked in a PBS solution containing 5% non-fat milk and 0.1% Triton X-100 for 1 h
252 with orbital agitation. Hereupon, the membrane was incubated with the primary
253 antibody overnight, at 4 °C, with agitation. The anti-BiP mouse monoclonal antibody
254 was used at a working dilution of 1:1250, while the anti-GAPDH rabbit monoclonal
255 antibody was used at a dilution of 1:10000.

256 A washing step was then performed with PBS 0.1% Triton X-100 for 30 min.
257 Then, the secondary antibody was incubated for 2 h at room temperature. The anti-
258 mouse secondary antibody was used at 1:2000, and the anti-rabbit secondary antibody at
259 1:3000. At this point, two more washing steps were performed with PBS 0.1% Triton
260 X-100 for 15 min and then with PBS, again twice for 15 min. Finally, bands were
261 detected by addition of a chemiluminescent substrate, WesternBright ECL HRP. The
262 resulting bands were visualized on a ChemiDoc[™] Imaging System (Bio-Rad, Hercules,
263 CA, USA). Their relative optical density was calculated by densitometry resorting to the
264 Fiji/ImageJ software version 1.51 (Fiji, Madison, WI, USA) and normalized against the

loading control (GAPDH). Results are presented as mean $\pm$ SEM of, at least, three independent experiments.

2.9. Statistical analysis

The statistical analysis was performed using GraphPad Prism 8 software. We resorted to the *t*-test with a level of significance of $p < 0.05$.

3. Results and Discussion

3.1. Chemical space of the library

We compiled a library of 134 molecules of natural origin (**Table S2**). The average molecular weight was 358 g/mol, the inferior limit being 110.1 g/mol (catechol and *p*-hydroquinone) and the upper limit 1131.3 (β -escin) (**Fig. 1A**). The computed natural product-likeness score, a parameter that tries to quantify how much a given molecule is “natural product-like” [27] had an average of 1.29 (min: -0.4, max: 2.72, **Fig. 1A**). Over 50% of the molecules displayed no violations of Lipinski’s Rule of 5 with over 30% displaying 2 or more (**Fig. 1A**), a relevant trait for candidate drugs.

For chemical ontology, we have used the computed chemical classes generated by ClassyFire [28]. From a chemical point of view, the distribution of the molecules by chemical superclass can be found in **Fig. 1B**. Phenylpropanoids and polyketides corresponded to the majority of the entries (65.9%), followed by benzenoids (14.4%)

289 and lipids or lipid-like molecules (6.1%). Within phenylpropanoids, flavonoids were the
 290 most prevalent molecules (**Fig. 1C**), followed by cinnamic acids and their derivatives.
 291 In the case of benzenoids, simple phenols were predominant, while in the case of lipids,
 292 prenol lipids were the most representative (**Fig. 1C**). The chemical structures of all of
 293 the molecules are represented in **Fig. S1**, separated according to the herein mentioned
 294 chemical superclasses.

296 **Fig. 1.** Physico-chemical properties of the library used, and distribution of the chemical
297 (super)classes of the molecules present in the library. [2-column]

298

299

300 **3.2. Impact of molecules upon cell viability**

301

302 Having performed the characterization of our in-house library of over 130 natural
303 products, considering that new candidates for countering ER stress should be devoid of
304 toxicity, we proceeded to the evaluation of the impact of all the molecules in the
305 viability of fibroblasts, as assessed by the MTT reduction assay. Fibroblasts present a
306 highly developed endoplasmic reticulum, since their function relates to the production
307 of components of the extracellular matrix, and thus are particularly susceptible to ER
308 stress, reason for which they were chosen as experimental model in this work [29].

309 Given the high number of molecules involved, all compounds were tested at a single
310 concentration (50 μ M). Statistically significant decreases in MTT reduction were
311 interpreted as negative impact on cellular viability and the corresponding molecules
312 excluded from subsequent experiments. As evidenced by **Fig. 2**, 37 molecules (depicted
313 in **Table S2**) were dropped from the pipeline for their toxicity.

314

Fig. 2. Impact of a library of natural products on MRC-5 fibroblasts viability, as determined by MTT reduction assays. Results represent the percentage of the control and correspond to, at least, three independent experiments, each performed in triplicate. [2-column]

The remaining 97 compounds were screened for potentially protective capacity against ER stress. The legend for the heatmap presented in **Fig. 2** can be consulted in **Table S2**, while **Fig. S2** contains the individual results for each molecule represented in the bar charts.

3.3. Effect of the library upon cytosolic calcium levels

The next step involved the screening of the nontoxic molecules for potential protection against the onset of ER stress. Towards this goal, and considering the high number of molecules still in the pipeline, we selected changes on calcium levels as an indicator of compromised ER homeostasis. The maintenance of the high concentration

332 of calcium in the ER lumen creates an appropriate environment for protein folding, and
 333 thus changes on its levels heavily impact ER function [30, 31]. This organelle is able to
 334 maintain its calcium levels due to the abundance of resident calcium-buffering proteins,
 335 like calnexin and calreticulin [32]. Under stress conditions, the ER may release calcium
 336 through channels, such as IP₃R and RyR. Eventually, this may lead to increased calcium
 337 in mitochondria, indirectly inducing the generation of ROS and triggering the
 338 dissipation of the mitochondrial membrane potential, ultimately leading to the release of
 339 resident proapoptotic factors [33].

340 To evaluate the potential protective effect of the molecules under study, we
 341 conducted experiments with the fluorescent probe Fura-2/AM. Calcium ions were
 342 detected employing the divalent cation ionophore A23187 (5 μ M) as a positive control
 343 for calcium leakage into the cytosol and molecules that could inhibit or counter its effect
 344 were searched. Calcium ionophore A23187, or calcimycin, is a calcium-binding
 345 molecule that increases the cellular permeability of ionic calcium [34]. There are
 346 multiple examples in the literature of the use of this molecule to increase cytosolic
 347 calcium levels, inducing ER stress, UPR activation and eventual apoptosis or autophagy
 348 [35-37].

349 Under these experimental conditions, we were able to identify several molecules
 350 that significantly prevented or ameliorated the calcium overload caused by A23187, as
 351 displayed in **Fig. 3**. Such molecules included six polyphenols (5-deoxykaempferol,
 352 delphinidin, fisetin, naringenin, naringin, quercetin-3-*O*- β -D-glucoside), one
 353 anthraquinone (sennoside B), one fatty acid (myristic acid) and one biogenic polyamine
 354 (spermine). The full list of molecules tested in this assay can be consulted in **Table S3**
 355 while **Fig. S3** represents these data in the bar charts.

356

357

358 **Fig. 3.** Cytosolic calcium levels in MRC-5 fibroblasts in response to co-incubation of
 359 calcium ionophore A23187 at 5 μM and nontoxic molecules (50 μM), as determined by
 360 the fluorescence of the probe Fura-2/AM. **A)** Heatmap representing all molecules tested;
 361 **B)** Molecules that caused a statistically significant decrease in calcium levels when
 362 compared to the positive control. Results correspond to the mean of, at least, three
 363 independent experiments, each performed in triplicate. [2-column]

364 3.4. ER stress counter candidates inhibit thapsigargin-induced UPR signaling 365 in MRC-5 fibroblasts

366

367 The non-toxic molecules that successfully lowered Ca^{2+} levels were considered
 368 potential candidates for countering ER stress. As so, they were evaluated for their effect
 369 upon UPR signaling pathways.

370 Thapsigargin, a natural sesquiterpene lactone that is widely used as UPR inducer,
 371 was the positive control. This molecule acts as an irreversible SERCA pump inhibitor,
 372 leading to an impaired calcium homeostasis and subsequent onset of organized cell
 373 death mechanisms [2]. Accordingly, as evidenced by **Fig. 4**, we can see that
 374 thapsigargin (3 μM) significantly increased the expression of all the genes of interest,
 375 namely the transcription factors *DDIT3* and *ATF4*, the chaperones *HSPA5* and
 376 *HSP90B1* (BiP and GRP94 proteins, respectively), and *EDEM1*, a gene coding for the

377 ER degradation-enhancing alpha-mannosidase-like protein 1, an enzyme involved in
378 ER-associated degradation.

379 5-Deoxykaempferol at 50 μ M inhibited the expression of *DDIT3*, *HSP90B1*,
380 *EDEMI* and *HSPA5*, even though it did not significantly decrease *ATF4* expression.
381 Fisetin was the only molecule to prevent the increase of all target genes in a significant
382 manner. Delphinidin negatively impacted the expression of *ATF4*, *HSP90B1* and
383 *EDEMI*. Myristic acid and quercetin-3-O- β -D-glucoside displayed a similar pattern in
384 the protection against UPR activation, by modulating *HSP90B1* and *EDEMI*
385 expressions. Sennoside B acted upon *ATF4* and *EDEMI*, while naringenin, naringin and
386 spermine failed to inhibit any of the selected genes.

387

388

389 **Fig. 4.** Effect of 5-deoxykaempferol, delphinidin, fisetin, myristic acid, naringenin,
390 naringin, sennoside B, spermine and quercetin-3-O-β-D-glucoside on Tg-induced UPR
391 signaling, as determined by RT-qPCR. Results correspond to the mean ± standard error
392 of the mean of, at least, four independent experiments, each performed in duplicate.
393 *GAPDH* was used as a reference gene for normalization of expression. [2-column]

394

395 In light of these results, we show that 6 out of the 9 molecules that effectively
396 countered the calcium overload were, simultaneously, capable of downregulating UPR-
397 associated genes, which suggests that the assessment of cytosolic calcium levels is a
398 reliable method to screen of the capacity to counter ER stress in this experimental
399 model. Considering that 5-deoxykaempferol, delphinidin, fisetin, myristic acid,

400 sennoside B and quercetin-3-*O*- β -D-glucoside were capable of significantly preventing
401 the Tg-induced increased expression of, at least, two UPR-related genes, they were
402 selected for subsequent studies. In contrast, naringin, naringenin and spermine failed to
403 inhibit the expression of any of the analyzed genes, thus they were dropped from the
404 pipeline.

405

406 **3.5. Molecules that counter ER stress ameliorate protein aggregation in MRC-5** 407 **fibroblasts and attenuate UPR activation at the protein level**

408

409 As discussed earlier, activation of the UPR affects the capacity of cells to properly
410 fold proteins, which frequently results in the formation of protein aggregates and may
411 lead to proteinopathies [38, 39]. Conversely, several diseases, notably those of
412 neurodegenerative nature, are associated with these aggregates [40]. As so, new
413 molecules capable to countering ER stress are expected to be able to prevent or lower
414 the amounts of protein aggregates in a biological context. To confirm this, we
415 proceeded to an experimental model of ER stress-triggered protein aggregation using
416 A β ₂₅₋₃₅ as a protein aggregation inducer. A β ₂₅₋₃₅ is an active fragment of A β ₄₂ [41], a
417 peptide that is associated to the pathogenesis of Alzheimer's disease (AD) and has been
418 characterized in experimental models, where it can lead to the formation of protein
419 aggregates *in vitro* and AD *in vivo* [42].

420 As shown in **Fig. 5A**, incubation of MRC-5 cells with A β ₂₅₋₃₅ elicits protein
421 aggregation, as assessed with thioflavin T, a benzothiazole that presents high
422 fluorescence when bound to β -sheet-rich structures common in protein aggregates, both
423 in a microplate-based (**Fig. 5A**) and an immunocytochemistry method (**Fig. 5B**). In the

424 same conditions, delphinidin, fisetin, myristic acid, sennoside B and quercetin-3-*O*- β -D-
425 glucoside significantly inhibited this effect. These results show that the molecules that
426 had been selected on the basis of their ability to attenuate UPR pathways were able to
427 act upon the endpoint biological effect of this cascade, namely protein aggregation.

428 As mentioned before, the UPR relies on three major signaling branches. They are all
429 governed by the major chaperone BiP, coded by the gene *HSPA5*. Under homeostatic
430 conditions, BiP remains bound to the three major UPR players (PERK, IRE1 and
431 ATF6), releasing them whenever it recognizes the presence of misfolded proteins. This
432 extends the permanence of these proteins in the ER lumen, in order to promote their
433 correct processing. For these reasons, BiP is a key regulator of UPR signaling and its
434 activation prevents protein aggregation [43]. Furthermore, it is described that its
435 expression is increased throughout the course of neurodegenerative disease [44]. Our
436 Western blotting results show that the exposure of Tg-insulted MRC-5 cells to
437 delphinidin and fisetin clearly reduced BiP expression, and, consequently, we presume
438 that the UPR activation in the presence of these molecules is mitigated.

439

Fig. 5. A: Impact of delphinidin, fisetin, myristic acid, sennoside B and quercetin-3-O-β-D-glucoside on Aβ₂₅₋₃₅-induced protein aggregation in MRC-5 cells, determined by the fluorescence of thioflavin T. Results correspond to the mean ± standard error of the mean of, at least, three independent experiments, individually performed in triplicate. **B:** The protective effect of bioactive molecules was captured under a fluorescence microscope. The fluorescent signal of thioflavin T is represented in the histograms. **C:** Effect of delphinidin on Tg-induced BiP expression in MRC-5 cells, as evaluated by Western blotting. Results correspond to the mean ± standard error of the mean of, at least, three independent experiments. **D:** Images obtained through Western blotting analysis. [2-column]

3.6. Selected molecules counter protein aggregation and UPR activation at the translational level in neuronal cells

Given the promising results on MRC-5 fibroblasts, we repeated the experiment, this time with SH-SY5Y cells, in order to analyze whether these compounds could also be protective on this neuronal cell line. The SH-SY5Y cell line has been widely used and described as a good *in vitro* model for the study of protein aggregation [45-48], a process that has been shown to be both consequence and cause of misfolded proteins in the ER and subsequent activation of the UPR [49].

Relevantly, in the case of AD it has been shown that increased levels of phosphorylated PERK and IRE1 α are found in the hippocampus of the patients and that they colocalize with phosphorylated tau. Furthermore, treatment of cells with A β ₄₂, the major component of amyloid plaques, induces CHOP expression, which is a product of the PERK and ATF6 branches of the UPR [44, 50, 51]. For this assay, the concentrations of the molecules under study had to be adjusted to the highest nontoxic concentration towards this cell line. Fisetin was tested at 25 μ M, while the remaining molecules were tested at the concentrations used in MRC-5 cells (50 μ M). The effect of this compound was not replicated on SH-SY5Y cells. We hypothesize that this may be due to the reduction of the concentration that was necessary in order not to impact cell viability in this model. On the other hand, the effect of delphinidin translates to these cells, virtually nullifying the advent of protein aggregates, as evidenced by **Fig. 6A** and **B**.

Fig. 6. A: Effect of delphinidin and fisetin on Aβ₂₅₋₃₅-induced protein aggregation in SH-SY5Y cells, as evaluated by determination of the fluorescence of thioflavin T. Results correspond to the mean ± standard error of the mean of, at least, three independent experiments, each performed in triplicate. **B:** The effect of bioactive molecules was observed under a fluorescence microscope. The fluorescent signal of thioflavin T is represented in the histograms. **C:** Effect of delphinidin on Tg-induced BiP expression in SH-SY5Y cells, as evaluated by Western blotting. Results correspond to the mean ± standard error of the mean of, at least, three independent experiments. **D:** Example of the obtained Western blotting results. [2-column]

3.7. Defining a chemical space for future drug development

Considering all the information generated during this work, together with the chemical, structural and topographical information available for the molecules under study, we were interested in understanding if the physico-chemical properties of the library could, *per se*, be a good indicator for its activity. To this end, we took all the molecules used and computed multiple pairs of their properties, sorting them as active

498 or inactive according to the results of the aggregation assays depicted in **Fig. 5**. While
 499 there seemed to be a tendency of active molecule to be apart from inactive molecules in
 500 some cases (**Fig. S4**), it soon became apparent that a pairwise comparison could leave
 501 behind important features of the molecules, as it reduces high dimensional data to just
 502 two dimensions. As so, we have decided to use other methodologies that could take into
 503 account all the features of the library, namely dimensionality reduction algorithms,
 504 which allows the combination of different features into principal components that can
 505 be more easily graphed and understood. We first conducted a correlation study in order
 506 to identify molecular features that were highly correlated, which could negatively
 507 impact the performance of the statistical analysis given the redundancy it would add.
 508 **Fig. 7** shows the original features, where it is clear that many are highly correlated, for
 509 example, the features “total atom number” and “number of carbons” (0.98), as one
 510 directly affects the other. Such features were dropped, and the remaining ones were
 511 used. We also decided to assess the robustness of the methods in identifying the
 512 chemical space occupied by the molecule that was shown to be active in both cell lines,
 513 namely delphinidin.

514

515

516 **Fig. 7.** Correlation matrix for the features used for the chemical library used in this
 517 work. [2-column]

518

519 We initially used the classical algorithm for principal component analysis (PCA),
 520 which is widely used both for chemometric and biological data [52, 53]. As shown,
 521 PCA was unable to identify a chemical space occupied solely by the molecule that was
 522 shown to be active in all the assays used (**Fig. 8A**), the same being true for
 523 multidimensional scaling (MDS, **Fig. 8B**). In light of this, we applied a suite of other
 524 algorithms, both individually and in tandem with PCA, namely uniform manifold
 525 approximation and projection (UMAP) and t-distributed stochastic neighbor embedding
 526 (t-SNE).

527 Differently from PCA and MSD, the combined used of PCA+UMAP led to a 2-
 528 principal component-based space, in which delphinidin clearly occupies an outlier
 529 space. We have tried distinct combinations of parameters, as stated in the Materials
 530 section, the number of neighbors of 45 giving the best performance, both for minimum
 531 distance of 0.01 and 0.1. In order to confirm this result, we have employed an additional
 532 algorithm, namely PCA+t-SNE. Again, it was shown that delphinidin, together with an
 533 outlier, occupies a space that is distinct from over 98% of the library.

534 Taken together, these results show that chemometric tools, in tandem with
 535 biological data generated from significant number of samples, can be used for future
 536 selection of drug candidates relying on data science-based approaches. As so, the
 537 composition of the principal components identified can be used for the subsequent
 538 selection of novel molecules capable of countering ER stress by screening large
 539 databases of molecules for ideal chemical descriptors.

Fig. 8. Different dimensionality reduction methods applied to chemical library under study. The active molecule delphinidin is presented in orange and inactive molecules in blue. [2-column]

548 **4. Conclusions**

549 Our framework blending data science-based methods to biological data resulted in
550 the identification of several potentially active molecules against the onset of ER stress.
551 Delphinidin stood out as the most promising candidate against UPR activation and
552 protein aggregation in a cellular model of neurodegenerative disease, being capable to
553 simultaneously modulate calcium levels and downregulate UPR pathways with
554 meaningful biological endpoints.

555 In light of our results, it is safe to assume that the natural product chemical space
556 may provide molecules that ultimately lead to the development of novel
557 pharmacological strategies to counter ER stress-related pathologies, as is the case of
558 neurodegenerative diseases.

559

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566

567 **References**

568

569 [1] D.C. da Silva, P. Valentão, P.B. Andrade, D.M. Pereira, Endoplasmic reticulum
570 stress signaling in cancer and neurodegenerative disorders: Tools and strategies to

571 understand its complexity, *Pharmacological Research* 155 (2020) 104702.
572 <https://doi.org/10.1016/j.phrs.2020.104702>.

573

574 [2] D.M. Pereira, P. Valentao, G. Correia-da-Silva, N. Teixeira, P.B. Andrade,
575 Translating endoplasmic reticulum biology into the clinic: a role for ER-targeted natural
576 products?, *Natural Product Reports* 32(5) (2015) 705-722.
577 <https://doi.org/10.1039/C4NP00102H>.

578

579 [3] C. Xiang, Y. Wang, H. Zhang, F. Han, The role of endoplasmic reticulum stress in
580 neurodegenerative disease, *Apoptosis* 22(1) (2017) 1-26.
581 <https://doi.org/10.1007/s10495-016-1296-4>.

582

583 [4] Y.M. Taalab, N. Ibrahim, A. Maher, M. Hassan, W. Mohamed, A.A. Moustafa, M.
584 Salama, D. Johar, L. Bernstein, Mechanisms of disordered neurodegenerative function:
585 concepts and facts about the different roles of the protein kinase RNA-like endoplasmic
586 reticulum kinase (PERK), *Rev Neurosci* 29(4) (2018) 387-415.
587 <https://doi.org/10.1515/revneuro-2017-0071>.

588

589 [5] L. Plate, C.B. Cooley, J.J. Chen, R.J. Paxman, C.M. Gallagher, F. Madoux, J.C.
590 Genereux, W. Dobbs, D. Garza, T.P. Spicer, L. Scampavia, S.J. Brown, H. Rosen, E.T.
591 Powers, P. Walter, P. Hodder, R.L. Wiseman, J.W. Kelly, Small molecule proteostasis
592 regulators that reprogram the ER to reduce extracellular protein aggregation, *eLife* 5
593 (2016) e15550. <https://doi.org/10.7554/eLife.15550>.

594

- 595 [6] M. Cissé, E. Duplan, F. Checler, The transcription factor XBP1 in memory and
596 cognition: Implications in Alzheimer disease, *Mol Med* 22 (2017) 905-917.
597 <https://doi.org/10.2119/molmed.2016.00229>.
- 598
- 599 [7] C. Duran-Aniotz, V.H. Cornejo, S. Espinoza, O. Ardiles Á, D.B. Medinas, C.
600 Salazar, A. Foley, I. Gajardo, P. Thielen, T. Iwawaki, W. Scheper, C. Soto, A.G.
601 Palacios, J.J.M. Hoozemans, C. Hetz, IRE1 signaling exacerbates Alzheimer's disease
602 pathogenesis, *Acta Neuropathol* 134(3) (2017) 489-506. [https://doi.org/10.1007/s00401-](https://doi.org/10.1007/s00401-017-1694-x)
603 [017-1694-x](https://doi.org/10.1007/s00401-017-1694-x).
- 604
- 605 [8] T. O'Connor, K.R. Sadleir, E. Maus, R.A. Velliquette, J. Zhao, S.L. Cole, W.A.
606 Eimer, B. Hitt, L.A. Bembinster, S. Lammich, S.F. Lichtenthaler, S.S. Hébert, B. De
607 Strooper, C. Haass, D.A. Bennett, R. Vassar, Phosphorylation of the translation
608 initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis,
609 *Neuron* 60(6) (2008) 988-1009. <https://doi.org/10.1016/j.neuron.2008.10.047>.
- 610
- 611 [9] G.M. Cragg, D.J. Newman, Natural product drug discovery in the next millennium,
612 *Pharmaceutical Biology* 39(sup1) (2001) 8-17.
613 <https://doi.org/10.1076/phbi.39.s1.8.0009>.
- 614
- 615 [10] B. Shen, A new golden age of natural products drug discovery, *Cell* 163(6) (2015)
616 1297-1300. <https://doi.org/10.1016/j.cell.2015.11.031>.
- 617
- 618 [11] C. Navarrete, R. Sancho, F.J. Caballero, F. Pollastro, B.L. Fiebich, O. Sterner, G.
619 Appendino, E. Muñoz, Basiliolides, a class of tetracyclic C19 dilactones from *Thapsia*

620 *garganica*, release Ca^{2+} from the endoplasmic reticulum and regulate the activity of the
621 transcription factors nuclear factor of activated T cells, nuclear factor- κB , and activator
622 protein 1 in T lymphocytes, *Journal of Pharmacology and Experimental Therapeutics*
623 319(1) (2006) 422.

624

625 [12] D.N. Syed, R.K. Lall, J.C. Chamcheu, O. Haidar, H. Mukhtar, Involvement of ER
626 stress and activation of apoptotic pathways in fisetin induced cytotoxicity in human
627 melanoma, *Archives of biochemistry and biophysics* 563 (2014) 108-117.
628 <https://doi.org/10.1016/j.abb.2014.06.034>.

629

630 [13] Y.-L. Shih, F.-M. Hung, C.-H. Lee, M.-Y. Yeh, M.-H. Lee, H.-F. Lu, Y.-L. Chen,
631 J.-Y. Liu, J.-G. Chung, Fisetin induces apoptosis of HSC3 human oral cancer cells
632 through endoplasmic reticulum stress and dysfunction of mitochondria-mediated
633 signaling pathways, *In Vivo* 31(6) (2017) 1103-1114.
634 <https://doi.org/10.21873/invivo.11176>.

635

636 [14] K.A. Kang, M.J. Piao, S.R.K. Madduma Hewage, Y.S. Ryu, M.C. Oh, T.K. Kwon,
637 S. Chae, J.W. Hyun, Fisetin induces apoptosis and endoplasmic reticulum stress in
638 human non-small cell lung cancer through inhibition of the MAPK signaling pathway,
639 *Tumor Biology* 37(7) (2016) 9615-9624. <https://doi.org/10.1007/s13277-016-4864-x>.

640

641 [15] Z.-s. Wang, F.-e. Lu, L.-j. Xu, H. Dong, Berberine reduces endoplasmic reticulum
642 stress and improves insulin signal transduction in Hep G2 cells, *Acta Pharmacologica*
643 *Sinica* 31(5) (2010) 578-584. <https://doi.org/10.1038/aps.2010.30>.

644

- 645 [16] Z. Zhang, B. Li, X. Meng, S. Yao, L. Jin, J. Yang, J. Wang, H. Zhang, Z. Zhang,
646 D. Cai, Y. Zhang, G. Ning, Berberine prevents progression from hepatic steatosis to
647 steatohepatitis and fibrosis by reducing endoplasmic reticulum stress, *Scientific Reports*
648 6 (2016) 20848. <https://doi.org/10.1038/srep20848>.
- 649
- 650 [17] X. Hao, A. Yao, J. Gong, W. Zhu, N. Li, J. Li, Berberine ameliorates pro-
651 inflammatory cytokine-induced endoplasmic reticulum stress in human intestinal
652 epithelial cells *in vitro*, *Inflammation* 35(3) (2012) 841-849.
653 <https://doi.org/10.1007/s10753-011-9385-6>.
- 654
- 655 [18] M.J. Choi, E.J. Park, K.J. Min, J.-W. Park, T.K. Kwon, Endoplasmic reticulum
656 stress mediates withaferin A-induced apoptosis in human renal carcinoma cells,
657 *Toxicology in Vitro* 25(3) (2011) 692-698. <https://doi.org/10.1016/j.tiv.2011.01.010>.
- 658
- 659 [19] S. Khan, A.W. Rammeloo, J.J. Heikkila, Withaferin A Induces Proteasome
660 Inhibition, Endoplasmic Reticulum Stress, the Heat Shock Response and Acquisition of
661 Thermotolerance, *PLOS ONE* 7(11) (2012) e50547.
662 <https://doi.org/10.1371/journal.pone.0050547>.
- 663
- 664 [20] A.A. Pimentel, P. Felibertt, F. Sojo, L. Colman, A. Mayora, M.L. Silva, H. Rojas,
665 R. Dipolo, A.I. Suarez, R.S. Compagnone, F. Arvelo, I. Galindo-Castro, J.B. De
666 Sanctis, P. Chirino, G. Benaïm, The marine sponge toxin agelasine B increases the
667 intracellular Ca^{2+} concentration and induces apoptosis in human breast cancer cells
668 (MCF-7), *Cancer Chemotherapy and Pharmacology* 69(1) (2012) 71-83.
669 <https://doi.org/10.1007/s00280-011-1677-x>.

670

671 [21] N. López-Antón, A. Rudy, N. Barth, L.M. Schmitz, G.R. Pettit, K. Schulze-
672 Osthoff, V.M. Dirsch, A.M. Vollmar, The marine product cephalostatin 1 activates an
673 endoplasmic reticulum stress-specific and apoptosome-independent apoptotic signaling
674 pathway, *Journal of Biological Chemistry* 281(44) (2006) 33078-33086.
675 <https://doi.org/10.1074/jbc.M607904200>.

676

677 [22] L.H. Tahtamouni, M.M. Nawasreh, Z.A. Al-Mazaydeh, R.A. Al-Khateeb, R.N.
678 Abdellatif, R.M. Bawadi, J.R. Bamburg, S.R. Yasin, Cephalostatin 1 analogues activate
679 apoptosis via the endoplasmic reticulum stress signaling pathway, *European Journal of*
680 *Pharmacology* 818 (2018) 400-409. <https://doi.org/10.1016/j.ejphar.2017.11.025>.

681

682 [23] E. Giordano, A. Davalos, N. Nicod, F. Visioli, Hydroxytyrosol attenuates
683 tunicamycin-induced endoplasmic reticulum stress in human hepatocarcinoma cells,
684 *Molecular Nutrition & Food Research* 58(5) (2013) 954-962.
685 <https://doi.org/10.1002/mnfr.201300465>.

686

687 [24] M. Sorokina, P. Merseburger, K. Rajan, M.A. Yirik, C. Steinbeck, COCONUT
688 online: Collection of Open Natural Products database, *J Cheminform* 13(1) (2021) 2.
689 <https://doi.org/10.1186/s13321-020-00478-9>.

690

691 [25] F. Pedregosa, G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M.
692 Blondel, P. Prettenhofer, R. Weiss, V. Dubourg, J. Vanderplas, A. Passos, D.
693 Cournapeau, M. Brucher, M. Perrot, É. Duchesnay, Scikit-learn: Machine Learning in
694 Python, *J. Mach. Learn. Res.* 12(null) (2011) 2825–2830.

695

696 [26] L.H. McInnes, J., UMAP: Uniform manifold approximation and projection for
697 dimension reduction, ArXiv e-prints 1802.03426 (2018).

698

699 [27] K. Vanii Jayaseelan, P. Moreno, A. Truszkowski, P. Ertl, C. Steinbeck, Natural
700 product-likeness score revisited: an open-source, open-data implementation, BMC
701 Bioinformatics 13(1) (2012) 106. <https://doi.org/10.1186/1471-2105-13-106>.

702 [28] Y. Djoumbou Feunang, R. Eisner, C. Knox, L. Chepelev, J. Hastings, G. Owen, E.
703 Fahy, C. Steinbeck, S. Subramanian, E. Bolton, R. Greiner, D.S. Wishart, ClassyFire:
704 automated chemical classification with a comprehensive, computable taxonomy, J
705 Cheminform 8 (2016) 61-61. <https://doi.org/10.1186/s13321-016-0174-y>.

706

707 [29] J. Weidner, L. Jarenbäck, I. Åberg, G. Westergren-Thorsson, J. Ankerst, L.
708 Bjermer, E. Tufvesson, Endoplasmic reticulum, Golgi, and lysosomes are disorganized
709 in lung fibroblasts from chronic obstructive pulmonary disease patients, Physiol Rep
710 6(5) (2018) e13584. <https://doi.org/10.14814/phy2.13584>.

711

712 [30] A. Kraus, M. Michalak, Endoplasmic reticulum dynamics and calcium signaling,
713 in: J. Krebs, M. Michalak (Eds.), New Comprehensive Biochemistry, Elsevier2007, pp.
714 199-218. [https://doi.org/10.1016/S0167-7306\(06\)41008-5](https://doi.org/10.1016/S0167-7306(06)41008-5).

715

716 [31] A. Carreras-Sureda, P. Pihán, C. Hetz, Calcium signaling at the endoplasmic
717 reticulum: fine-tuning stress responses, Cell Calcium 70 (2018) 24-31.
718 <https://doi.org/10.1016/j.ceca.2017.08.004>.

719

720 [32] H. Coe, M. Michalak, Calcium binding chaperones of the endoplasmic reticulum,
721 Gen Physiol Biophys 28 Spec No Focus (2009) F96-f103.

722

723 [33] K.R. Bhattarai, T.A. Riaz, H.-R. Kim, H.-J. Chae, The aftermath of the interplay
724 between the endoplasmic reticulum stress response and redox signaling, Experimental
725 & Molecular Medicine 53(2) (2021) 151-167. [https://doi.org/10.1038/s12276-021-](https://doi.org/10.1038/s12276-021-00560-8)
726 [00560-8](https://doi.org/10.1038/s12276-021-00560-8).

727

728 [34] A. Verma, A.N. Bhatt, A. Farooque, S. Khanna, S. Singh, B.S. Dwarakanath,
729 Calcium ionophore A23187 reveals calcium related cellular stress as “I-Bodies”: An old
730 actor in a new role, Cell Calcium 50(6) (2011) 510-522.
731 <https://doi.org/10.1016/j.ceca.2011.08.007>.

732

733 [35] E. Castillero, H. Akashi, K. Pendrak, H. Yerebakan, M. Najjar, C. Wang, Y. Naka,
734 D. Mancini, H.L. Sweeney, J. D Armiento, Z.A. Ali, P.C. Schulze, I. George,
735 Attenuation of the unfolded protein response and endoplasmic reticulum stress after
736 mechanical unloading in dilated cardiomyopathy, Am J Physiol Heart Circ Physiol
737 309(3) (2015) H459-H470. <https://doi.org/10.1152/ajpheart.00056.2015>.

738

739 [36] V. Shinde, P. Kotla, C. Strang, M. Gorbatyuk, Unfolded protein response-induced
740 dysregulation of calcium homeostasis promotes retinal degeneration in rat models of
741 autosomal dominant retinitis pigmentosa, Cell death & disease 7(2) (2016) e2085-
742 e2085. <https://doi.org/10.1038/cddis.2015.325>.

743

- 744 [37] E.S. Jung, H. Hong, C. Kim, I. Mook-Jung, Acute ER stress regulates amyloid
745 precursor protein processing through ubiquitin-dependent degradation, Scientific
746 Reports 5(1) (2015) 8805. <https://doi.org/10.1038/srep08805>.
- 747
- 748 [38] K.M. Doyle, D. Kennedy, A.M. Gorman, S. Gupta, S.J. Healy, A. Samali,
749 Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders, J
750 Cell Mol Med 15(10) (2011) 2025-39. [https://doi.org/10.1111/j.1582-
751 4934.2011.01374.x](https://doi.org/10.1111/j.1582-4934.2011.01374.x).
- 752
- 753 [39] N. Ogen-Shtern, T. Ben David, G.Z. Lederkremer, Protein aggregation and ER
754 stress, Brain Research 1648 (2016) 658-666.
755 <https://doi.org/10.1016/j.brainres.2016.03.044>.
- 756
- 757 [40] D.-K. Kim, T.H. Kim, S.-J. Lee, Mechanisms of aging-related proteinopathies in
758 Caenorhabditis elegans, Experimental & Molecular Medicine 48(10) (2016) e263-e263.
759 <https://doi.org/10.1038/emm.2016.109>.
- 760
- 761 [41] W. Zhou, L. Chang, Y. Fang, Z. Du, Y. Li, Y. Song, F. Hao, L. Lv, Y. Wu,
762 Cerebral dopamine neurotrophic factor alleviates A β 25-35-induced endoplasmic
763 reticulum stress and early synaptotoxicity in rat hippocampal cells, Neuroscience
764 Letters 633 (2016) 40-46. <https://doi.org/10.1016/j.neulet.2016.09.008>.
- 765
- 766 [42] Y.-Q. Liu, M.-Q. Jia, Z.-H. Xie, X.-F. Liu, Y. Hui, X.-L. Zheng, H.-Q. Yuan, J.-Z.
767 Bi, Arrestins contribute to amyloid beta-induced cell death via modulation of autophagy

768 and the $\alpha 7$ nACh receptor in SH-SY5Y cells, Scientific reports 7(1) (2017) 3446-3446.
769 <https://doi.org/10.1038/s41598-017-01798-x>.

770

771 [43] M.S. Gorbatyuk, O.S. Gorbatyuk, The molecular chaperone GRP78/BiP as a
772 therapeutic target for neurodegenerative disorders: a mini review, J Genet Syndr Gene
773 Ther 4(2) (2013) 128. <https://doi.org/10.4172/2157-7412.1000128>.

774

775 [44] R. Ghemrawi, M. Khair, Endoplasmic reticulum stress and unfolded protein
776 response in neurodegenerative diseases, International journal of molecular sciences
777 21(17) (2020) 6127. <https://doi.org/10.3390/ijms21176127>.

778

779 [45] T. Nonaka, S.T. Watanabe, T. Iwatsubo, M. Hasegawa, Seeded aggregation and
780 toxicity of α -synuclein and tau, Journal of Biological Chemistry 285(45) (2010) 34885-
781 34898. <https://doi.org/10.1074/jbc.M110.148460>.

782

783 [46] H. Xicoy, B. Wieringa, G.J.M. Martens, The SH-SY5Y cell line in Parkinson's
784 disease research: a systematic review, Molecular Neurodegeneration 12(1) (2017) 10.
785 <https://doi.org/10.1186/s13024-017-0149-0>.

786

787 [47] A.N. Rcom-H'cheo-Gauthier, A.C. Meedeniya, D.L. Pountney, Calcipotriol inhibits
788 α -synuclein aggregation in SH-SY5Y neuroblastoma cells by a Calbindin-D28k-
789 dependent mechanism, J Neurochem 141(2) (2017) 263-274.
790 <https://doi.org/10.1111/jnc.13971>.

791

- 792 [48] J.C. Sang, E. Hidari, G. Meisl, R.T. Ranasinghe, M.G. Spillantini, D. Klenerman,
793 Super-resolution imaging reveals α -synuclein seeded aggregation in SH-SY5Y cells,
794 Commun Biol 4(1) (2021) 613. <https://doi.org/10.1038/s42003-021-02126-w>.
795
- 796 [49] B.D. Roussel, A.J. Kruppa, E. Miranda, D.C. Crowther, D.A. Lomas, S.J.
797 Marciniak, Endoplasmic reticulum dysfunction in neurological disease, Lancet Neurol
798 12(1) (2013) 105-18. [https://doi.org/10.1016/s1474-4422\(12\)70238-7](https://doi.org/10.1016/s1474-4422(12)70238-7).
799
- 800 [50] J.H. Lee, S.M. Won, J. Suh, S.J. Son, G.J. Moon, U.J. Park, B.J. Gwag, Induction
801 of the unfolded protein response and cell death pathway in Alzheimer's disease, but not
802 in aged Tg2576 mice, Exp Mol Med 42(5) (2010) 386-94.
803 <https://doi.org/10.3858/emmm.2010.42.5.040>.
804
- 805 [51] L.E. Santos, S.T. Ferreira, Crosstalk between endoplasmic reticulum stress and
806 brain inflammation in Alzheimer's disease, Neuropharmacology 136 (2018) 350-360.
807 <https://doi.org/10.1016/j.neuropharm.2017.11.016>.
808
- 809 [52] P. Boileau, N.S. Hejazi, S. Dudoit, Exploring high-dimensional biological data
810 with sparse contrastive principal component analysis, Bioinformatics 36(11) (2020)
811 3422-3430. <https://doi.org/10.1093/bioinformatics/btaa176>.
812
- 813 [53] R.R. Meglen, Examining large databases: a chemometric approach using principal
814 component analysis, Marine Chemistry 39(1) (1992) 217-237.
815 [https://doi.org/10.1016/0304-4203\(92\)90103-H](https://doi.org/10.1016/0304-4203(92)90103-H).
816