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Research paper

Novel anti-Alzheimer phenol-lipoyl hybrids: Synthesis, physico-chemical characterization, and biological evaluation

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ABSTRACT

To date, drugs that hit a single target are inadequate for the treatment of neurodegenerative diseases, such as Alzheimer's or Parkinson's diseases. The development of multitarget ligands, able to interact with the different pathways involved in the progression of these disorders, represents a great challenge for medicinal chemists.

In this context, we report here the synthesis and biological evaluation of phenol-lipoyl hybrids (**SV1-13**), obtained via a linking strategy, to take advantage of the synergistic effect due to the antioxidant portions and anti-amyloid properties of the single constituents present in the hybrid molecule. Biological results showed that **SV5** and **SV10** possessed the best protective activity against $A\beta_{1-42}$ induced neurotoxicity in differentiated SH-SY5Y cells. **SV9** and **SV10** showed remarkable antioxidant properties due to their ability to counteract the damage caused by H_2O_2 in SHSY-5Y-treated cells. However, **SV5**, showing moderate antioxidant and good neuroprotective activities, resulted the best candidate for further experiments since it also resulted stable both simulated and plasma fluids.

1. Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by several pathological hallmarks: deposition of Aβ oligomers, neurofibrillary tangles, and hyperphosphorylated tau proteins which lead to loss of memory and cognitive impairment [1,2]. The etiology of AD is not yet clear, but it is certain that the simultaneous presence of oxidative stress, metal dishomeostasis, neuroinflammation, and proteins accumulation contribute to the development of the disorder [3]. To date, the current pharmacological treatment (Acetylcholinesterase inhibitors and N-metyl-D-aspartate antagonist) is only symptomatic so there is no effective cure to reduce or block the progression of AD [4].

Recently, natural compounds gained much attention for their intrinsic therapeutic potential [5]. In this context, phenolic acids, such as caffeic or ferulic acids, are strong antioxidants able to scavenge reactive oxygen species (ROS) thus reducing oxidative damage that characterizes AD [6–10]. Notably, like other polyphenols, they can interfere with the deposition of the amyloid peptide destabilizing the β-sheet conformation in *in vitro* models of AD [11–13].

Although lipoic acid (LA) and its reduced form (dihydrolipoic acid, DHLA) are structurally different from polyphenols, they can act as multifunctional antioxidant against ROS and chelator for metal ions such as zinc or copper involved in the pathogenesis of AD [14]. Moreover, they increase the level of reduced glutathione, downregulate the inflammatory process, scavenge lipid peroxidation products, and increase acetylcholinesterase production by activation of choline acetyltransferase [15].

Abbreviations: DMAP, 4-Dimethylaminopyridine; EDC·HCl, *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; HOBt, 1-Hydroxybenzotriazole; TEA, triethylamine; TFA, trifluoroacetic acid.

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Small molecules targeting Aβ and ROS concurrently could be effective in the prevention and/or treatment of AD. In our previous papers we reported the design and synthesis of different lipoyl hybrids as multifunctional agents for management of neurodegenerative diseases [16,17]. Hybrid molecules can be obtained by linking two or more pharmacophoric portions into a single compound able to interact with multiple targets [18].

Recently, hybrid compounds combining polyphenols and lipoic acid have been reported in the literature acting as selective butyrylcholinesterase (BuChE) inhibitors, an enzyme which, along with acetylcholinesterase (AChE), has an important role in the development and progression of AD [19–22].

Based on these data, our study describes the development of phenol-lipoyl hybrids (**SV1-13**) via a linking strategy (Fig. 1). The phenol-lipoyl hybrids differ by a) the phenolic acid and b) the linkage between the two units; since they incorporate antioxidant and anti-amyloid portions, **SV1-13** could provide a synergistic effect against the neurodegenerative process in patients affected by AD. The polyamines were chosen as linkers for two main reasons: a) they were recently proposed as universal template for the design of multitarget-directed ligands for the management of multifactorial neurodegenerative diseases, such as Alzheimer's disease [23]; b) pharmacological evidences reported that polyamines exerts beneficial effects on learning and memory in the therapeutic approach of cognitive pathologies [24].

Thus, the synthesis and *in vitro* capabilities of **SV1-13** against neurotoxicity and apoptosis induced by $A\beta_{1-42}$ in SHSY-5Y neuroblastoma cells were analyzed. The antioxidant properties of SV hybrids were also investigated considering the presence of different antioxidant portions in each hybrid. Finally, **SV1-13** were also subjected to stability studies in simulated gastric, intestinal, and plasma fluids.

2. Results and discussion

2.1. Synthesis of SV1-13

Phenol-lipoyl hybrids (**SV1-13**) were synthesized following a sequence of reactions depicted in Schemes 1–4. Several Boc-aminoalcohols and Boc-diamines **2a-g**, prepared as previously described [25–28], were used as linkers (Schemes 1–4). The 3,4-Dimethoxyphenylacetic acid (**1**) was coupled to the suitable Boc-aminoalcohols (**2a**, **2c**, or **2f**) or Boc-diamines (**2b**, **2d**, or **2e**) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and catalytic amount of 4-dimetylaminopyridine (DMAP) to obtain ester derivatives **3a**, **3c**, and **3f** or 1-hydroxybenzotriazole (HOBt) to afford amide derivatives **3b**, **3d**, and **3e**, respectively. Mild TFA-mediated removal of the Boc group followed by coupling with (±) α-lipoic acid resulted in hybrids **4a-b**, **4e-f**, and **SV1-2**. Removal of di-methoxy groups with boron trifluoride dimethylsulfide complex ($BF_3 \cdot SMe_2$) provided lipoyl hybrids **SV7-10** and **SV12–13** in good yields.

Reagents and reaction conditions: i) **2a, 2c,** or **2f**, EDC·HCl, 10% DMAP, CH_2Cl_2, 0 °C→ rt, 18 h, or **2b, 2d, 2e**, or **2g**, HOBt, CH_2Cl_2, 0 °C→ rt, 18 h; ii) 5%TFA/CH_2Cl_2, 0 °C→ rt, 1.5 h; iii) EDC·HCl, lipoic acid, TEA, HOBt, CH_2Cl_2, 0.5% cysteine, 0 °C→ rt, 18 h; iv) $BF_3 \cdot SMe_2$, CH_2Cl_2, 0 °C→ rt, 22 h.

For the synthesis of **SV4** (Scheme 2), caffeic acid **5** was transformed to the corresponding dimethoxy derivative **6** using dimethylsulfate [29]. Standard acylation reaction using the linker **2e** in the presence of EDC·HCl gave the derivative **7e**. Boc group deprotection with 5%TFA/CH_2Cl_2 followed by coupling with lipoic acid furnished **SV4** in good yield.

Reagents and reaction conditions: i) Dimethyl sulfate, NaOH, H_2O, rt, 18 h; ii) **2e**, EDC·HCl, HOBt, CH_2Cl_2, 0 °C→ rt, 18 h; iii) 5% TFA/CH_2Cl_2, 0 °C→ rt, 1.5 h; iv) EDC·HCl, lipoic acid, TEA, HOBt, CH_2Cl_2, 0.5% cysteine, 0 °C→ rt, 18 h.

In a similar manner, starting from ferulic acid **8**, hybrids **SV5-6** were obtained in satisfactory yields (Scheme 3). Following the same synthetic procedure, **SV3** and **SV11** were synthetized as reported in Scheme 4.

Reagents and reaction conditions: i) **2b** or **2d**, EDC·HCl, HOBt, CH_2Cl_2, 0 °C→ rt, 18 h; ii) 5%TFA/CH_2Cl_2, 0 °C→ rt, 1.5 h; iii) EDC·HCl, lipoic acid, TEA, HOBt, CH_2Cl_2, 0.5% cysteine, 0 °C→ rt, 18 h.

Reagents and reaction conditions: i) EDC·HCl, HOBt, CH_2Cl_2,

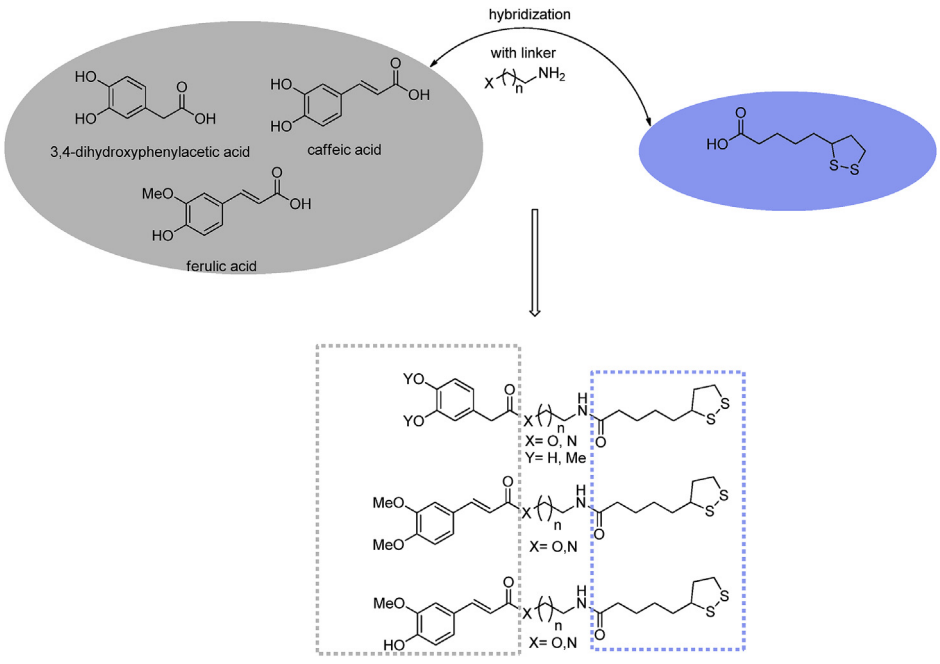
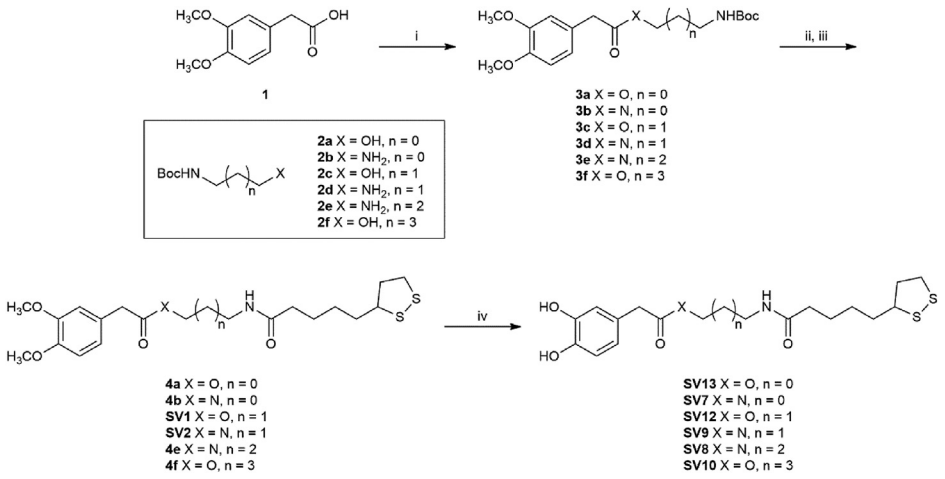
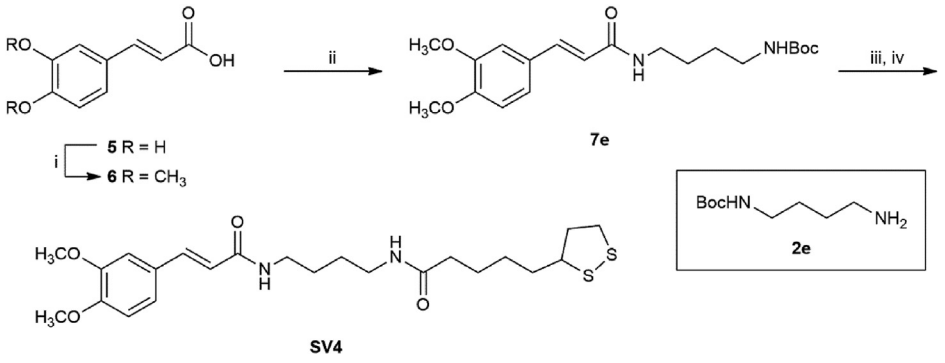


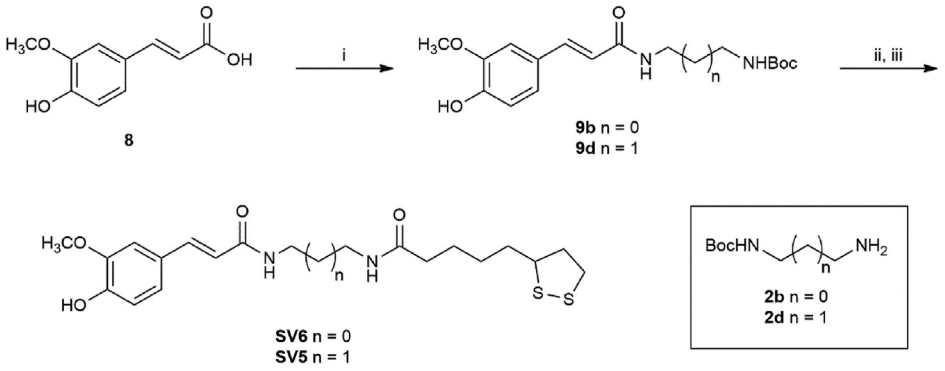
Fig. 1. Design strategy towards phenolic acid – lipoic acid hybrids (**SV1-13**).



Scheme 1. Synthesis of **SV1-2, 8–10, 12–13**.



Scheme 2. Synthesis of **SV4**.



Scheme 3. Synthesis of **SV5-6**.

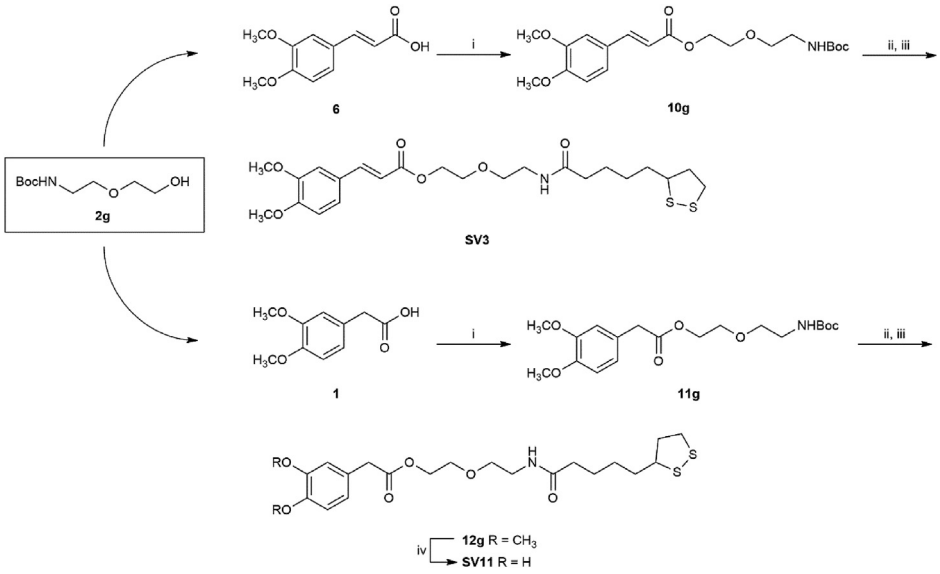
0 °C→ rt, 18 h; ii) 5%TFA/CH_2Cl_2, 0 °C→ rt, 1.5 h; iii) EDC·HCl, lipoic acid, TEA, HOBt, CH_2Cl_2, 0.5% cysteine, 0 °C→ rt, 18 h; iv) $BF_3 \cdot SMe_2$, CH_2Cl_2, 0 °C→ rt, 22 h.

The structure of all synthesized compounds was confirmed by ^1H, ^{13}C NMR and Mass spectrometry. The purity of the new hybrid compounds was verified by HPLC analysis and was found >95% (Supporting Information).

2.2. Physico-chemical characterization of SV1-13

Physico-chemical properties of all synthetized **SV1-13** were analyzed by HPLC. Results showed that all hybrids possessed a poor water solubility even if derivatives with free catechol groups resulted slightly water-soluble compared to compounds with the protected catechol ring (Table 1). Moreover, poor water-soluble hybrids **SV1-5** showed LogP values higher than 3 suggesting that these compounds are quite lipophilic while hybrids **SV7-9**, showing LogP values lower than 2, could be better absorbed by gastrointestinal tract [30].

Stability studies performed in simulated gastric fluid (SGF) at pH 1.2 in presence of pepsin revealed that **SV2** and **SV5-7** are the most stable compounds (Table 2). Similar results were obtained in simulated intestinal fluid (SIF) at pH 6.8 suggesting that these hybrids, containing two amide bonds and a double bond (**SV5-6**), are



Scheme 4. Synthesis of **SV3** and **SV11**.

Table 1
Physicochemical properties of **SV 1–13.**

Compound	Water Solubility (mg/mL)[a]	Classification[b]	cLogP[c]
SV1	0.110 (±0.001)	VSS	3.63
SV2	0.042 (±0.002)	PI	2.73
SV 3	0.010 (±0.003)	PI	3.95
SV 4	–	PI	3.33
SV 5	0.049 (±0.002)	PI	3.19
SV 6	0.023 (±0.001)	PI	2.92
SV 7	2.688 (±0.026)	SS	1.59
SV 8	0.081 (±0.001)	PI	1.29
SV 9	0.235 (±0.010)	VSS	1.81
SV 10	–	PI	2.95
SV 11	2.484 (±0.017)	SS	2.49
SV 12	0.933 (±0.087)	VSS	2.71
SV 13	0.736 (±0.002)	VSS	2.43

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] FS (Freely Soluble), S (Soluble), SPS (Sparingly Soluble), SS (Slightly Soluble), VSS (Very Slightly Soluble), PI (Practically Insoluble).
[c] Values are calculated with ACD LogP software package, version 4.55.

hardly attackable by proteolytic enzymes (Table 2). Plasma stability studies confirmed these data (Table 3). On the other hand, hybrids **SV1**, **SV10**–**13** were not very stable at pH 1.2 and underwent to immediate hydrolysis in SIF at pH 6.8 (Table 2). Probably, the introduction of an ester bond between the catechol residue and the linker is detrimental for the stability of the compounds. In human plasma the same hybrids showed a half-life less than 1 h suggesting that they do not possess physico-chemical properties suitable for further studies (Table 3). The structure-activity relationships highlighted the larger effect of the amide bond on the stability of the hybrids than the ester junction.

2.3. Biological studies

2.3.1. Effect of ***SV1-13*** on neurotoxicity induced by $A\beta_{1-42}$ in SH-SY5Y cells

In the current study, we aimed to test the protective effects of **SV1-13** on Aβ-induced differentiated SH-SY5Y cell death. First, as a preliminary study, the toxicity of **SV1-13** on SH-SY5Y cells was investigated in a wide range of concentrations (1–250 mg/L). Results showed that they did not cause any significant cytotoxicity compared to control cells (Fig. 1S). Afterwards, we examined the protective effects of **SV1-13** on viability of SHSY-5Y cells exposed to $A\beta_{1-42}$, a pathogenic inducer of AD. Based on our previous study [31], the viability of differentiated SH-SY5Y cells decreased with increasing concentration of $A\beta_{1-42}$, and the 50% decrease in cell proliferation was determined when treated with 20 μM of $A\beta_{1-42}$. Therefore, this concentration was used in all subsequent experiments.

The viability of differentiated SH-SY5Y cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT). The cell viability was elevated in a concentration-dependent manner when the cells, exposed to $A\beta_{1-42}$, were treated with SV hybrids. Similar results were obtained using lactate dehydrogenase (LDH). Fig. 2 shows that the increased cytotoxicity in the cells exposed to $A\beta_{1-42}$ was decreased after treatment with SV hybrids. These results indicated that these compounds have exhibited different protection levels on cellular toxicity caused by $A\beta_{1-42}$. Based on these data, **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** were chosen for further biological experiments due to their higher potential protective activity against $A\beta_{1-42}$ induced neurotoxicity in differentiated SH-SY5Y cells (Fig. 2).

2.3.2. Effect of ***SV2***, ***SV3***, ***SV5***, ***SV9***, and ***SV10*** on $A\beta_{1-42}$ -induced apoptosis in SH-SY5Y cells

Because of their neuroprotective activity on $A\beta_{1-42}$-induced cell death, **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** were chosen for further studies. To examine their effect on the apoptotic cell death of SH-SY5Y cells, Hoechst 33258 staining was performed to reveal DNA fragmentation or chromatin condensation. The blue stained cells, after exposure to $A\beta_{1-42}$ with or without SV hybrids for 48 h, were reported in Fig. 3. The cells in the control group (Panel A) have regular shape while exposure to $A\beta_{1-42}$ significantly affect their morphology including the presence of condensed chromatin (seen as a bright blue) and fragmented nuclear DNA (observed as blue spots) (Panel B). On the contrary, fragmentation and condensation of DNA were decreased when the cells exposed to $A\beta_{1-42}$ plus SV hybrids, particularly with **SV2** (Panel C), **SV5** (Panel E), and **SV10** (Panel G).

Furthermore, the effect of **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** on apoptotic cell death caused by $A\beta_{1-42}$ in differentiated SH-SY5Y

Table 2
Stability of **SV 1**–**13** in simulated gastric fluid at pH 1.2 (SGF) and in intestinal fluid at pH 6.8 (SIF) with Pepsin and Pancreatin, respectively.

	Time point (min)	SV1	SV2	SV3	SV4	SV5	SV6	SV7	SV8	SV9	SV10	SV11	SV12	SV13
SGF	0	–	–	–	–	–	–	–	–	–	–	–	–	–
	15	15.23 (±0.53)	2.90 (±0.03)	5.60 (±0.34)	4.67 (±0.19)	0.36 (±0.01)	0.25 (±0.01)	0.63 (±0.02)	10.37 (±0.94)	15.27 (±0.14)	35.42 (±0.76)	45.21 (±0.84)	72.4 (±0.64)	12.34 (±0.88)
	30	35.89 (±0.75)	1.89 (±0.56)	7.35 (±0.24)	8.23 (±0.53)	0.43 (±0.02)	1.28 (±0.06)	0.99 (±0.01)	37.63 (±0.74)	47.45 (±0.15)	79.35 (±0.40)	63.52 (±0.54)	86.54 (±0.63)	46.72 (±0.71)
	60	60.98 (±0.53)	4.03 (±0.38)	30.76 (±0.82)	22.16 (±0.99)	1.78 (±0.76)	3.45 (±0.54)	2.74 (±0.15)	57.89 (±0.65)	65.48 (±0.15)	100 (±0.05)	78.90 (±0.75)	100 (±0.50)	67.34 (±0.82)
SIF	0	Immediate	–	–	–	–	–	–	–	–	Immediate	–	Immediate	–
	15	hydrolysis	0.45 (±0.07)	12.01 (±0.86)	3 (±0.89)	0.54 (±0.01)	2.52 (±0.62)	1.89 (±0.03)	1.50 (±0.07)	5.60 (±0.36)	hydrolysis	3.87 (±0.19)	hydrolysis	2.50 (±0.08)
	30		0.73 (±0.21)	21.06 (±0.95)	3.64 (±0.27)	0.86 (±0.05)	3.01 (±0.21)	2.17 (±0.04)	20.44 (±0.87)	30.40 (±0.17)		45.89 (±0.75)		35.40 (±0.49)
	60		0.90 (±0.01)	27.20 (±0.50)	11.79 (±0.23)	1.04 (±0.45)	5.20 (±0.20)	4.02 (±0.30)	42.70 (±0.05)	56.30 (±0.09)		60.78 (±0.42)		54.62 (±0.60)
	120		1.32 (±0.09)	31.38 (±0.50)	22.21 (±0.81)	2.50 (±0.09)	4.15 (±0.28)	3.78 (±0.17)	63.00 (±0.86)	70.00 (±0.16)		89.78 (±0.92)		70.45 (±0.84)
	180		2.09 (±0.11)	40.30 (±0.75)	34.75 (±0.86)	3.46 (±0.04)	4.80 (±0.37)	4.32 (±0.20)	88.91 (±0.86)	90.20 (±0.66)		100 (±0.08)		100 (±0.47)

[a] Values are means of three experiments; standard deviation is given in parentheses.
[b] unstable RD% > 5%, stable RD% < 5%.

Table 3
Human plasma stability of **SV 1**–**13.**

		SV1	SV2	SV3	SV4	SV5	SV6	SV7	SV8	SV9	SV10	SV11	SV12	SV13
Human plasma	$t_{½}$ (h) [a]	0.62 (±0.12)	>12 (±)	17.23 (±4.84)	36.44 (±2.88)	72.37 (±1.46)	57.44 (±1.82)	23.14 (±7.21)	1.49 (±0.36)	9.21 (±0.05)	0.03 (±0.01)	2.16 (±0.02)	0.75 (±0.01)	1.13 (±0.05)
	k_{obs} (h^{-1}) [a]	1.146 (±0.224)	–	0.042 (±0.012)	0.019 (±0.002)	0.010 (±0.001)	0.012 (±0.001)	0.031 (±0.010)	0.479 (±0.117)	0.08 (±0.001)	20.56 (±2.631)	0.32 (±0.004)	0.93 (±0.003)	0.613 (±0.025)

[a] Values are means of three experiments; standard deviation is given in parentheses.[a] Va.

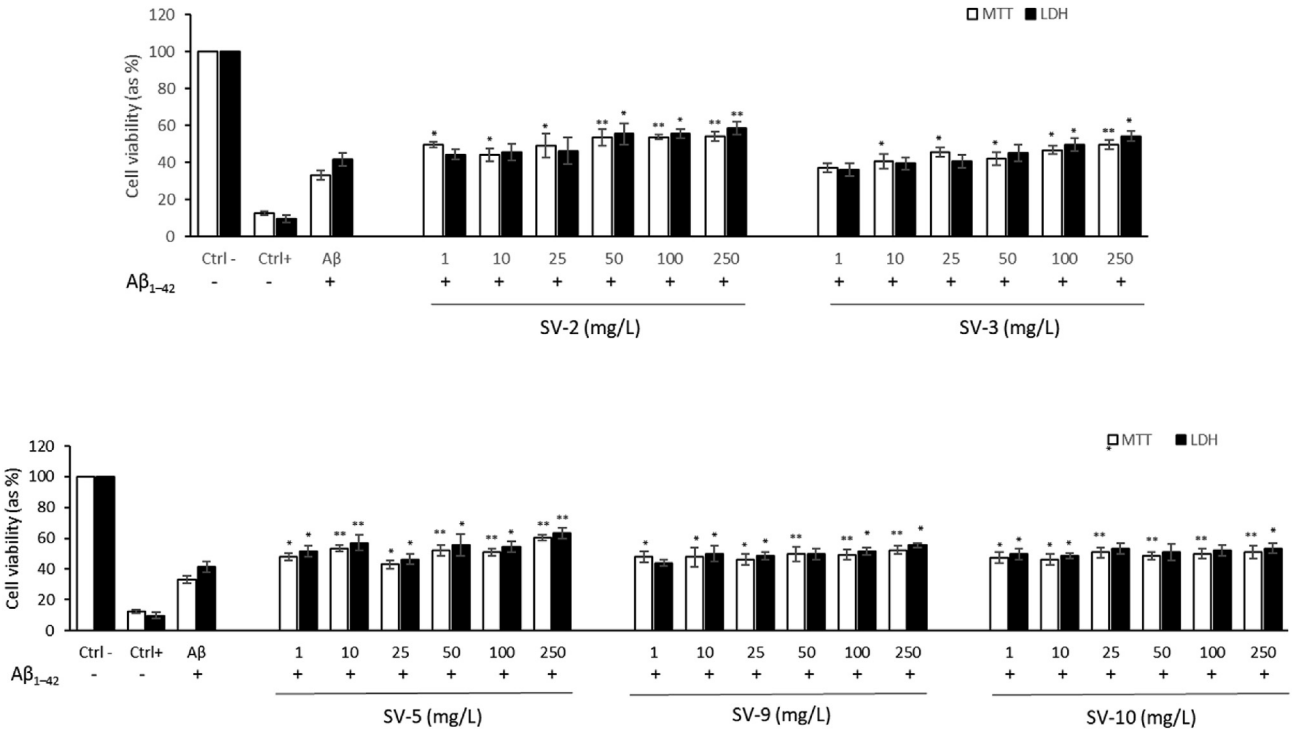


Fig. 2. The neuroprotective effects of SV derivatives against $A\beta_{1-42}$ at a concentration of 20 μM was examined by MTT and LDH assay. Values are shown as the percentage of cell viability and represented as mean ± SEM of five independent experiments. **p = 0.001; *p = 0.05; n.s. p > 0.05.

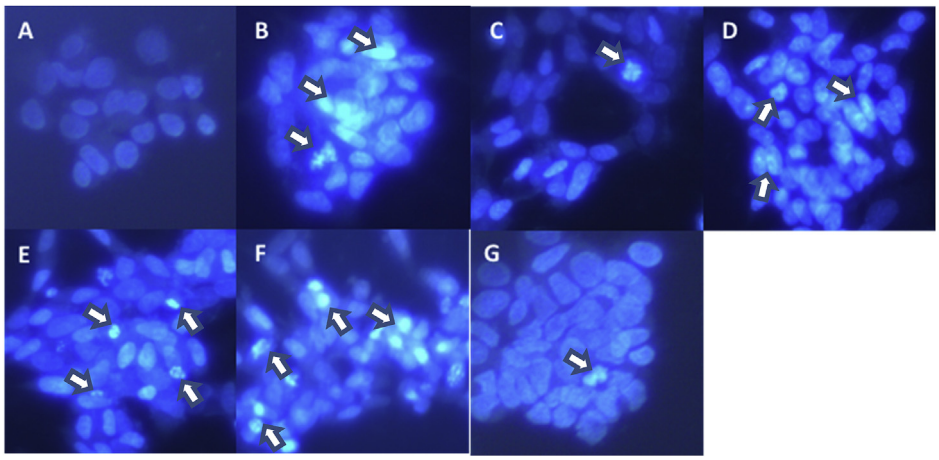


Fig. 3. SH-SY5Y cells stained with Hoechst 33258 showing the antiapoptotic effects of SV derivatives against $A\beta_{1-42}$ (20 μM). **A)** Control, **B)** $A\beta_{1-42}$, **C)** $A\beta_{1-42}$ + **SV2**, **D)** $A\beta_{1-42}$ + **SV3**, **E)** $A\beta_{1-42}$ + **SV5**, **F)** $A\beta_{1-42}$ + **SV9**, **G)** $A\beta_{1-42}$ + **SV10**; Original magnification, × 200.

cells was assessed by Annexin V-FITC and propidium iodide (PI) double staining (Fig. 4). Cells were incubated with $A\beta_{1-42}$ with or without hybrids for 48 h, and then analyzed by flow cytometry. As shown in Fig. 4, $A\beta_{1-42}$ caused an increase in cell death (apoptosis 34.46%, necrosis 6.99%) in SH-SY5Y cells. On the other hand, SV hybrids provided protection of differentiated SH-SY5Y cells against $A\beta_{1-42}$ induced cell death.

2.3.3. Effect of ***SV2***, ***SV3***, ***SV5***, ***SV9***, and ***SV10*** on gene expression profile in SH-SY5Y cells exposed to $A\beta_{1-42}$

To further explore the mechanism underlying the protective effects of SV hybrids on $A\beta_{1-42}$-induced neurotoxicity of differentiated SH-SY5Y cells, the mRNA levels of 12 different AD related genes were analyzed. Cells were treated with $A\beta_{1-42}$ with or without SV hybrids and changes in the gene expression levels of treated cells compared to control group were shown in Fig. 5. It was observed that $A\beta_{1-42}$ in SH-SY5Y cell line led to an increase in the gene expressions of *APOE, NCTSN, APH1α, PSENEN, APP, TNF-α, EGFR, MAPT, BDNF, BACE1* and a decrease in the gene expressions of *PSEN* and *ADAM10*. On the other hand, SV hybrids caused different ameliorative changes in the gene expression profile of cells exposed to $A\beta_{1-42}$. Among all tested SV hybrids, **SV5** and **SV10** showed the best activity against $A\beta_{1-42}$ induced neurotoxicity in SH-SY5Y cells.

2.3.4. Cytotoxic effects of ***SV2***, ***SV3***, ***SV5***, ***SV9***, and ***SV10*** on human whole blood cells

Human whole blood cells were treated with **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** and tested to evaluate their potential cytotoxic effects. A wide range of hybrids concentrations were screened and incubated with the blood cells for 24 h. The cytotoxicity of each hybrid was determined using both MTT and LDH release assays. The positive control, that was treated with Triton-X and H_2O_2 (50 μM), showed

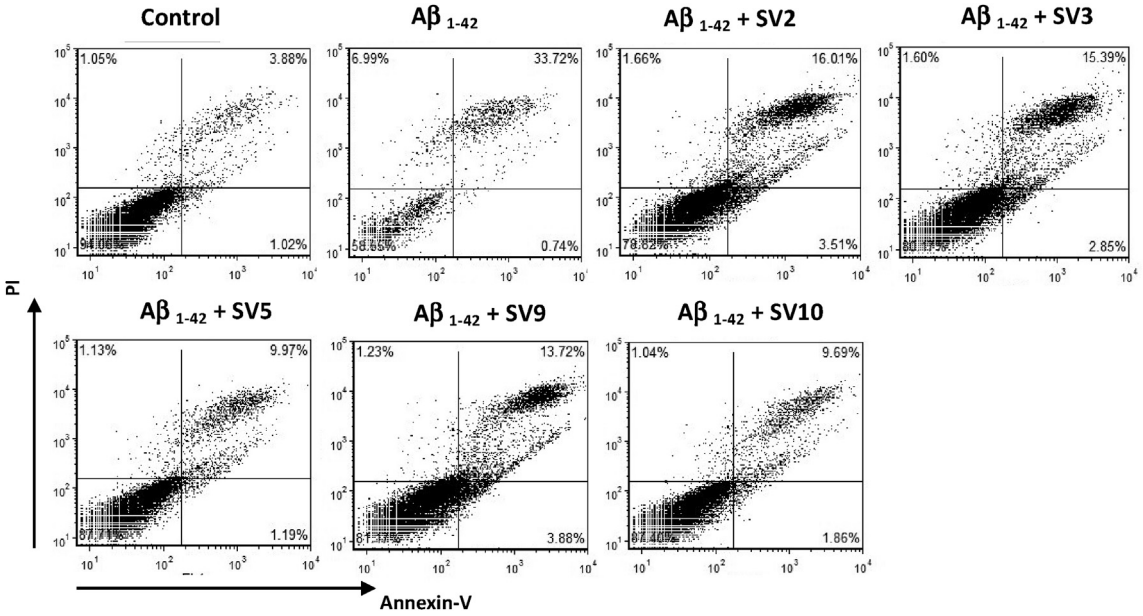


Fig. 4. Representative images of flow cytometry evaluation of cell apoptosis after treatment with SV derivatives against $A\beta_{1-42}$ (20 μM) induced toxicity in SH-SY5Y cells. The apoptotic rate was determined by annexin V/PI staining. The lower left quadrant I represents viable cells (Annexin V−/PI −); the lower right quadrant II represents the cells in the early stage of apoptosis (Annexin V+/PI −); the upper left quadrant III represents the cells in the late stage of apoptosis (Annexin V+/PI +); and the upper right quadrant IV represents necrotic cells (Annexin V−/PI +).

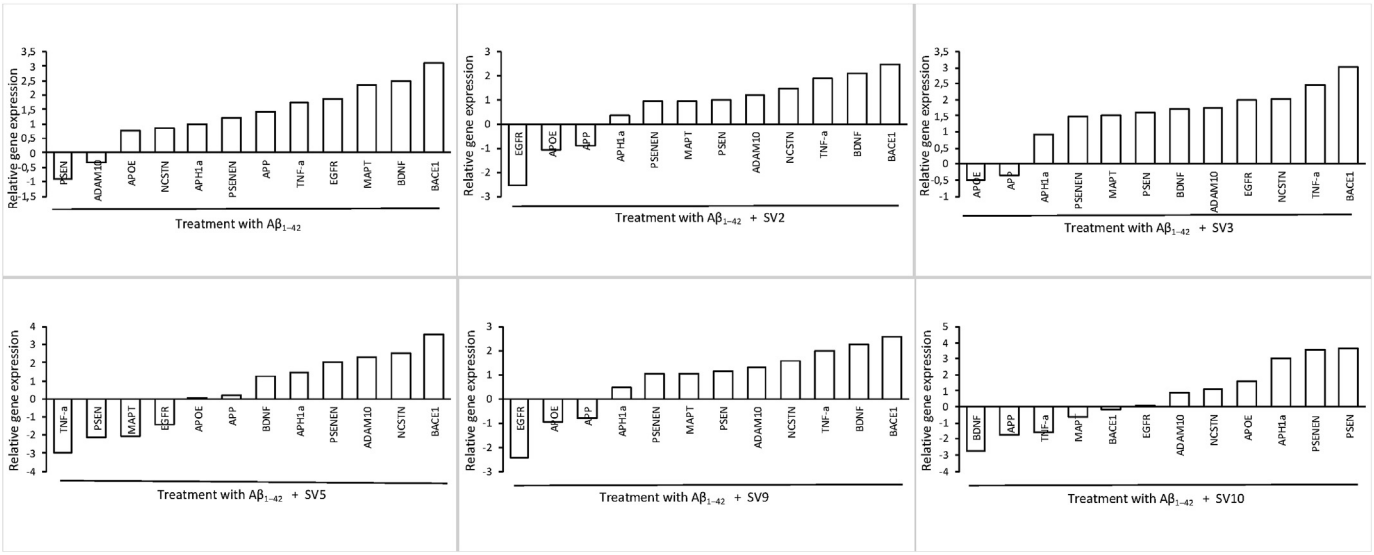


Fig. 5. Gene expression profile in differentiated SH-SY5Y cells treated with **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** against $A\beta_{1-42}$ (20 μM). Up-regulation and down-regulation of each gene are represented as a fold-change relative to untreated cells.

the lowest cell viability rates (<20% and <50%, respectively) as compared to untreated (control (−)) cells. On the contrary, after 24 h of treatments, the cultures showed excellent viability even up to the concentration of 400 μM of SV hybrids. Although the minimum cell viability rates were observed after treatment with 400 μM of **SV3**, the determined cell viability rates were still higher than 70%. Likewise, it was observed in LDH release assay that a 24 h treatment of blood cells to **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** concentrations led no decrease of the number of viable cells, whereas there was a slight reduction in cell viability at the concentration of 400 μM of **SV3** (data not shown). For all that, this reduction did not reflect cytotoxic potential since the observed values were above 75%. Eventually, the cytotoxicity analysis revealed that **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** hybrids have non-cytotoxic nature on human blood cells.

2.3.5. *Antioxidant properties of **SV2**, **SV3**, **SV5**, **SV9**, and **SV10***

SV2, **SV3**, **SV5**, **SV9**, and **SV10** were also evaluated for their potential antioxidant activity using TAC assay (Fig. 6). Significant differences were observed in the supporting capacity of SV hybrids. Maximum % increases of TAC levels decreased in the following order: **SV10** (32.3%) > **SV9** (26.5%) > **SV5** (6.7%) > **SV2** (4.2%) > **SV3** (2.0%) as compared to control (−) level. Ascorbic acid (AA) increased the TAC level in 10.7%.

Taking account of cell viability rates (>95%) and results obtained by TAC assay, two effective concentrations were selected as 50 and 100 μM for further cyto-protectivity evaluations. So, the blood cells were simultaneously incubated with 50 or 100 μM of selected SV hybrids and H_2O_2 to reveal whether these derivatives conferred cytoprotection against oxidative damages of cells. Blood cells treated with H_2O_2 caused 56.9% and 52.2% decreases in cell viability when compared to control (−) rates (MTT and LDH release assays), respectively. However, **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** led to improvement of the cell viability. A clear concentration-dependent cyto-protection was monitored, and the cell viability was increased between the ranges of 41.6 and 86.8% by SV hybrids indicating 0.5 and 42.0% recovery from H_2O_2-induced cell death in MTT analysis. Similarly, the cell viability was increased between the ranges of 48.2 and 89.3% by SV hybrids indicating 0.4% and 41.5% recovery from H_2O_2-induced cell death in LDH release analysis (Fig. 7 and Table 1S).

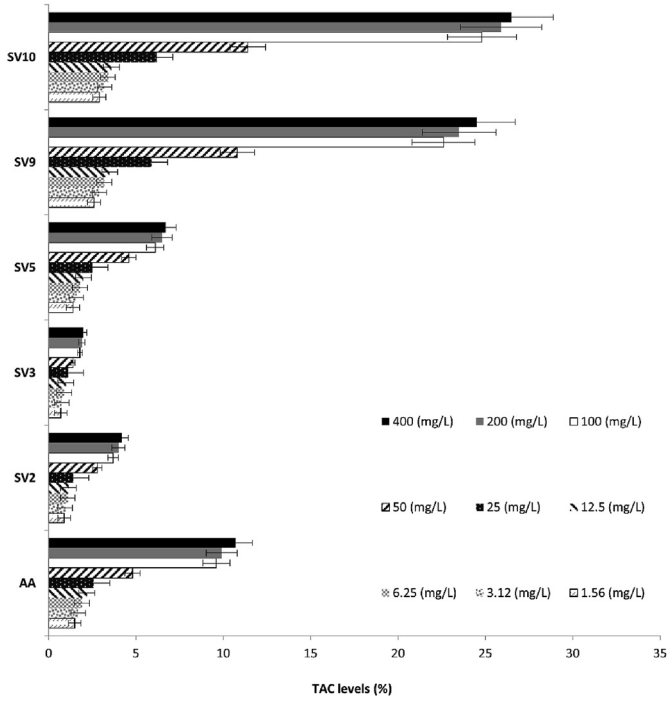


Fig. 6. The increases of TAC levels (%) as compared to level of control (−) group after treatment with different concentrations (0–400 mg/L) of **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** for 72 h in whole blood cultures. (AA: Ascorbic acid).

Fig. 8 reflects the observed TAC levels in cultures after incubation with selected SV hybrids and H_2O_2, simultaneously. The TAC levels significantly ($p < 0.05$) decreased when blood cells were treated with H_2O_2. In fact, the exposure to H_2O_2 caused 83.3% decrease in TAC level as compared to untreated cultures. By contrast with, SV hybrids provided antioxidative restoration and minimized these decreases in clear compound type and concentration manners. Nominately, 50 μM and 100 μM **SV3** exhibited the weakest (12.5% and 29.2%) but **SV9** and **SV10** provided the strongest

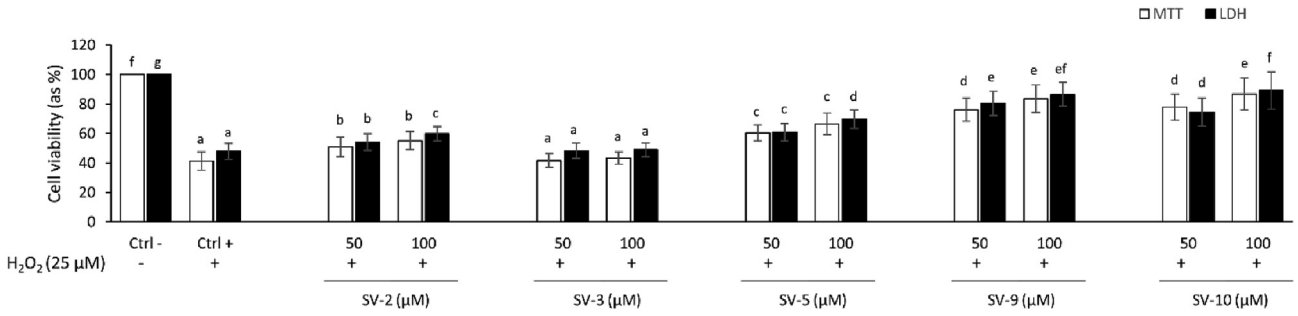


Fig. 7. Protective effect of **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** on human blood cells against H_2O_2 induced cell damage as analyzed by MTT and LDH release assays. Different letters in the same column indicate statistically significant differences ($P < 0.05$) from each other, as analyzed by Dunnett's multiple comparison test.

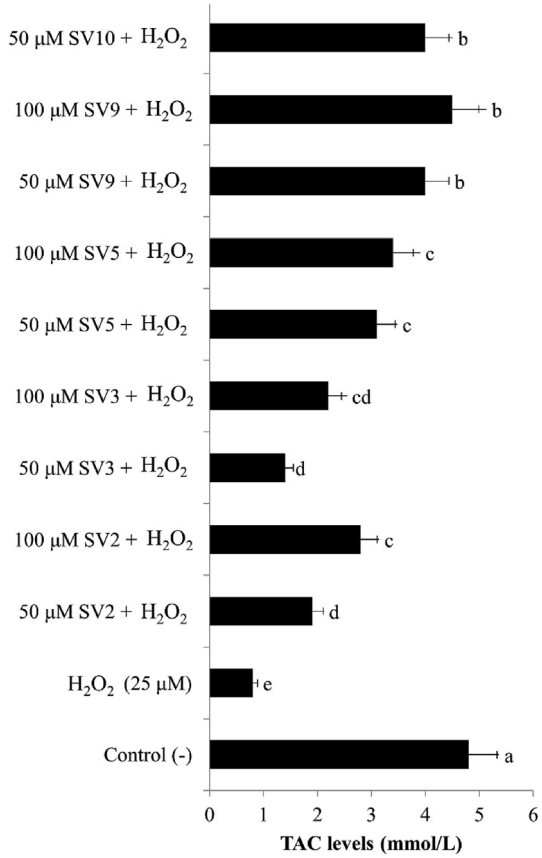


Fig. 8. The effects of concomitant treatments of **SV2**, **SV3**, **SV5**, **SV9**, or **SV10** and H_2O_2 on TAC levels (as mmol/L) of cultured human blood cells. Bars with different letters are considered statistically different ($P < 0.05$), as analyzed by Dunnett's multiple comparison test.

protection (reaching to 79.2%) against H_2O_2-induced oxidative insults.

In summary, we reported the synthesis and biological characterization of 13 novel hybrids containing phenols and lipoic acid as novel multifunctional antioxidants for the development of potential anti-Alzheimer agents. Taking together all the obtained data, the present study demonstrated that **SV2**, **SV3**, **SV5**, **SV9**, and **SV10** derivatives exhibited non-cytotoxic and cyto-protective actions. The *in vitro* assays showed that most of the hybrids had significant neuroprotective activity against H_2O_2-induced oxidative stress in SHSY-5Y cells. Notably, **SV9** and **SV10** showed the highest antioxidant properties but they resulted unstable, especially **SV10**, in simulated fluids and human plasma. In fact, stability studies in simulated and plasma fluids confirmed that the introduction of ester bond - between phenolic portion and the linker (**SV10**) - compared to the amide linkage is detrimental for the stability of the synthetized hybrids.

The best activity against neurotoxicity induced by $A\beta_{1\text{-}42}$ in SHSY-5Y-treated cells was exhibited by **SV5** and **SV10**; the presence of ferulic and 3,4-dihydoxyphenilacetic acids as phenolic portions, respectively in **SV5** and **SV10**, represent important structural motifs for a strong free radical scavenging character and anti-amyloidogenic properties. Hydroxyl groups of SV hybrids could form non-covalent interactions with the β-sheet structures interfering with their assembling.

In conclusion, the moderate antioxidant properties of **SV5** (compared to **SV9** and **SV10**) but its good potential as neuroprotective compound against $A\beta_{1\text{-}42}$ and its high stability in simulated fluids and human plasma (hal-life > 72 h), render this hybrid a potential multifunctional candidate for further studies to the development of new anti-Alzheimer agents.

3. Experimental part

3.1. General

All used reagents were purchased at the highest quality from commercial suppliers and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel plates (silica gel $60F_{254}$) and components were visualized by UV light absorbance. Purification of compounds by column chromatography was carried out on silica gel (70–230 mesh) with the indicated solvents. ^1H and ^{13}C spectra were recorded on a Varian VXR-300 spectrometer (Varian Medical Systems, Inc., Palo Alto, CA, USA). ^1H and ^{13}C spectra are referenced according to the residual peak of the solvent based on literature data. ^{13}C spectra are fully proton decoupled. ESI mass spectral analyses were performed on a mass spectrometer (Thermo Finnigan), using direct sample injection. Negative or positive ion ESI spectra were acquired by adjusting the needle and cone voltages accordingly. The spectroscopy and analytical data were obtained in the Laboratory of Organic Chemistry of the University of Athens. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

3.1.1. General esterification procedure (method A)

To a stirred solution of the appropriate carboxylic acid (1.00 mmol), Boc-protected aminoalcohol (1.00 mmol) and DMAP (0.10 mmol) in CH_2Cl_2 (5 mL) at 0 °C, EDC·HCl (1.00 mmol) was added. After stirred at room temperature overnight, the mixture was diluted with CH_2Cl_2, washed with citric acid 5%, H_2O, $NaHCO_3$ 5%, H_2O and brine. The organic phase was dried over Na_2SO_4,

filtered, and concentrated to obtain yellow oil, which was further purified by column chromatography on silica gel using appropriate mixture of CH_2Cl_2/MeOH or AcOEt/Petroleum ether.

3.1.1.1. 2-((tert-butoxycarbonyl)amino)ethyl 2-(3,4-dimethoxyphenyl)acetate (3a). Following the general method **A**, compound **3a** was obtained as thicky oil in 77% yield. R_f (CH_2Cl_2/MeOH 9.8:0.2) = 0.29. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.77 (s, 3H), 4.80 (br s, 1H), 4.10 (t, J = 5.4 Hz, 2H), 3.82 (dd, J = 3.6, 2.1 Hz, 6H), 3.53 (s, 2H), 3.32 (d, J = 5.4 Hz, 2H), 1.40 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.5, 155. 6, 148.7, 148.0, 126.1, 121.2, 112.2, 111.05, 79.3, 63.7, 55.6, 40.5, 39.4, 28.1.

3.1.1.2. 3-((tert-butoxycarbonyl)amino)propyl 2-(3,4-dimethoxyphenyl)acetate, 3c. Following the general method **A**, compound **3c** was obtained as thicky oil in 75% yield. R_f (CH_2Cl_2/MeOH 9.8:0.2) = 0.38. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.69 (s, 3H), 4.93 (br s, 1H), 4.02 (t, J = 6.2 Hz, 2H), 3.72 (d, J = 4.1 Hz, 6H), 3.43 (s, 2H), 3.02 (q, J = 6.4 Hz, 2H), 1.67 (p, J = 6.4 Hz, 2H), 1.31 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.4, 155.5, 148.5, 147.7, 126.0, 120.9, 112.01, 110.8, 78.5, 61.8, 55.3, 40.3, 36.8, 28.6, 27.9.

3.1.1.3. 5-((tert-butoxycarbonyl)amino)pentyl 2-(3,4-dimethoxyphenyl)acetate (3f). Following the general method **A**, compound **3f** was obtained as thicky oil in 83% yield. R_f (CH_2Cl_2/MeOH 9.8:0.2) = 0.45. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.68 (d, J = 2.9 Hz, 3H), 4.80 (br s, 1H), 3.93 (td, J = 6.2, 3.8 Hz, 2H), 3.71 (t, J = 3.9 Hz, 6H), 3.41 (d, J = 3.8 Hz, 2H), 2.95 (d, J = 5.9 Hz, 2H), 1.48 (dd, J = 15.7, 8.9 Hz, 2H), 1.36–1.11 (m, 13H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.4, 155.6, 148.3, 147.6, 126.1, 120.9, 111.9, 110.7, 78.3, 64.2, 55.3, 40.4, 39.9, 29.2, 27.9, 27.8, 22.7.

3.1.1.4. 2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethyl (E)-3-(3,4-dimethoxyphenyl)acrylate (10g). Following the general method **A**, compound **10g** was obtained as thicky oil in 57% yield. R_f(AcOEt/P.E. 1:1) = 0.60. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.43 (dd, J = 15.9, 2.4 Hz, 1H), 6.93–6.79 (m, 2H), 6.64 (dd, J = 8.0, 2.3 Hz, 1H), 6.14 (dd, J = 15.9, 2.3 Hz, 1H), 5.02 (br s, 1H), 4.12 (d, J = 2.7 Hz, 2H), 3.68 (d, J = 2.1 Hz, 6H), 3.52 (s, 2H), 3.40–3.30 (m, 2H), 3.12 (d, J = 3.6 Hz, 2H), 1.22 (d, J = 2.1 Hz, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.5, 155.5, 150.7, 148.7, 144.6, 126.8, 122.3, 114.9, 110.5, 109.2, 78.5, 69.7, 68.5, 62.9, 55.4, 55.3, 39.8, 27.9.

3.1.1.5. 2-(2-((tert-butoxycarbonyl)amino)ethoxy)ethyl 2-(3,4-dimethoxyphenyl)acetate (11g). Following the general method **A**, compound **11g** was obtained as thicky oil in 74% yield. R_f (CH_2Cl_2/MeOH 9.8:0.2) = 0.35. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.70 (t, J = 2.7 Hz, 3H), 4.98 (br s, J = 2.7 Hz, 1H), 4.15–4.04 (m, 2H), 3.72 (t, J = 3.6 Hz, 6H), 3.50 (dt, J = 7.4, 4.0 Hz, 4H), 3.40–3.32 (m, 2H), 3.21–3.07 (m, 2H), 1.32 (d, J = 3.3 Hz, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.2, 155.5, 148.5, 147.7, 125.9, 121.0, 112.1, 110.8, 78.7, 69.6, 68.3, 63.3, 55.4, 40.2, 39.8, 27.9.

3.1.2. General amidation procedure (method B)

To a stirred solution of the appropriate carboxylic acid (1.00 mmol), Boc-protected diamine (1.00 mmol) and HOBt (1.00 mmol) in CH_2Cl_2 (5 mL) at 0 °C, EDC·HCl (1.00 mmol) was added. After stirred at room temperature overnight, the mixture was diluted with CH_2Cl_2, washed with citric acid 5%, H_2O, $NaHCO_3$ 5%, H_2O and brine. The organic phase was dried over Na_2SO_4, filtered, and concentrated to obtain yellow oil, which was further purified by column chromatography on silica gel using appropriate mixture of CH_2Cl_2/MeOH.

3.1.2.1. tert-butyl (2-(2-(3,4-dimethoxyphenyl)acetamido)ethyl) carbamate (3b). Following the general method **B**, compound **3b** was obtained as white solid in 83% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.61. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.76 (s, 3H), 6.36 (br s, 1H), 5.12 (br s, 1H), 3.82 (s, 6H), 3.44 (s, 2H), 3.26 (s, 2H), 3.17 (s, 2H), 1.38 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.0, 156.5, 148.9, 148.0, 127.1, 121.3, 112.2, 111.2, 79.3, 55.7, 43.1, 40.4, 40.1, 28.2.

3.1.2.2. tert-butyl (3-(2-(3,4-dimethoxyphenyl)acetamido)propyl) carbamate (3d). Following the general method **B**, compound **3d** was obtained as thicky oil in 85% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.46. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.65 (t, J = 4.4 Hz, 4H), 5.30 (d, J = 5.0 Hz, 1H), 3.73–3.61 (m, 2H), 3.30 (d, J = 4.9 Hz, 6H), 3.15–3.00 (m, 2H), 2.91 (d, J = 5.4 Hz, 2H), 1.39 (d, J = 5.3 Hz, 2H), 1.24 (d, J = 5.0 Hz, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.5, 156.1, 148.5, 147.5, 127.3, 120.9, 111.9, 110.9, 78.4, 55.3, 42.7, 36.5, 35.6, 29.5, 27.9.

3.1.2.3. tert-butyl (4-(2-(3,4-dimethoxyphenyl)acetamido)butyl) carbamate (3e). Following the general method **B**, compound **3e** was obtained as white solid in 89% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.43. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.69 (s, 3H), 6.17 (br s, 1H), 4.87 (br s, 1H), 3.73 (s, 6H), 3.36 (s, 2H), 3.09 (d, J = 5.4 Hz, 2H), 2.95 (d, J = 5.2 Hz, 2H), 1.31 (s, 13H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.1, 155.8, 148.7, 147.7, 127.3, 121.1, 112.0, 111.1, 78.6, 55.5, 55.5, 42.8, 39.7, 38.9, 28.0, 27.1, 26.2.

3.1.2.4. tert-butyl (E)-(4-(3-(3,4-dimethoxyphenyl)acrylamido)butyl) carbamate (7e). Following the general method **B**, compound **7e** was obtained as white solid in 82% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.41. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.44 (d, J = 15.6 Hz, 1H), 7.01 (br s, 1H), 6.95–6.83 (m, 2H), 6.66 (d, J = 8.1 Hz, 1H), 6.36 (d, J = 15.6 Hz, 1H), 5.01 (br s, 1H), 3.72 (d, J = 8.6 Hz, 6H), 3.28 (d, J = 5.4 Hz, 2H), 3.02 (d, J = 5.7 Hz, 2H), 1.46 (s, 4H), 1.32 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.2, 156.0, 150.0, 148.6, 139. 8, 127.6, 121.4, 118.9, 110.7, 109.4, 78.7, 55.5, 55.4, 39.8, 39.0, 28.1, 27.3, 26.4.

3.1.2.5. tert-butyl (E)-(2-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)ethyl)carbamate (9b). Following the general method **B**, compound **9b** was obtained as white solid in 76% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.33. ^{1}H NMR (200 MHz, CD_3OD-d4) δ 7.42 (d, J = 15.7 Hz, 1H), 6.96 (dd, J = 11.5, 3.2 Hz, 2H), 6.75 (dd, J = 8.0, 3.8 Hz, 1H), 6.39 (d, J = 15.7 Hz, 1H), 4.94 (br s, 3H), 3.80–3.71 (m, 3H), 3.41–3.33 (m, 1H), 3.26–3.14 (m, 3H), 1.37 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD-d4) δ 169.2, 158.3, 149.6, 149.0, 142.0, 127.9, 123.0, 118.5, 116.3, 111.3, 80.0, 56.2, 40.9, 40.5, 28.7.

3.1.2.6. tert-butyl (E)-(3-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)propyl)carbamate (9d). Following the general method **B**, compound **9d** was obtained as white solid in 69% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.39. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.48 (d, J = 15.5 Hz, 1H), 7.02 (br s, 1H), 6.95–6.79 (m, 3H), 6.31 (d, J = 15.6 Hz, 1H), 5.33 (br s, 1H), 3.75 (s, 3H), 3.37 (d, J = 5.2 Hz, 2H), 3.14 (d, J = 5.2 Hz, 2H), 1.63 (s, 2H), 1.40 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.9, 156.7, 147.6, 147.0, 140.7, 126.9, 121.9, 118.0, 114.9, 109.7, 79.2, 55.6, 36.9, 36.0, 29.9, 28.2.

3.1.3. General procedure for the lipoyl amides synthesis

Boc-protected compound (1 mmol) was suspended in CH_2Cl_2 (10 mL), and then trifluoroacetic acid (0.5 mL) was added at 0 °C. After stirring at room temperature for 1.5 h, the volatiles were removed under reduced pressure to obtain the crude product which was dissolved in CH_2Cl_2 (7 mL) and directly coupled with lipoic acid (1 mmol) at 0 °C, in the presence of Et_3N (1.10 mmol), EDC·HCl (1.2 mmol), HOBt (1.00 mmol) and 0.5% cysteine to avoid

polymerization. After stirred at room temperature overnight, in the dark, the reaction mixture was taken up with CH_2Cl_2 and washed with HCl 0.5 N, water, 5% $NaHCO_3$, water, and brine. The organic phase was dried over Na_2SO_4, filtered, and concentrated to obtain the crude product, which was further purified by column chromatography on silica gel (CH_2Cl_2/MeOH).

3.1.3.1. 2-(5-(1,2-dithiolan-3-yl)pentanamido)ethyl 2-(3,4-dimethoxyphenyl)acetate (4a). Following the general method, compound **4a** was obtained as thick oil in 59% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.56. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.76 (d, J = 1.5 Hz, 3H), 5.92 (br s, 1H), 4.12 (t, J = 5.4 Hz, 2H), 3.82–3.76 (m, 6H), 3.55–3.37 (m, 5H), 3.18–2.96 (m, 2H), 2.39 (td, J = 12.4, 6.4 Hz, 1H), 2.07 (t, J = 7.1 Hz, 2H), 1.93–1.74 (m, 1H), 1.60 (ddd, J = 13.9, 9.1, 2.2 Hz, 4H), 1.38 (dd, J = 14.2, 6.1 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.7, 171.6, 148.6, 147.9, 126.0, 121.1, 112.1, 110.9, 63.2, 56.2, 55.6, 40.5, 40.0, 38.3, 38.2, 36.0, 34.3, 28.6, 25.0.

3.1.3.2. N-(2-(2-(3,4-dimethoxyphenyl)acetamido)ethyl)-5-(1,2-dithiolan-3-yl)pentanamide (4b). Following the general method, compound **4b** was obtained as white solid in 45% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.55. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (br s, 1H), 6.79–6.66 (m, 4H), 3.78 (t, J = 1.4 Hz, 6H), 3.54–3.42 (m, 1H), 3.39 (s, 2H), 3.24 (s, 4H), 3.16–2.94 (m, 2H), 2.46–2.28 (m, 1H), 2.06 (t, J = 7.2 Hz, 2H), 1.82 (dq, J = 13.7, 6.9 Hz, 1H), 1.65–1.44 (m, 4H), 1.43–1.25 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.6, 172.4, 148.7, 147.8, 127.1, 121.1, 112.2, 111.1, 56.2, 55.6, 55.6, 42.8, 40.0, 39.6, 39.5, 38.2, 35 9, 34.3, 28.6, 25.1.

3.1.3.3. N-(4-(2-(3,4-dimethoxyphenyl)acetamido)butyl)-5-(1,2-dithiolan-3-yl)pentanamide (4e). Following the general method, compound **4e** was obtained as white solid in 40% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.45. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82–6.69 (m, 3H), 6.29 (br s, 1H), 6.11 (br s, 1H), 3.80 (d, J = 3.5 Hz, 6H), 3.52 (dd, J = 13.7, 6.9 Hz, 1H), 3.43 (s, 2H), 3.21–2.97 (m, 6H), 2.39 (td, J = 12.4, 6.4 Hz, 1H), 2.11 (t, J = 7.3 Hz, 2H), 1.83 (dq, J = 13.8, 6.9 Hz, 1H), 1.69–1.50 (m, 4H), 1.41 (t, J = 6.4 Hz, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.9, 171.4, 148.9, 147.9, 127.3, 121.3, 112.2, 111.2, 56.3, 55.7, 43.0, 40.0, 39.0, 38.8, 38.3, 36.1, 34.4, 28.7, 26.7, 26.5, 25.2.

3.1.3.4. 5-(5-(1,2-dithiolan-3-yl)pentanamido)pentyl 2-(3,4-dimethoxyphenyl)acetate (4f). Following the general method, compound **4f** was obtained as white solid in 70% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.71. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.80–6.73 (m, 3H), 5.76 (br s, 1H), 4.04 (t, J = 6.5 Hz, 2H), 3.85–3.78 (m, 6H), 3.60–3.45 (m, 3H), 3.24–3.00 (m, 4H), 2.41 (td, J = 12.4, 6.4 Hz, 1H), 2.12 (t, J = 7.2 Hz, 2H), 1.85 (dq, J = 13.8, 6.9 Hz, 1H), 1.58 (dt, J = 14.0, 6.7 Hz, 6H), 1.49–1.20 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.6, 171.7, 148.6, 147.8, 126.3, 121.2, 112.2, 110.9, 64.4, 56.2, 55.7, 55.7, 40.7, 40.0, 39.1, 38.3, 36.2, 34.4, 29.0, 28.7, 28.0, 25.2, 23.0.

3.1.3.5. 2-(2-(5-(1,2-dithiolan-3-yl)pentanamido)ethoxy)ethyl 2-(3,4-dimethoxyphenyl)acetate (12g). Following the general method, compound **12g** was obtained as thick oil in 72% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.62. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.79 (d, J = 1.9 Hz, 3H), 5.92 (br s, 1H), 4.23 (dd, J = 5.5, 3.8 Hz, 2H), 3.83 (dd, J = 4.1, 2.0 Hz, 6H), 3.66–3.60 (m, 2H), 3.57 (d, J = 1.9 Hz, 2H), 3.49 (t, J = 5.6 Hz, 3H), 3.43–3.32 (m, 2H), 3.20–2.99 (m, 2H), 2.42 (td, J = 12.4, 6.4 Hz, 1H), 2.13 (t, J = 7.2 Hz, 2H), 1.96–1.77 (m, 1H), 1.73–1.53 (m, 4H), 1.49–1.32 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.6, 171.7, 148.7, 148.0, 126.1, 121.2, 112.2, 111.0, 69.6, 68.7, 63.5, 56.3, 55.7, 40.6, 40.1, 38.9, 38.3, 36.1, 34.5, 28.7, 25.2.
34.3, 28.5, 28.4, 25.1.

3.1.3.6. 3-(5-(1,2-dithiolan-3-yl)pentanamido)propyl 2-(3,4-dimethoxyphenyl)acetate (SV1). Following the general method, **SV1** was obtained as white solid in 61% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.67. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.75 (d, J = 5.6 Hz, 3H), 6.09 (t, J = 5.6 Hz, 1H), 4.07 (t, J = 6.2 Hz, 2H), 3.78 (d, J = 3.1 Hz, 6H), 3.55–3.39 (m, 3H), 3.18 (q, J = 6.4 Hz, 2H), 3.03 (dt, J = 6.5, 4.9 Hz, 2H), 2.36 (td, J = 12.4, 6.1 Hz, 1H), 2.06 (t, J = 7.3 Hz, 2H), 1.89–1.68 (m, 3H), 1.67–1.48 (m, 4H), 1.37 (dd, J = 13.9, 7.1 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.6, 171.7, 148.5, 147.7, 126.0, 121.0, 112.0, 110.9, 62.0, 56.1, 55.5, 40.5, 39.9, 38.1, 36.0, 35.7, 34.3, 28.5, 28.4, 25.1. MS (ESI) m/z calculated for $C_{21}H_{30}NO_4S_2^-$ $[M-H]^-$ 440.1, found 440.0. Anal. Calcd for $C_{21}H_{31}NO_5S_2$: C, 57.12; H, 7.08; N, 3.17; O, 18.12; S, 14.52. Found: C, 57.20; H, 7.15; N, 3.09; O, 18.04; S, 14.55.

3.1.3.7. N-(3-(2-(3,4-dimethoxyphenyl)acetamido)propyl)-5-(1,2-dithiolan-3-yl)pentanamide (SV2). Following the general method, **SV2** was obtained as off-white solid in 42% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.43. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.81–6.57 (m, 5H), 3.76–3.70 (m, 6H), 3.51–3.32 (m, 3H), 3.20–2.90 (m, 6H), 2.34 (td, J = 12.4, 6.4 Hz, 1H), 2.08 (t, J = 7.2 Hz, 2H), 1.78 (td, J = 13.7, 7.0 Hz, 1H), 1.65–1.23 (m, 8H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.1, 171.8, 148.6, 147.7, 127.2, 121.0, 112.0, 111.1, 56.1, 55.5, 55.5, 42.9, 39.9, 38.1, 36.0, 35.7, 35.3, 34.2, 29.2, 28.5, 25.1. MS (ESI) m/z calculated for $C_{21}H_{33}N_2O_4S_2^+$ $[M+H]^+$ 441.2, found 441.2. Anal. Calcd for $C_{21}H_{31}N_2O_4S_2$: C, 57.37; H, 7.11; N, 6.37; O, 14.56; S, 14.59. Found: C, 57,35; H, 7,17; N, 6,41; O, 14,52; S, 14,55.

3.1.3.8. 2-(2-(5-(1,2-dithiolan-3-yl)pentanamido)ethoxy)ethyl (E)-3-(3,4-dimethoxyphenyl)acrylate (SV3). Following the general method, compound **SV3** was obtained as yellowish solid in 70% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.63. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.46 (d, J = 15.9 Hz, 1H), 6.97–6.81 (m, 2H), 6.69 (d, J = 8.2 Hz, 1H), 6.27–6.06 (m, 2H), 4.23–4.09 (m, 2H), 3.71 (t, J = 2.4 Hz, 6H), 3.60–3.51 (m, 2H), 3.41 (dd, J = 10.2, 5.4 Hz, 2H), 3.28 (dd, J = 11.7, 6.9 Hz, 3H), 3.02–2.82 (m, 2H), 2.23 (dt, J = 18.9, 6.4 Hz, 1H), 2.02 (t, J = 7.4 Hz, 2H), 1.67 (dt, J = 19.7, 6.9 Hz, 1H), 1.45 (dd, J = 10.3, 4.9 Hz, 4H), 1.26 (dd, J = 8.2, 5.8 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.4, 166.6, 150.7, 148.6, 144.7, 126.6, 122.3, 114.7, 110.6, 109.1, 769.2, 68.5, 62.8, 55.9, 55.4, 55.4, 39.7, 38.6, 37.9, 35.7, 34.1, 28.4, 24.9. MS (ESI) m/z calculated for $C_{23}H_{34}NO_6S_2^-$ $[M+H]^+$ 484.2, found 484.2. Anal. Calcd for $C_{23}H_{33}NO_6S_2$: C, 57.12; H, 6.88; N, 2.90; O, 19.85; S, 13.26. Found: C, 57,11; H, 6.88; N, 2,87; O, 19.95; S, 13.19.

3.1.3.9. (E)-N-(4-(3-(3,4-dimethoxyphenyl)acrylamido)butyl)-5-(1,2-dithiolan-3-yl)pentanamide (SV4). Following the general method, compound **SV4** was obtained as yellowish solid in 65% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.29. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.56 (d, J = 15.5 Hz, 1H), 7.14–6.98 (m, 2H), 6.84 (d, J = 8.2 Hz, 1H), 6.43–6.29 (m, 2H), 6.21 (br s, 1H), 3.89 (s, 6H), 3.62–3.48 (m, 1H), 3.41 (d, J = 5.4 Hz, 2H), 3.30 (d, J = 5.7 Hz, 2H), 3.13 (dd, J = 12.9, 6.1 Hz, 2H), 2.54–2.31 (m, 1H), 2.21 (t, J = 7.3 Hz, 2H), 1.88 (td, J = 13.8, 7.0 Hz, 1H), 1.73–1.57 (m, 7H), 1.50 (d, J = 8.3 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.2, 166.5, 150.5, 149.0, 140.7, 127.7, 121.9, 118.5, 111.0, 109.6, 56.4, 55.9, 55.8, 40.2, 39.2, 39.1, 38.4, 36.3, 34.6, 28.9, 26.9, 26.8, 25.4. MS (ESI) m/z calculated for $C_{23}H_{35}N_2O_4S_2^+$ $[M+H]^+$ 467.2, found 467.2. Anal. Calcd for $C_{23}H_{34}N_2O_4S_2$: C, 59.20; H, 7.34; N, 6.00; O, 13.71; S, 13.74. Found: C, 59.18; H, 7.35; N, 6.02; O, 13.75; S, 13,70.

3.1.3.10. (E)-5-(1,2-dithiolan-3-yl)-N-(3-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)propyl)pentanamide (SV5). Following the general method, compound **SV5** was obtained as white solid in 66% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.34. ^{1}H NMR (200 MHz, $(CD_3)_2CO$) δ 7.75–7.42 (m, 3H), 7.19–6.96 (m, 2H), 6.86 (d, J = 8.1 Hz, 1H), 6.56 (d, J = 15.6 Hz, 1H), 3.84 (s, 3H), 3.52 (dd,

J = 13.4, 7.1 Hz, 1H), 3.42–3.23 (m, 4H), 3.20–2.98 (m, 2H), 2.40 (dt, J = 18.8, 6.4 Hz, 1H), 2.22 (t, J = 7.2 Hz, 2H), 1.94–1.50 (m, 8H), 1.48–1.30 (m, 2H). ^{13}C NMR (50 MHz, $(CD_3)_2CO$) δ 173.7, 167.1, 149.2, 148.6, 140.7, 127.8, 122.6, 119.5, 116.2, 111.2, 57.1, 56.1, 40.8, 39.0, 37.3, 37.1, 36.6, 35.3, 30.6, 29.6, 26.2. MS (ESI) m/z calculated for $C_{21}H_{29}N_2O_4S_2^-$ $[M-H]^-$ 437.2, found 437.2. Anal. Calcd for $C_{21}H_{30}N_2O_4S_2$: C, 57.51; H, 6.89; N, 6.39; O, 14.59; S, 14.62. Found: C, 57.55; H, 6.91; N, 6.35; O, 14.64; S, 14.58.

3.1.3.11. (E)-5-(1,2-dithiolan-3-yl)-N-(2-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)ethyl)pentanamide (SV6). Following the general method, compound **SV6** was obtained as white solid in 66% yield. R_f (CH_2Cl_2/MeOH 9.5:0.5) = 0.27. ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.48 (s, 1H), 7.95 (d, J = 25.1 Hz, 2H), 7.32 (d, J = 15.8 Hz, 1H), 7.16–6.93 (m, 2H), 6.78 (d, J = 7.5 Hz, 1H), 6.41 (d, J = 15.9 Hz, 1H), 3.79 (s, 3H), 3.17 (s, 6H), 2.37 (d, J = 6.6 Hz, 1H), 2.06 (s, 2H), 1.84 (s, 1H), 1.50 (s, 5H), 1.35 (s, 2H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 172.4, 165.7, 148.3, 147.9, 139.2, 126.4, 121.6, 118.9, 115.7, 110.8, 56.2, 55.6, 40.0, 38.5, 38.2, 36.2, 35.4, 34.2, 28.4, 25.1. MS (ESI) m/z calculated for $C_{20}H_{27}N_2O_4S_2^-$ $[M-H]^-$ 423.1, found 423.2. Anal. Calcd for $C_{20}H_{28}N_2O_4S_2$: C, 56.58; H, 6.65; N, 6.60; O, 15.07; S, 15.10. Found: C, 56.60; H, 6.68; N, 6.57; O, 15.01; S, 15.14.

3.1.4. General procedure for the phenolic hydroxyl group deprotection

The methoxy-containing compound (0.50 mmol) was dissolved in 5 mL of dry CH_2Cl_2 and cooled to 0 °C. A solution of $BF_3{\cdot}SMe_2$ (10 mmol/methoxy) was added to the mixture. The solution was allowed to slowly warm to room temperature and stirred for a total of 18 h. The reaction was stopped by the addition of 1 mL of methanol. The volatiles were removed *in vacuo*, the residue was purified with column chromatography using CH_2Cl_2/MeOH as eluent.

3.1.4.1. N-(2-(2-(3,4-dihydroxyphenyl)acetamido)ethyl)-5-(1,2-dithiolan-3-yl)pentanamide (SV7). Following the general method, **SV7** was obtained as white solid in 22% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.41. ^{1}H NMR (200 MHz, CD_3OD) δ 6.83 (dd, J = 5.0, 3.0 Hz, 2H), 6.70 (dd, J = 8.1, 2.0 Hz, 1H), 3.67 (dt, J = 12.5, 6.2 Hz, 1H), 3.48–3.45 (m, 2H), 3.42 (dd, J = 3.3, 1.7 Hz, 2H), 3.39 (s, 2H), 3.31–3.12 (m, 2H), 2.56 (td, J = 12.4, 6.5 Hz, 1H), 2.26 (t, J = 7.3 Hz, 2H), 1.99 (td, J = 13.6, 7.0 Hz, 1H), 1.72 (td, J = 13.5, 6.6 Hz, 4H), 1.55 (dd, J = 13.4, 7.2 Hz, 2H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.4, 175.1, 146.4, 145.4, 128.0, 121.5, 117.2, 116.4, 57.5, 43.4, 41.3, 40.1, 39.9, 39.3, 36.9, 35.7, 29.9, 26.6. MS (ESI) m/z calculated for $C_{18}H_{26}N_2O_4S_2^-$ $[M-H]^-$ 397.1, found 396.7. Anal. Calcd for $C_{18}H_{26}N_2O_4S_2$: C, 54.25; H, 6.58; N, 7.03; O, 16.06; S, 16.09. Found: C, 54.28; H, 6.55; N, 7.01; O, 16.03; S, 16.13.

3.1.4.2. N-(4-(2-(3,4-dihydroxyphenyl)acetamido)butyl)-5-(1,2-dithiolan-3-yl)pentanamide (SV8). Following the general method, **SV8** was obtained as white solid in 50% yield. R_f (CH_2Cl_2/MeOH 8:2) = 0.54 ^{1}H NMR (200 MHz, CD_3OD) δ 8.05–7.82 (m, 1H), 6.70 (d, J = 7.9 Hz, 2H), 6.58 (dd, J = 8.1, 1.9 Hz, 1H), 3.55 (dt, J = 12.5, 6.4 Hz, 1H), 3.33 (s, 2H), 3.21–3.06 (m, 6H), 2.44 (td, J = 12.4, 6.4 Hz, 1H), 2.18 (t, J = 7.2 Hz, 2H), 1.86 (dt, J = 19.8, 6.9 Hz, 1H), 1.62 (dt, J = 15.4, 7.8 Hz, 4H), 1.49 (s, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.0, 174.7, 146.3, 145.3, 128.2, 121.4, 117.1, 116.3, 57.5, 43.4, 41.3, 40.1, 40.0, 39.3, 36.9, 35.7, 29.9, 27.8, 27.7, 26.8. MS (ESI) m/z calculated for $C_{20}H_{31}N_2O_4S_2^+$ $[M+H]^+$ 427.1, found 426.9.

Anal. Calcd for $C_{20}H_{30}N_2O_4S_2$: C, 56.31; H, 7.09; N, 6.57; O, 15.00; S, 15.03. Found: C, 56.33; H, 7.11; N, 6.58; O, 14.98; S, 15.00.

3.1.4.3. N-(3-(2-(3,4-dihydroxyphenyl)acetamido)propyl)-5-(1,2-dithiolan-3-yl)pentanamide (SV9). Following the general method, compound **SV9** was obtained as thick oil in 62% yield. R_f (CH_2Cl_2/MeOH 8:2) = 0.42. ^{1}H NMR (200 MHz, CD_3OD) δ 7.90 (br s, 1H), 6.71–6.61 (m, 2H), 6.60–6.50 (m, 1H), 4.84 (s, 3H), 3.49 (d, J = 3.9 Hz, 1H), 3.26 (s, 2H), 3.19–3.00 (m, 6H), 2.50–2.30 (m, 1H), 2.14 (t, J = 7.2 Hz, 2H), 1.91–1.73 (m, 1H), 1.70–1.50 (m, 6H), 1.49–1.33 (m, 2H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.1, 174.9, 146.4, 145.4, 128.2, 121.4, 117.1, 116.4, 57.5, 43.4, 41.3, 39.4, 37.9, 37.7, 36.9, 35.7, 30.2, 29.9, 26.7. MS (ESI) m/z calculated for $C_{19}H_{27}N_2O_4S_2^-$ $[M-H]^-$ 441.1, found 441.1. Anal. Calcd for $C_{19}H_{28}N_2O_4S_2$: C, 55.31; H, 6.84; N, 6.79; O, 15.51; S, 15.54. Found: C, 55.38; H, 6.88; N, 6.74; O, 15.48; S, 15.52.

3.1.4.4. 5-(5-(1,2-dithiolan-3-yl)pentanamido)pentyl 2-(3,4-dihydroxyphenyl)acetate (SV10). Following the general method, **SV10** was obtained as thick oil in 42% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.34. ^{1}H NMR (200 MHz, CD_3OD) δ 6.73–6.66 (m, 3H), 6.56 (d, J = 8.1 Hz, 2H), 4.07 (t, J = 6.4 Hz, 2H), 3.65 (s, 2H), 3.53 (dd, J = 13.4, 5.9 Hz, 1H), 3.12 (dt, J = 8.2, 6.8 Hz, 4H), 2.45 (dt, J = 12.9, 6.6 Hz, 1H), 2.18 (t, J = 7.1 Hz, 2H), 1.86 (dt, J = 19.8, 7.0 Hz, 1H), 1.70–1.53 (m, 6H), 1.52–1.31 (m, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.0, 174.1, 146.3, 145.4, 127.0, 121.6, 117.3, 116.3, 65.7, 57.6, 41.6, 41.3, 40.2, 39.3, 36.9, 35.7, 30.0, 29.9, 29.3, 26.8, 24.4. MS (ESI) m/z calculated for $C_{21}H_{30}NO_5S_2^-$ $[M-H]^-$ 440.1, found 440.1. Anal. Calcd for $C_{21}H_{31}NO_5S_2$: C, 57.12; H, 7.08; N, 3.17; O, 18.12; S, 14.52. Found: C, 57.18; H, 7.06; N, 3.21; O, 18.07; S, 14.48.

3.1.4.5. 2-(2-(5-(1,2-dithiolan-3-yl)pentanamido)ethoxy)ethyl 2-(3,4-dihydroxyphenyl)acetate (SV11). Following the general method, **SV11** was obtained as white solid in 32% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.35. ^{1}H NMR (200 MHz, CD_3OD) δ 6.73–6.66 (m, 4H), 6.60–6.52 (m, 2H), 4.28–4.13 (m, 2H), 3.68–3.61 (m, 5H), 3.50–3.44 (m, 4H), 3.11 (dd, J = 13.5, 6.7 Hz, 2H), 2.43 (dd, J = 12.4, 5.9 Hz, 1H), 2.17 (d, J = 6.9 Hz, 2H), 1.96–1.77 (m, 1H), 1.74–1.50 (m, 4H), 1.45 (s, 2H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.2, 173.9, 146.3, 145.4, 126.9, 121.6, 117.3, 116.3, 70.5, 69.9, 65.0, 57.5, 41.4, 41.2, 40.3, 39.3, 36.8, 35.7, 29.8, 26.7. MS (ESI) m/z calculated for $C_{20}H_{28}NO_6S_2^-$ $[M-H]^-$ 442.1, found 442.1. Anal. Calcd for $C_{20}H_{29}NO_6S_2$: C, 54.15; H, 6.59; N, 3.16; O, 21.64; S, 14.46. Found: C, 54.18; H, 6.55; N, 3.18; O, 21.61; S, 14.49.

3.1.4.6. 3-(5-(1,2-dithiolan-3-yl)pentanamido)propyl 2-(3,4-dihydroxyphenyl)acetate (SV12). Following the general method, **SV12** was obtained as thick oil in 49% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.34. ^{1}H NMR (200 MHz, CD_3OD) δ 6.74–6.65 (m, 3H), 6.61–6.50 (m, 2H), 4.10 (t, J = 6.3 Hz, 2H), 3.61–3.50 (m, 1H), 3.47 (s, 2H), 3.29–3.07 (m, 4H), 2.43 (dd, J = 12.7, 6.3 Hz, 1H), 2.18 (t, J = 7.2 Hz, 2H), 1.83 (td, J = 13.1, 6.6 Hz, 4H), 1.72–1.45 (m, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.1, 174.0, 146.3, 145.4, 126.9, 121.6, 117.3, 116.2, 63.4, 57.5, 41.4, 41.2, 39.3, 37.0, 36.9, 35.7, 29.8, 29.5, 26.7. MS (ESI) m/z calculated for $C_{19}H_{28}NO_5S_2^+$ $[M+H]^+$ 431.2, found 431.2. Anal. Calcd for $C_{19}H_{27}NO_5S_2$: C, 55.18; H, 6.58; N, 3.39; O, 19.34; S, 15.51. Found: C, 55.21; H, 6.56; N, 3.41; O, 19.30; S, 15.53.

3.1.4.7. 2-(5-(1,2-dithiolan-3-yl)pentanamido)ethyl 2-(3,4-dihydroxyphenyl)acetate (SV13). Following the general method, **SV13** was obtained as white solid in 23% yield. R_f (CH_2Cl_2/MeOH 9:1) = 0.34. ^{1}H NMR (200 MHz, CD_3OD) δ 6.72–6.66 (m, 4H), 6.60–6.51 (m, 2H), 4.13 (t, J = 5.4 Hz, 2H), 3.66 (s, 3H), 3.41 (d, J = 5.4 Hz, 2H), 3.12 (dd, J = 13.8, 7.0 Hz, 2H), 2.44 (dd, J = 12.3, 6.1 Hz, 1H), 2.15 (t, J = 7.3 Hz, 2H), 1.86 (dd, J = 12.9, 6.9 Hz, 1H), 1.58 (d, J = 7.5 Hz, 4H), 1.45 (s, 2H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.5, 173.9, 146.3, 145.4, 126.9, 121.6, 117.3, 116.3, 64.2, 57.6, 41.3, 41.2, 39.4, 39.3, 36.8, 35.7, 29.8, 26.7. MS (ESI) m/z calculated for $C_{18}H_{24}NO_5S_2^-$ $[M-H]^-$ 398.1, found 398.0. Anal. Calcd for $C_{18}H_{25}NO_5S_2$: C, 54.11; H, 6.31; N, 3.51; O, 20.02; S, 16.05. Found: C,

54.10; H, 6.33; N, 3.57; O, 20.11; S, 15.98.

3.1.5. HPLC-UV assays

Analytical HPLC apparatus was a Waters 600 HPLC pump (Waters Corporation, Milford, MA, USA), equipped with a Waters 2996 photodiode array detector. The mobile phase, consisting in a mixture of Water and Acetonitrile +0.1% of trifluoroacetic acid, was flushed with a flow of 0.8 mL/min in a Hypersil GOLD C18 column (250 × 4.6 mm).

3.1.6. Solubility

Solubility was determined as previously reported [30].

3.1.7. Stability in gastrointestinal fluids

Simulated gastric fluid (SGF) and intestinal fluids (SIF), containing Pepsin (3.2 mg/mL) and Pancreatin (10 mg/mL), respectively, were prepared following the USP specifications. A drug stock solution, prepared in organic solvent, was added to pre-heated fluids placed in a 37 °C water bath. At fixed time points, 100 μL of sample was picked up and deproteinised with 100 μL of ice-cold acetonitrile. Each sample, after centrifugation at 4 °C and 12000 rpm for 10 min, was filtered and analyzed by HPLC. The relative difference (RD%), measured with the following equation, was used to estimate the amount of degraded compound in different gastrointestinal medium.

$$RD = \frac{C_i - C_f}{C_i} \times 100\%$$

C_i is the amount of drug found at zero time point and C_f is the quantity found at the end of each incubation time [32].

3.1.8. Human plasma stability

Human plasma was purchased by 3H Biomedical, Uppsala, Sweden. Enzymatic hydrolysis was evaluated adding a drug stock solution to a pre-heated (37 °C) plasma fraction, previously diluted with 0.02 M phosphate buffer (pH 7.4) to give a final sample containing 80% plasma. Samples of 100 μL were taken at various times and 200 μL of cold acetonitrile were used to stop the enzymatic process. After centrifugation, the supernatant was analyzed by HPLC [33].

3.1.9. Cell culture and treatments

The human neuroblastoma SH-SY5Y cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). SH-SY5Y cells were seeded in growth medium containing DMEM/F12 (1:1) medium (Hyclone, Inc.) with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Thermo Fisher Scientific) and were incubated in a humidified atmosphere containing 5% CO_2 atmosphere at 37 °C. The SH-SY5Y cells were differentiated with using retionoic acid (RA, Sigma-Aldrich, 10 μM) dissolved in ethanol (Merck, 10 mM).

3.1.10. Cell proliferation assay

Cell proliferation was determined using the MTT assay. Differentiated SH-SY5Y cells were placed onto 96-well plates (1×10^4 cells/well) and cultured for 24 h before beginning the experiment. The cells were exposed to $A\beta_{1-42}$ (20 μM), with or without various concentrations of SV hybrids (1−250 mg/L) for 48 h. The MTT assay was carried out by adding MTT solution (5 mg/mL in PBS) and incubating 4 h at 37 °C. The formazan was dissolved by mixing with DMSO and the optical density of reaction products was read at 570 nm and 630 nm by Epoch™ spectrophotometer (BioTek Instruments, Inc., Vermont, USA). All data were presented as mean ± S.D. from five independent experiments. Untreated cells used as negative control and 1% Triton-X was used as the positive control.

3.1.11. LDH assay

The measurement of LDH release was performed using the CytoSelect LDH Assay Kit (Cell BioLabs, San Diego, CA) following to the providers' manual. Differentiated SH-SY5Y cells were placed onto 96-well plates (1×10^4 cells/well) and cultured for 24 h before beginning the experiment. The cells were exposed to $A\beta_{1-42}$ (20 μM), with or without various concentrations of SV hybrids (1−250 mg/L) for 48 h. After incubation, 90 μl of the supernatant from each well was mixed with 10 μl of LDH reagent and incubated for 30 min. The optical density was read at 450 nm by Epoch™ spectrophotometer (BioTek Instruments, Inc., Vermont, USA).

3.1.12. Detection of morphological alterations with Hoechst 33258 staining

Apoptotic morphological evaluation of SH-SY5Y cells was examined by Hoechst 33258 staining. Briefly, the 4×10^4 cells/well were seeded in 24-well plates. After 48 h of treatment of cells exposed to $A\beta_{1-42}$ with or without SV derivatives (IC_{50}), cells were incubated in fixation solution (4% paraformaldehyde in PBS) for 30 min, and then rinsed with PBS. The cells were incubated with Hoechst 33258 fluorescent dye for 15 min. Nuclear morphology was viewed and photographed under a fluorescence microscopy (Leica® DM IL LED).

3.1.13. Annexin V-FITC/propidium iodide staining

The rate of apoptosis was examined using a BioVision annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA) following the provides' instructions. Following 48 h of treatment as described above, differentiated SH-SY5Y cells were collected and rinsed with PBS. The cells were centrifuged for 3 min at 500×*g* and resuspended in buffer solution (0.5 mL). Then, the cells were incubated with annexin V (5 μL) and propidium iodide (5 μL) for 5 min in dark. Fluorescence was detected with FL-1 (530 nm) and FL-2 filters (585 nm) using a CyFlow® Cube 6 flow cytometer (Partec; Münster, Germany) to evaluate apoptosis.

3.1.14. RNA isolation and quantitative RT-qPCR

Differentiated SH-SY5Y cells in 6-well plates (2×10^7 cells/well) were treated as described above for 48 h. After treatment, total RNA was isolated by a PureLink RNA Mini kit (Invitrogen). cDNA synthesis was performed with equal amounts of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturers' protocol. Then, SYBR Green PCR Master Mix (Applied Biosystems) and primers designed with Primer 3 software were used to quantitatively detect the gene expression on a Rotor-Gene Q (Qiagen). Expression data were normalized to β-actin. The sequences of primer pairs were listed in Table 2S. Each experiment was performed in triplicate.

3.1.15. Cytotoxicity/cytoprotectivity testing

Human whole blood cultures were set up according to a previous report [34]. Briefly, the heparinized blood (0.6 mL) was cultured in 7 mL culture medium (Chromosome Medium B; Biochrom, Germany) containing 5 μg/L of phytohemagglutinin. Different SV hybrids concentrations (1.56, 3.12, 6.25, 12.5, 25 50, 100, 200 and 400 μM) were applied into culture tubes. After addition of SV hybrids, the whole blood samples were incubated for 72 h at 37 °C. Hydrogen peroxide (H_2O_2; Merck, Germany; 25 μM) was used to induce oxidative damage. Cells of the positive control were treated with 1% triton-X100 and ascorbic acid (AA; $C_6H_8O_6$; Sigma-Aldrich) containing medium in testing cytotoxicity and oxidative alterations. In addition, individual culture without SV hybrids, H_2O_2 was

studied as a negative control (Control (−)) group [35]. All experiments have been performed according to the Declaration of Helsinki.

MTT assay was performed to assess cytotoxicity by SV hybrids. Isolated lymphocytes (2×10^5) were seeded in a 96-well plate and treated with SV hybrids at certain concentrations (0–400 μM) for 24 h in the presence or absence of H_2O_2. At the end of incubation period MTT solution was added into each well and incubated at 37 °C for additional 4 h. The resulting formazan crystals were solubilized with DMSO for 30 min. Then, the absorbance was spectrophotometrically measured at 570 nm [33].

LDH amount released from damaged cells in culture medium was determined via LDH assay kit (Cayman Chemical, USA) according to the provider's guide. The cell medium samples (100 μL) from each group was used for LDH analysis.

3.1.16. Oxidative/antioxidative analysis

The automated total antioxidant capacity (TAC) assays were performed by commercially available kits (Rel Assay Diagnostics, Turkey) on medium samples from whole blood cultures for 24 h. TAC provides reliable measurement of the antioxidant capacity of all antioxidants in a biological sample and not just of a single antioxidant [36].

3.1.17. Statistical analysis

All data presented in this study were expressed as the mean ± SD of and obtained from at least five independent experiments. Statistical analysis was carried out using SPSS statistical software version 22.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan's test was used for statistical comparisons. A p-value < 0.05 was regarded as statistically significant.

Notes

The authors declare no conflicts of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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