ROS

RESEARCH ARTICLES

Oxidative Stress and Neuronal Damages: Estrogenic Compounds, Anti-Apoptotic Factors, and Amyloidogenesis

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ABSTRACT | In stroke, increased oxidative stress (OS), mediated by reactive oxygen species (ROS) including H_2O_2 release, can induce changes that lead to neural production of amyloid-β (Aβ), a hallmark protein in the brains of Alzheimer's disease (AD) patients. Aβ peptide can also induce OS by itself or by activating microglia to release ROS. Estrogenic compounds can protect neurons against Aβ- and OS- induced cell death. Aβ- and OS-induced cell death is another hallmark of AD. We have studied OS-related cell damage by exposing rat primary hippocampal neurons and differentiated human SH-SY5Y neuroblastoma cells to H_2O_2 or $Aβ_{1–42}$ and evaluated the neuroprotective potential of 17β-estradiol (E2), estrone (E1), tamoxifen (Tam), 4-OH-tamoxifen (4-OH-Tam), diethylstilbestrol (Des) and genistein (Gen) against OS. These compounds have differences in estrogen receptor (ER) binding affinities and their number of antioxidative –OH groups varies. The cell damage indicator was the lactate dehydrogenase release into culture medium. Treatment with 5 nM E2, Gen, or 4-OH-Tam for 24 h before and after the H_2O_2 insult was neuroprotective in both hippocampal and SH-SY5Y cultures. E2 and Gen were neuroprotective against $Aβ_{1–42}$-mediated toxicity. Protection by E2 was partially mediated by Bcl-2, Bcl-xL, and BAG-1. Tam also increased Bcl-2 and Bcl-xL but was much less neuroprotective. Gen increased amyloid precursor protein (APP) synthesis, but γ-secretase component PS-1 was reduced, suggesting that Gen can increase the production of neurotrophic soluble APPα. Des increased Aβ production. In conclusion, Gen shows comparable neuroprotective efficacy to E2, and seems also to reduce Aβ production in our study. However, other neuroprotective mechanisms may exist, and further studies on this subject will enhance our understanding in this respect.

KEYWORDS | Amyloid-β; BAG-1; Bcl-2; Bcl-xL; Estrogen; Oxidative stress; Presenilin-1; Selective estrogen receptor modulator

ABBREVIATIONS | AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; BDNF, brain-derived neurotrophic factor; CAV, caveolin; Des, diethylstilbestrol; E1, estrogen; E2, 17β-estradiol; E3, estriol; ERRγ, estrogen-related receptor-γ; Gen, genistein; LDH, lactate dehydrogenase; NFT, neurofibrillary tangles; NTF, N-terminal fragment; 4-OH-Tam, 4-hydroxytamoxifen; α-OH-Tam, α-hydroxytamoxifen; OS,

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oxidative stress; PS-1, presenilin-1; ROS, reactive oxygen species; SERM, selective estrogen receptor modulator; VDAC, voltage-dependent anion channel

CONTENTS

1. INTRODUCTION

Oxidative stress (OS), with increased lipid peroxidation and free radical production that mediate cell death, has been linked to many neurodegenerative diseases, including Alzheimer's disease (AD) and, to a lesser degree, aging [1–3]. In AD brains, several pathological changes can be found. These include neuro-inflammation that involves activated microglia and excitotoxic damage, extracellular deposition of amyloid-β (Aβ)-containing plaque and intracellular deposition of neurofibrillary tangles (NFT) composed of hyperphosphorylated tau (p-tau), and most importantly, a loss of neurons. Increased presence of OS markers, such as 8-hydroxyguanosine and 4-hydroxynonenal adducts, becomes detectable in the early stages of AD pathology [4]. Reactive oxygen species (ROS) are normally produced as by-products of oxidative metabolism including mitochondrial respiration, but they are generated also during pathological events, including inflammation. Common ROS include superoxide, hydroxyl radicals, hydrogen peroxide (H_2O_2), and nitric oxide (NO). Superoxide, if not scavenged, reacts with NO, resulting in peroxynitrite production. ROS can act as free radicals or they can react with other molecules to form free radicals, capable of oxidizing a wide range of substrates and causing many types of biological damage [1, 5].

Brain metabolism requires relatively large amounts of oxygen, resulting in production of ROS. Some of the generated ROS is required in memory formation, development in general, or as defense against pathogens. But when unregulated, ROS are generally harmful [6–8]. Nonetheless, oxidative burden increases during aging, especially in the central nervous system (CNS), sensitizing it to many pathologies [2]. Neurons are especially sensitive to oxidants as their normal antioxidant content is low and neuronal membranes contain a great amount of oxidant-sensitive polyunsaturated fatty acids. OS can, for instance, promote Aβ generation, as well as act as inducers for kinases involved in hyperphosphorylation of the microtubule associated protein tau, resulting in NFT [9, 10]. Neurons also have high levels of iron that can initiate the Fenton reaction, producing hydroxyl radicals [11]. For instance, Aβ can enhance the production of toxic H_2O_2 and liberation of hydroxyl radicals in the presence of minor amounts of Cu^{2+} or Fe^{3+} in vitro [9].

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Microglia, the principal defense cells in the CNS, display a quiescent phenotype in the normal adults. In the context of killing invading pathogens or brain injury in vivo, their activation lead to production of ROS, including H_2O_2, as well as reactive nitrogen intermediates, which are released as oxidative bursts causing an external oxidative and nitrosative stress to surrounding cells. Active microglia, as well as reactive astrocytes, also surround Aβ-plaques and dead or damaged neurons in AD. This suggests a clearance effort of Aβ and cell debris by these active cells, and the potential to cause further damage, since Aβ deposition or fibrils can also promote microglial activation, leading to ROS production [12–14]. Nevertheless, overload of internal or external ROS contributes to brain injury by inducing lipid peroxidation, DNA fragmentation, and protein oxidation in neurons and their supporting cells, and causes also leakage of the blood-brain barrier. ROS can lead to activation of proapoptotic pathways. They can also activate different defense systems and antiapoptotic signaling programs, as evidenced by a role of ROS in synaptic plasticity [6, 7].

Estrogens have been proven to increase the viability of different neural cell cultures as well as to protect neural cells against various insults, including OS and Aβ protein [15–19]. For instance, estrogen regulates several neurotrophic genes, including brain-derived neurotrophic factor (BDNF), neutrophins 3 and 4, and nerve growth factor [20, 21], as well as many genes involved in neuroprotection, such as anti-apoptotic proteins Bcl-2 [22, 23] and Bcl-xL [24], and caspase inhibitors [25]. The neuroprotective features of estrogens are likely transmitted by a spectrum of independent processes, and not only via classic estrogen receptors (ERs), ERα and ERβ. However, ERα gene variants have been shown to be associated with AD susceptibility [26].

The most important endogenous estrogens are fat soluble estrone (E1), estradiol (17β-estradiol, E2), and estriol (E3) that readily pass through cell membranes. Other well-known estrogens include synthetic diethylstilbestrol (Des) and genistein (Gen; 4′,5,7-trihydroxyisoflavone). Selective estrogen receptor modulators (SERMs) are synthetic ER ligands with different kinds of properties. Different SERMs can have distinct agonist/antagonist profiles in different tissues [27–29]. Some of their actions can also be unwanted. The mixed agonist/antagonist activity of SERMs is a consequence of the specific cell and promoter context, depending of the variability and availability, for instance, of transcription factors, co-factors, and members of kinase pathways [29]. One of the SERMs approved in clinical use is tamoxifen (Tam), a nonsteroidal antiestrogenic and antiproliferative drug, which can act as a tissue specific agonist or antagonist. Tam is metabolized in the liver and its oxidative metabolites include α-hydroxytamoxifen (α-OH-Tam) and 4-hydroxytamoxifen (4-OH-Tam). 4-OH-Tam is also an ER ligand, being the most potent but the least common of Tam's metabolites.

Our study aimed to compare the neuroprotective potential of estrogens E1, E2, isoflavone Gen, and SERMs (including Des, Tam, and 4-OH-Tam) (**Figure 1**) against H_2O_2 and $A\beta_{1–42}$-induced cell damage. H_2O_2 was chosen, since it can be detected in the rat brain at micromolar concentrations after ischemia/reperfusion [30]. H_2O_2 is also a relatively stable compound among ROS, and it can diffuse freely inside cells and across membranes, and furthermore, it reacts with Fe^{2+} to give rise to the highly reactive hydroxyl radicals [30]. $A\beta_{1–42}$ is more toxic than $A\beta_{1–40}$ [31]. We also elucidated the impact of E2 and SERMs on anti-apoptotic targets Bcl-xL, Bcl-2, and BAG-1 (Bcl-2-associated athanogene/BAG family molecular chaperone regulator 1), as well as on amyloidogenic pathway members amyloid precursor protein (APP) and presenilin-1 (PS-1) [32, 33].

2. MATERIALS AND METHODS

2.1. Rat Primary Hippocampal Neurons

Primary neuronal cultures of hippocampus were obtained from 17-day-old embryos (E17) of outbred Harlan Spraque Dawley (Hsd:SD) rats [34]. Briefly, the 17-day-old embryos were removed from the euthanized mothers and placed in an isolation buffer (IB) containing 1 mg/ml bovine serum albumin (Roche, Mannheim, Germany), 10 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (BioWhittaker/Cambrex, East Rutherford, NJ, USA). The hippocampal areas of the pups were isolated and digested in an IB supplemented with 0.5 mg/ml papain (Sigma-Aldrich), at 37°C for 10–15 min. The cells were dissociated in deoxyribonuclease I (Roche)-supplemented IB by trituration. The cells were plated 6×10^4 cells/cm^2 onto the poly-D-lysine (Sigma-Aldrich) coated 24-well plates

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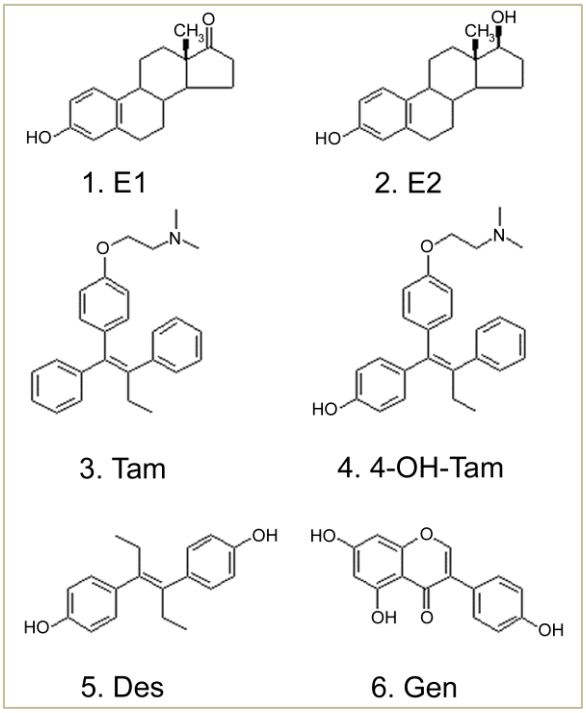

FIGURE 1. The chemical structures of the estrogenic compounds. (1) Estrone (E1, MW: 270.366 g/mol) is the main estrogen form in post-menopausal women. E1 concentration is 17–200 pg/ml in premenopausal women, whereas in postmenopausal women it is around 7–40 pg/ml [35]. The most potent estrogen is (2) 17β-estradiol (E2, MW: 272.388 g/mol). Both these estrogens are involved in many functions of the brain, including neuronal survival, growth and differentiation, synaptic plasticity and long-term potentiation. They can also function as antioxidants [36, 37]. The physiological concentration of E2 varies depending on age and individual; the average is around 130–200 pg/ml in serum in adult premenopausal women, but the concentration can also be much higher [35]. (3) Synthetic Tamoxifen (Tam, MW: 371.515 g/mol) is a nonsteroidal antiestrogenic and antiproliferative SERM, which can also have neuroprotective properties [38]. However, some of its actions may also be mediated by OS [39]. Tam is metabolized in the liver and its oxidative metabolites include (4) 4-hydroxytamoxifen (4-OH-Tam (Afimoxifene), MW: 387.514 g/mol). 4-OH-Tam has antioxidant properties and can inhibit lipid peroxidation [40]. Tam and its metabolite levels varies in serum, depending what is the medical dosage, but the range is commonly 5–85 ng/ml [41]. (5) Diethylstilbestrol (Des, MW: 268.356 g/mol) is a synthetic estrogen with antioxidative properties, although it is also carcinogenic [42]. (6) Genistein (Gen (4′,5,7-trihydroxyisoflavone), MW: 270.241 g/mol) is one of the principal isoflavones in the soy bean, and isoflavones are considered as weak estrogens [43]. E2 and Tam have nearly the highest and equal binding affinity for ERα and ERβ, E1 has preferential binding affinity for ERα over ERβ, Des has slightly higher binding affinity to ERβ than to ERα, whereas Gen favors ERβ [44]. However, when bound, ER ligands induce distinct conformational changes in the ERs affecting their biological activity.

(Cellstar) in Neurobasal™ media (Gibco/Life Technologies/Thermo Fisher, Waltham, MA) supplemented with 0.5 mM L-glutamine (Sigma-Aldrich), 10 U/ml penicillin (Sigma-Aldrich), 10 mg/ml streptomycin (Sigma-Aldrich) and 5% heat inactivated fetal bovine serum (iFBS) (Hyclone/GE Healthcare Life Sciences, Chicago, IL, USA). Medium was replaced with 1× B-27 (Gibco) supplemented medium after a 24 h incubation. The cells were maintained at 37°C in a saturated humidity atmosphere, containing

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95% air and 5% CO_2. The cells were used for the experiments on the 8th day. A day before starting the preincubation with the test compounds, Neurobasal™ medium containing B-27 supplement was replaced by Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose, without phenol red; Sigma-Aldrich) containing 1× B-27 Supplement Minus AO (Gibco).

The animal procedures were conducted in accordance with the guidelines of the European Community Council (Directives 86/609/EEC) and Finnish guidelines and had the approval of the Animal Use and Care Committee of the University of Turku. All efforts were made to minimize the pain, discomfort, and number of experimental animals.

2.2. SH-SY5Y Cells

The human neuroblastoma SH-SY5Y cells (DSMZ, Germany) were grown in 50% Optimem I medium (Gibco) and 50% DMEM (4.5 g/L glucose, without phenol red; Gibco) supplemented with 2 mM L-glutamine, 10 U/ml penicillin, 10 mg/ml streptomycin and 5% twice dextran charcoal treated inactivated FBS (2× dciFBS, Autogen Bioclear, Wiltshire, UK). For the experiment, the cells were plated onto the 24-well plate (4.5×10^4 cells/cm^2) and the Optimem I/DMEM ratio were changed to 20%/80%. After 24 h, the SH-SY5Y cells were differentiated as described before [45]. Briefly, the cells were cultured for five days in the presence of 10 μM all trans retinoic acid (ATRA; Sigma-Aldrich), after which the cells were washed twice with DMEM and incubated for three days with 60 ng/ml human BDNF in DMEM without ATRA, serum, or phenol red. The cells were used for the experiment on the 8th day after the addition of ATRA.

2.3. Test Compounds

Estrone (IUPAC: 3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthrene-17-one; Hormos Medical, Turku, Finland)), 17β-estradiol (IUPAC: (17β)-estra-1,3,5(10)-triene-3,17-diol; Sigma-Aldrich), tamoxifen (IUPAC: (*Z*)-2-[4-(1,2-diphenylbut-1-enyl) phenoxy]-*N*,*N*-dimethylethanamine; Orion Pharma, Espoo, Finland), 4-OH-tamoxifen (Sigma-Aldrich), diethylstilbestrol (IUPAC: 4,4′-(3*E*)-hex-3-ene-3,4-diyldiphenol; Sigma-Aldrich) and genistein (IUPAC: 5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one; Hormos Medical) were dissolved in dimethyl sulfoxide (DMSO, Merck, Keniworth, NJ, USA) to make a 10 mM stock solution. The highest concentration of DMSO in the cultures was 0.01%. The cells were treated with 1 nM–2.5 μM concentrations of the substances (the stock was diluted in culture medium) for 24 h before and after the $A\beta_{1–42}$ or the H_2O_2 exposure.

2.4. H_2O_2 Exposure

In LDH-release experiments, the described cells were exposed to 0.5 mM (primary cells) or 1 mM H_2O_2 (neuronlike SH-SY5Y cells) for 5 or 10 min, respectively, in an exposure buffer containing 2 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 12.5 mM HEPES (Sigma-Aldrich), 10 mM glucose, and 0.1 M NaCl after a 24h preincubation with DMSO vehicle, estrogens, or SERMs. After the exposure to H_2O_2, the cultures were washed with the medium without supplementation, and fresh media containing the test compounds were added to the cultures. In Western blot studies, the pretreated SH-SY5Y cells were exposed to 0.25 mM H_2O_2 for 5 min in plain culture medium. The medium was removed, and the cells were washed once with the culture medium, after which the cells were maintained in fresh normal culture medium, supplemented with the compounds, for 24 h.

2.5. $A\beta_{1–42}$ Exposure

$A\beta_{1–42}$ (Biopeptide, San Diego, CA, USA) was dissolved in PBS and aggregated for 7 days at 37ºC on a shaker. After aggregation, Aβ was aliquoted and stored at –20ºC. The differentiated SH-SY5Y cultures and the rat primary hippocampal neuron cultures were preincubated with the test compounds for 24 h after which 5 μM of aggregated $A\beta_{1–42}$ was added to the cells in fresh culture media. The cells were incubated with Aβ for 48 h in the presence of the test compounds.

2.6. Cell Harvesting and Protein Concentration Measurement

Culture media were collected, and the cells were washed with PBS 24 h after the H_2O_2 or Aβ exposure. The whole cell lysates were obtained by homogenization in RIPA buffer containing 0.5 mM ethylene-

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diaminetetraacetic acid (EDTA), 1% Triton-X 100, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M Tris-HCl (pH 7.2) and Complete™ Mini protease inhibitor cocktail tablet (Roche). Media and lysates were stored at –80ºC. The protein concentrations of the lysates were determined by using Micro BCA™ protein assay reagent kit (Pierce/Thermo Fisher) following the manufacturer's protocol in 96-well plates (Cellstar) and measured (560 nm) by a Victor^2 multilabel counter (Perkin Elmer Wallac, Waltham, MA, USA).

2.7. Cytotoxicity Assay

The activity of lactate dehydrogenase (LDH), released from damaged cells, was determined from media and lysates by performing Modified Cytotox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI, USA). The assay was performed according to the manufacturer's protocol on 96-well plates (Cellstar) and measured (490 nm) by a Victor^2 multilabel counter. In the case of cell lysate, the Promega's lysis solution was replaced with the RIPA buffer with protease inhibitors, and the lysate was then used for determining the maximum LDH release.

2.8. Immunoblotting

Equal amounts of protein (35 µg) from lysates were separated in 12.5% gels in SDS-polyacrylamide gel electrophoresis and the proteins were transferred to a PVDF-membrane (Santa Cruz Biotech, Dallas, TX, USA). The antibodies were purchased from Santa Cruz Biotech: Actin (H-196, sc-7210); BAG-1 (FL-274, sc-8348); Bcl-2 (N-19, sc-492); Bcl-xL (S-18, sc-634); Nicastrin (N-19, sc-14369); PS-1 (H-70, sc-7860). Anti-Aβ [4–10] antibody (WO-2) was purchased from the Genetics (Schlieren, Switzerland) and anti-α-tubulin (clone B-5-1-2) antibody was obtained from Sigma-Aldrich.

As described earlier [46], the membranes were blocked with 3% non-fat milk (Valio; Finland) in Tris-buffered saline (TBS; pH 7.5)/0.1% Tween-20 (Sigma-Aldrich), and the antibodies were used at 1:400 dilution, with exception of WO-2, which was used at 0.5 µg/ml. For detection, the membranes were incubated with ECL™ donkey anti-rabbit IgG F(ab')2 peroxidase-linked detection antibody (from sheep) (GE Healthcare Life Sciences) or bovine anti-goat IgG-HRP mouse/human adsorbed (sc-2384; Santa Cruz Biotech) detection antibodies. Immobilon Western chemiluminescent HRP substrate (Millipore; Billerica, MA, USA) was used for detection and Fujifilm Super RX (Fuji Photo Film, Tokyo, Japan) for documentation. The detected bands were quantitated with a MCID M5-image analysis system (Imaging Research, Ontario, Canada).

2.9. Calculations and Statistical Analysis

The percentage cytotoxicity was calculated by dividing the experimental LDH release (medium) with the maximal LDH release (lysate). All the results are expressed as mean ± SD. When the number of repetitions was high enough (≥ 3), Mann–Whitney U-test and Student's t-test (paired) were performed. The results of the separate experiments and samples of immunoblots were analyzed with parametric paired Student's t-test (T-t) and non-parametric Mann–Whitney U-test (M–W).

3. RESULTS

3.1. H_2O_2-Induced Damage in Rat Primary Hippocampal Neurons and SH-SY5Y Cell Cultures

Prior to the study, the optimal concentration of H_2O_2 and exposure time were selected to fulfill a criterion of 60% survival rate. Visible cell death and nerve branch loss after 24 h of the exposure was estimated with light microscopy. The cells were subjected to 0.1–1.5 mM concentrations of H_2O_2 in the exposure buffer and DMEM, and exposure times between 5–15 min were tested (data not shown). Cell injury was assessed by measuring the levels of LDH release. The concentration of H_2O_2 and exposure time that fulfilled our criterion for the rat hippocampal neurons was 0.5 mM H_2O_2 for 5 min. For human differentiated SH-SY5Y neuron-like cells, the conditions were 1.0 mM H_2O_2 for 10 min of exposure. The neuroprotective effects of the test compounds (**Figure 1**) against oxidative damage were determined by using 5 and 100 nM concentrations of the compounds. The protective effects of the 5 nM treatments were visualized with light microscopic photographs (**Figure 2**).

E2 was an effective neuroprotectant against H_2O_2-induced oxidative damage in rat primary hippocampal neurons, a judgment based on the morphology of

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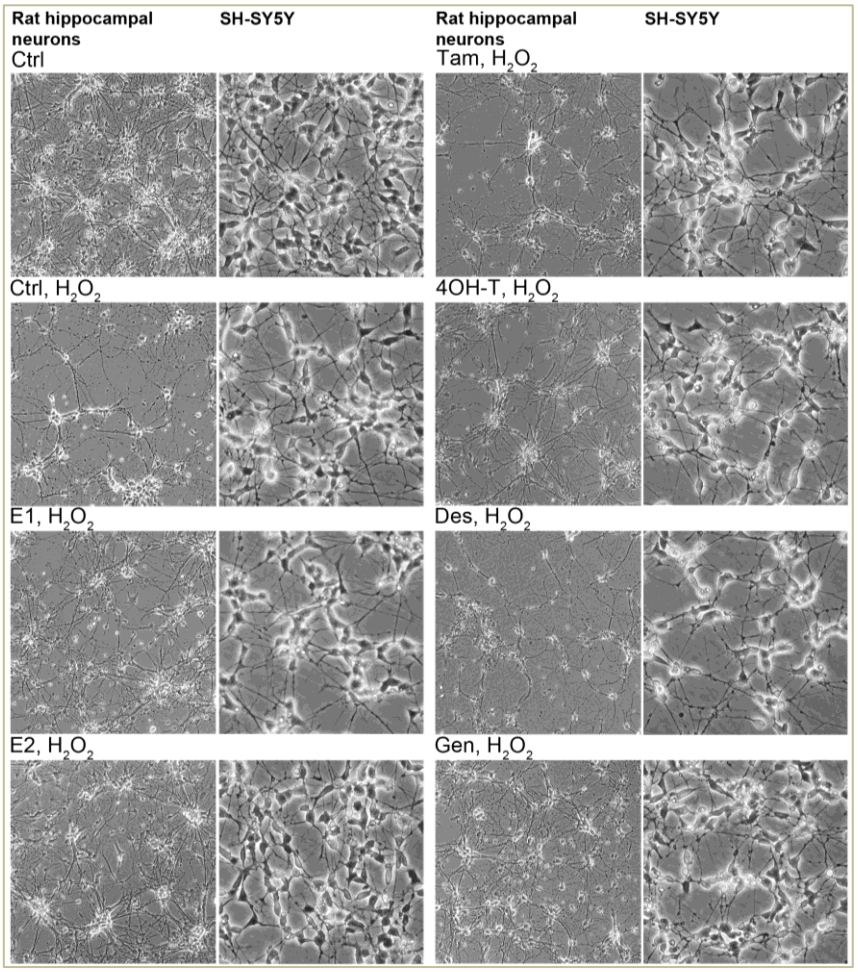

FIGURE 2. Morphological changes of rat hippocampal neurons and differentiated human SH-SY5Y neuron-like cells after the treatment with the various compounds. The cells were pretreated for 24 h with the respective compounds prior to the 0.5 mM H_2O_2 insult (5 min; hippocampal neurons) or 1.0 mM H_2O_2 exposure (10 min; SH-SY5Y). After the H_2O_2 exposure, the cells were incubated with or without respective fresh compound-containing media for 24 h. Ctrl denotes DMSO-diluter treated control cells; left, rat hippocampal neurons; right, SH-SY5Y cells. The rest denotes H_2O_2-exposed cells without (Ctrl) or with 5 nM estrogens or SERMs as described. The photographs were taken 24 h after the H_2O_2 exposure (100× magnification).

the neurites and the confluency of the cells after the treatments (**Figure 2**). Cell injury was verified in the LDH-release studies (**Table 1**). With 5 nM E2 treatment, the LDH activity was 16% less than that in H_2O_2 only-exposed cultures. The other test compounds also showed protective effects; 5 nM concentrations of 4-OH-Tam reduced LDH activity by 8%, Des by 15%, and Gen by 16%. E1 and Tam had no or little effects on LDH activity. However, the effects of the compounds were altered when 100 nM concentrations were used: treatments with Tam and 4-OH-Tam enhanced the cytotoxicity of H_2O_2; Tam increased LDH activity by 39% and 4-OH-Tam by 37%, whereas E2, Des, and Gen were still neuroprotective (**Table 1**).

In differentiated SH-SY5Y cultures, all tested compounds showed some protective effects, but the protection varied depending on the concentrations of

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TABLE 1. The percentage change of LDH release from rat hippocampal neurons

	Mean% ± SD	n	t-test, p	M–W, p
H_2O_2 Ctrl/Ctrl	37.3 ± 21.1	15	< 0.001***	< 0.001***
5 nM				
E1	–1.5 ± 14.1	12	0.711	0.421
E2	–16.3 ± 18.4	15	0.004**	0.020*
Tam	4.1 ± 11.2	14	0.194	0.481
4-OH-Tam	–7.8 ± 5.5	15	< 0.001***	< 0.001***
Des	–15.3 ± 22.4	15	0.019*	0.020*
Gen	–16.5 ± 23.4	14	0.021*	< 0.001***
100 nM				
E1	3.5 ± 25.7	5	0.774	0.550
E2	–25.6 ± 5.6	3	0.016*	0.006**
Tam	38.6 ± 22.9	5	0.020**	0.003**
4-OH-Tam	37.0 ± 13.9	6	0.001***	0.002**
Des	–21.6 ± 10.4	5	0.010**	0.003**
Gen	–7.3 ± 16.8	5	0.383	0.073

Note: The cells were treated with 5 nM concentrations of compounds or 100 nM concentrations of compounds 24 h before and after the 0.5 mM H_2O_2 insult (5 min). H_2O_2 Ctrl/Ctrl, H_2O_2 exposure control compared to untreated control; The rest, compound treatment + H_2O_2 exposure compared to H_2O_2 only exposure control; Negative value, reduction compared to respective control; Positive value, increase compared to respective value; SD, standard deviation; n, number of separate repeats; t-test, paired; M–W, Mann–Whitney U-test; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

the compounds (**Table 2**). When 5 nM treatment was used, LDH activity was decreased by 23% with E1, by 26% with 4-OH-Tam, and 30% by E2, when compared to H_2O_2 only-exposed group (**Table 2**). With 100 nM treatment, the protection improved in the case of Tam (12% → 31%) and Gen (11% → 21%) (**Table 2**). Thus, in contrary to hippocampal neurons, Tam was protective, as was E1 when a low concentration of each compound was used. In summary, among the compounds tested, E2 and Gen were the most effective neuroprotectants against H_2O_2-induced OS in both culture systems.

3.2. Aβ-Induced Cell Damage in Hippocampal Neurons and in Differentiated SH-SY5Y Cells

Preliminary tests for concentration and exposure time for $A\beta_{1–42}$ treatment were done with a concentration range of 1–20 μM, for 24 h or 48 h (data not shown). Exposure to 5 μM $A\beta_{1–42}$ for 48 h was selected for further studies, since it increased LDH activity in the culture medium in both cell culture models, although to a lesser extent compared with that induced by H_2O_2. The 48 h-treatment with 5 μM $A\beta_{1–42}$ and the estrogenic compounds showed that only 100 nM E2 and Gen were neuroprotective, both reducing LDH levels in the culture medium by almost 30% (**Table 3**).

Differentiated SH-SY5Y cultures, exposed to 5 μM $A\beta_{1–42}$ for 48 h, resulted in a 30% increase in LDH release into the culture medium compared to control cells (**Table 4**). All estrogenic compounds were neuroprotective against the $A\beta_{1–42}$ exposure (**Table 4**), although generally less potent than their protection against H_2O_2 insults in the same culture system (**Table 2**). The decrease in LDH activity was only 7% versus 23% with E1, 10% versus 26% with 4-OH-Tam, and 8% versus 30% with E2. The most protective compound was Gen (25%), as in hippocampal cultures (28%) (**Table 3**).

3.3. Gen Increased Protein Levels of Neuroprotective Bcl-2 and BAG-1 but not Bcl-xL

Anti-apoptotic Bcl-2 and Bcl-xL are known to be regulated by E2 [47, 48], but which specific ERs and additional regulatory factors are engaged in this regulation, remains poorly understood. In our earlier

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TABLE 2. The percentage change of LDH release from differentiated SH-SY5Y neuroblastomas

	Mean% ± SD	n	t-test, p	M–W, p
H_2O_2 Ctrl/Ctrl	65.2 ± 56.7	13	0.001***	<0.001***
5 nM				
E1	–23.0 ± 15.3	12	0.001***	<0.001***
E2	–29.7 ± 13.5	10	0.001***	<0.001***
Tam	–11.9 ± 13.0	11	0.003**	0.004**
4-OH-Tam	–26.5 ± 17.4	12	0.001***	<0.001***
Des	–4.5 ± 18.1	12	0.007**	0.057
Gen	–10.9 ± 23.4	10	0.008**	0.007**
100 nM				
E1	2.2 ± 12.3	3	0.487	0.790
E2	–18.9 ± 3.5	3	0.037*	0.011*
Tam	–31.2 ± 9.5	3	0.037*	0.029*
4-OH-Tam	–11.9 ± 6.0	3	0.037*	0.075
Des	8.6 ± 13.6	3	0.487	0.385
Gen	–21.0 ± 3.3	3	0.037*	0.008**

Note: The cells were treated with 5 nM or 100 nM concentrations of compounds 24 h before and after the 1.0 mM H_2O_2 insult (10 min). H_2O_2 Ctrl/Ctrl, H_2O_2 exposure control compared to untreated control; The rest, compound treatment + H_2O_2 exposure compared to H_2O_2 only exposure control; Negative value, reduction compared to respective control; Positive value, increase compared to respective value; SD, standard deviation; n, number of separate repeats; t-test, paired; M–W, Mann–Whitney U-test; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

TABLE 3. The percentage change of LDH release from rat hippocampal neurons

	Mean% ± SD	n	t-test, p
$A\beta_{42}$/Ctrl	19.6 ± 25.6	14	
E1	–19.6 ± 8.5	2	0.734
E2	–28.3 ± 6.7	3	0.028*
Tam	–26.7 ± 18.5	3	0.146
4-OH-Tam	–8.3 ± 20.2	3	0.604
Des	–6.1 ± 8.8	3	0.379
Gen	–29.7 ± 6.9	3	0.001***

Note: The cells were treated with 100 nM concentrations of compounds 24 h before and 48 h after the 5 μM $A\beta_{1-42}$ insult. $A\beta_{42}$/Ctrl, $A\beta_{42}$-treated control compared to untreated control; The rest, compound treatment + Aβ-treatment compared to Aβ-treated control; Negative value, reduction compared to respective control; Positive value, increase compared to respective value; SD, standard deviation; n, number of repeats; t-test, paired; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

studies, expression levels of ERα and ERβ were examined by polymerase chain reaction (PCR) assays in SH-SY5Y cells, and we found that ERβ was the main form in our SH-SY5Y cells, whereas expression of ERα was hardly detectable. We suggest, therefore, that the study employing SH-SY5Y cells

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TABLE 4. The percentage change of LDH release from differentiated SH-SY5Y-cells

	Mean% ± SD	n	t-test, p	M–W, p
$A\beta_{42}$/Ctrl	31.8 ± 16.4	9	<0.001***	<0.001***
E1	–7.3 ± 5.2	9	0.003**	<0.001***
E2	–7.9 ± 9.6	9	0.038*	0.034*
Tam	–17.7 ± 5.4	9	<0.001***	<0.001***
4-OH-Tam	–10.3 ± 13.1	9	0.046*	0.203
Des	–15.4 ± 9.2	9	0.001***	<0.001***
Gen	–24.7 ± 8.1	6	0.001***	<0.001***

Note: The cells were treated with 5 nM concentrations of compounds 24 h before and 48 h after the 5 μM $A\beta_{1-42}$ insult. $A\beta_{42}$/Ctrl, $A\beta_{42}$-treated control compared to untreated control; The rest, compound treatment + Aβ-treatment compared to Aβ-treated control; Negative value, reduction compared to respective control; Positive value, increase compared to respective value; SD, standard deviation; n, number of repeats; M–W, Mann–Whitney U-test; t-test, paired; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

more reflects on the role of ERβ in neuroprotection. Nevertheless, homodimeric BAG-1 functions as a co-chaperone for heat shock protein HSP70/HSC70 chaperone proteins, and these interactions seem to be crucial for its protective properties [49], including the regulation of ER function [50]. Overexpression of BAG-1 also increases HSP70 protein levels, as well as the ratio of p-AKT to total AKT, and reduces apoptosis in neurons after hypoxia/re-oxygenation [51]. BAG-1 is essential in the nervous system, since BAG-$1^{-/-}$ embryos display defects in the nervous system in addition to the liver [52]. Differentially spliced BAG-1 exists as multiple isoforms, such as isoform 1, ~39 kD; isoform 2, ~35 kD; isoform 3, ~31 kD; and isoform 4, ~26 kD (http://www.uniprot.org/uniprot/Q99933), of which isoforms 1, 3, and 4, but not 2, interact with Bcl-2 in an ATP-dependent manner [49]. However, additional, less-well characterized high molecular weight forms or complexes of BAG-1 seem to exist [53]. BAG-1 has also been linked to stability of microtubules, which are composed of tubulin, tau, and some additional proteins. BAG-1 can inhibit the degradation of tau by forming a complex with HSC70 and tau, which can lead to an increase in tau levels. BAG-1 binds also to APP and is found to be highly expressed in the same neurons that contain intracellular tau or Aβ in the hippocampal sections from patients with AD. In addition to tau, over-expression of BAG-1 in cell cultures have also been demonstrated to lead to increased APP protein levels [54, 55].

We wanted to analyze the impact of estrogens and SERMS on Bcl-2, Bcl-xL, and BAG-1 and evaluate their role as mediators of protection against H_2O_2 insult in differentiated SH-SY5Y cells. The immunoblot results were normalized with actin. E2 and Tam with similar affinity to ERα and ERβ [44] significantly increased the expression of Bcl-2 (68% and 64%, respectively) and Bcl-xL (79% and 65%, respectively), whereas E1 increased only Bcl-2 (127%). E2 also increased BAG-1 (39 kD) (50%) (**Table 5**). However, Gen that mainly binds to ERβ [44] significant increased production of Bcl-2 and BAG-1 (39 kD) as well (33% and 55%, respectively), whereas Des with a slightly higher ERβ than ERα affinity [44] reduced BAG-1 (26 kD) (–39%), but had no impact on its 39 kD form. The results suggest that binding properties of SERMs on ER forms do not play an important role in regulation of Bcl-2 or BAG-1. Instead, the impact of SERMs may be due to their chemical properties, which may affect, for instance, the splicing of BAG-1.

Similar study with H_2O_2 exposed cells gave more varied results (**Table 5** and **Figure 3**) due to the strong impact of H_2O_2 on cells (**Figure 2**). Bcl-2 and BAG-1 (39 kD) were rather unaffected, whereas Bcl-xL showed reduction (–30–36%). Tam reduced the levels of mainly cytoplasmic BAG-1 (26 kD) significantly (–45%) (**Table 5**). However, H_2O_2 exposure damaged the cells (**Figure 2**) and likely reduced protein synthesis and caused leakage of the cytoplasmic and unbound targets from the cells.

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TABLE 5. The effects of 5 nM estrogens and SERMs on protein expression of Bcl-2, Bcl-xL, and BAG-1 in neuron-like SH-SY5Y cells

		SERM-Ctrl/Ctrl, %				H_2O_2-SERM/H_2O_2-Ctrl, %			
	24 h + 24 h 5 nM	Mean% ± SEM	n	t-test	M–W	Mean% ± SEM	n	t-test	M–W
Bcl-xL	E1	0.8 ± 22.1	6	0.973	1.000	–36.4 ± 11.7	6	0.027*	0.036*
	E2	78.5 ± 30.6	9	0.033*	0.004**	–19.4 ± 13.1	9	0.178	0.258
	Tam	65.2 ± 28.3	9	0.050*	0.004**	–29.7 ± 8.4	9	0.008**	0.004**
	4-OH-Tam	43.4 ± 43.4	9	0.347	0.258	–30.9 ± 11.2	9	0.026*	0.050*
	Des	24.1 ± 32.3	9	0.477	0.050*	–32.2 ± 13.3	9	0.041*	0.004**
	Gen	69.3 ± 37.4	9	0.101	0.258	–34.9 ± 14.0	9	0.037*	0.004**
Bcl-2	E1	127.1 ± 53.6	6	0.064	0.036*	50.9 ± 32.5	6	0.178	0.328
	E2	68.1 ± 26.3	9	0.032*	0.004**	14.0 ± 31.6	9	0.669	0.258
	Tam	64.3 ± 25.3	9	0.035*	0.004**	19.5 ± 28.7	9	0.515	0.258
	4-OH-Tam	40.7 ± 26.9	9	0.169	0.258	8.2 ± 24.2	9	0.743	0.730
	Des	–4.8 ± 21.6	9	0.830	0.258	15.2 ± 17.1	9	0.401	0.258
	Gen	33.3 ± 24.2	9	0.206	0.050*	–9.1 ± 17.9	9	0.625	0.050*
BAG-1 39 kD	E1	32.7 ± 32.6	6	0.362	0.328	7.4 ± 18.4	6	0.702	1.000
	E2	49.9 ± 21.0	9	0.045*	0.050*	21.5 ± 29.1	9	0.482	0.258
	Tam	49.4 ± 29.3	9	0.130	0.730	1.3 ± 12.7	9	0.921	0.730
	4-OH-Tam	34.9 ± 31.2	9	0.295	0.730	5.0 ± 15.3	9	0.753	0.730
	Des	–8.0 ± 9.6	9	0.427	0.258	–0.2 ± 17.4	9	0.992	0.258
	Gen	54.8 ± 30.0	9	0.106	0.050*	10.1 ± 21.2	9	0.647	0.730
BAG-1 26 kD	E1	156.4 ± 1332.0	6	0.293	1.000	1.2 ± 38.4	9	0.977	0.050*
	E2	36.0 ± 32.9	9	0.305	0.730	–21.2 ± 30.8	9	0.512	0.050*
	Tam	31.6 ± 42.2	9	0.475	0.730	–44.6 ± 12.6	9	0.008*	0.004**
	4-OH-Tam	–22.4 ± 34.9	9	0.467	0.258	95.2 ± 135.4	9	0.502	0.050*
	Des	–39.4 ± 11.2	9	0.008**	0.004**	56.9 ± 114.6	9	0.633	0.050*
	Gen	85.0 ± 105.1	9	0.442	0.730	46.0 ± 70.5	6	0.543	1.000

Note: The cells were also exposed to 0.25 mM H_2O_2 for 5 min and the protein expression was analyzed by Western blot. The results were normalized against actin. SERM Ctrl/Ctrl, compound-treated control compared to untreated control; H_2O_2 SERM/H_2O_2 Ctrl, compound and H_2O_2 exposed compared to H_2O_2 exposed control; Positive value, increase compared to respective value; Negative value, reduction compared to respective control; SEM, standard error of mean; n, number of repeats; t-test, paired; M–W, Mann–Whitney U-test; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

3.4. E1 and Gen Increased APP Synthesis and Affected Also N-Terminal Fragment Levels of PS-1

In an effort to reveal the role of OS, estrogens, and SERMS in regulation of production of Aβ, we also examined the induction of APP and APP cleaving γ-secretase member PS-1, and in specific, the endoproteolytic cleavage product of PS-1, the 34 kD N-terminal fragment (NTF) in neuron-like SH-SY5Y cells. The activation of PS-1 requires this 34 kD NTF as well as the 18.5 kD C-terminal fragment, and when complexed with the additional proteins Pen-2, Nicastrin and Aph-1, they form a functional γ-secretase-complex [56]. In addition, we examined α-tubulin (**Table 6**), which is a part of the microtubule structure in neurites. Tau is an essential stabilizing protein of the microtubules. However, BAG-1 also interacts with tau and regulates its proteasomal degradation [57].

Gen treatment increased APP protein synthesis (168%) but decreased the levels of NTF of PS-1 (–44%) in non-H_2O_2-exposed cells. E1, the main estrogen in postmenopausal women, increased both the

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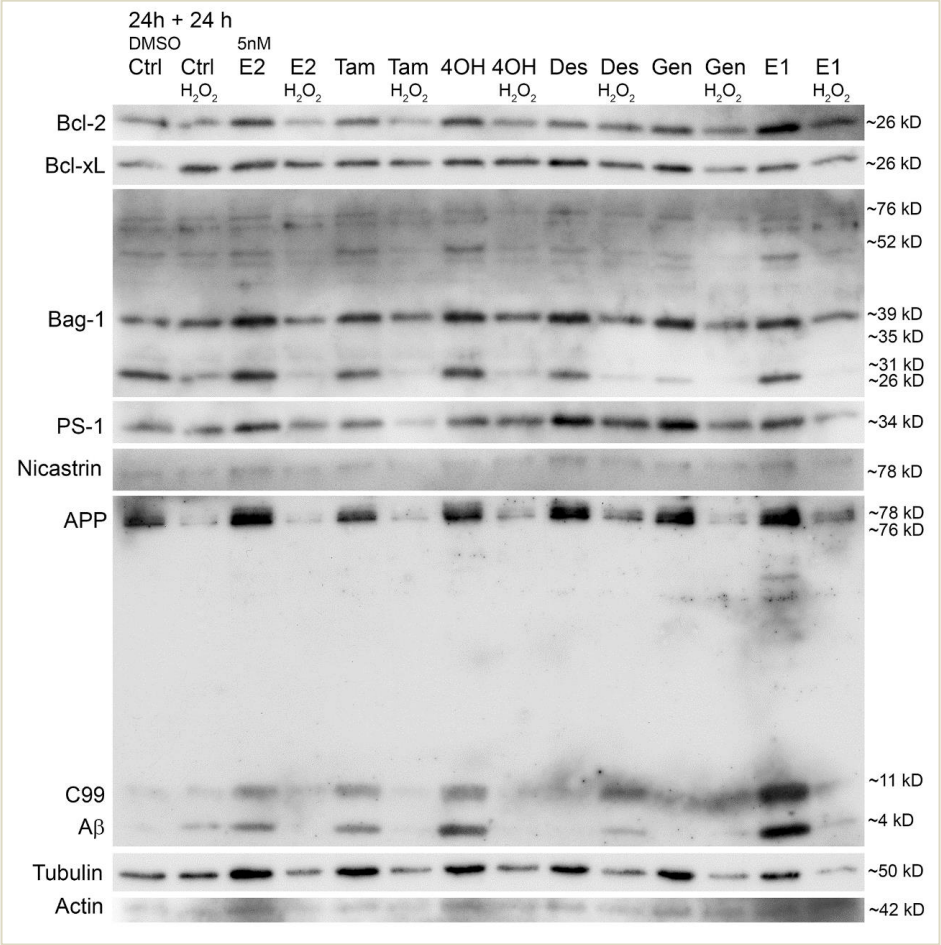

FIGURE 3. Representative Western blot analysis of the molecular targets (Tables 5-7). Differentiated SH-SY5Y cells were treated either with DMSO diluter (control, C) or with 5 nM estrogens or SERMs for 24 h, after which the cells were either unexposed or exposed to 0.25 mM H_2O_2 for 5 min (H_2O_2) and incubated for 24 h with the fresh media containing the described compounds. The targets include Bcl-2; Bcl-xL; BAG-1; PS-1 (NTF 34 kD); Nicastrin, additional part of γ-secretase complex; APP, C99, and Aβ (WO-2 antibody detects amino acids 4–10 from Aβ; G); α-tubulin; and actin. The results are from the same membrane. Actin was used for normalization during the numerical analyses.

APP protein levels, and NTF of PS-1 levels (**Table 6** and **Figure 3**). However, only synthetic estrogen Des increased production of APP cleaving products C99 and Aβ significantly (**Table 7** and **Figure 3**).

Neurites contain microtubules, composed of tubulin heterodimers, and these neuronal cell structures that cargo cell components to synapses are stabilized with tau-protein among other proteins [58, 59]. Tam treated cells showed increased tubulin levels (292%) (**Table 6** and **Figure 3**). E1, Gen, and 4-OH-Tam reduced α-tubulin levels in H_2O_2-exposed cells (–26–56%), indicating its release from the neurites due to damages in cells (**Figure 2**). However, E2 and SERMs seemed to have a tendency to increase α-tubulin levels compared to untreated control cells. Excess of tubulin may partially explain the release and can also function as a protective agent in neurite maintenance.

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TABLE 6. The effects of 5 nM estrogens and SERMs on protein expression of APP, PS-1 (34 kD), and α-tubulin in neuron-like SH-SY5Y cells

		SERM-Ctrl/Ctrl, %				H_2O_2-SERM/H_2O_2-Ctrl, %			
	24 h + 24 h 5 nM	Mean% ± SEM	n	t-test	M–W	Mean% ± SEM	n	t-test	M–W
APP	E1	312.4 ± 160.3	5	0.123	0.093	77.0 ± 60.7	6	0.260	0.036*
	E2	139.6 ± 69.0	8	0.083	0.442	–13.5 ± 30.8	8	0.674	0.093
	Tam	119.9 ± 98.1	8	0.261	0.442	–30.3 ± 22.3	8	0.216	0.008**
	4-OH-Tam	36.5 ± 22.0	7	0.149	0.189	3.3 ± 33.8	7	0.926	0.681
	Des	203.4 ± 91.5	8	0.061	0.105	92.7 ± 25.1	9	0.006**	0.004**
	Gen	167.6 ± 80.9	8	0.077	0.010**	7.1 ± 19.7	9	0.727	0.730
PS-1	E1	28.9 ± 21.5	6	0.237	0.036*	–11.4 ± 30.2	6	0.722	0.328
34 kD	E2	10.0 ± 20.4	8	0.639	1.000	2.0 ± 21.0	8	0.925	0.093
	Tam	3.2 ± 16.4	8	0.850	1.000	–28.4 ± 15.8	8	0.116	0.423
	4-OH-Tam	–12.6 ± 22.1	8	0.587	0.423	–1.7 ± 28.8	8	0.954	0.423
	Des	–34.8 ± 29.1	8	0.271	0.008**	0.4 ± 39.8	8	0.992	0.093
	Gen	–43.6 ± 20.3	8	0.069	0.008**	–32.7 ± 15.9	8	0.080	0.093
α-Tubulin	E1	5.2 ± 40.6	6	0.904	0.328	–66.7 ± 16.2	6	0.009**	0.036*
	E2	135.4 ± 89.9	9	0.170	0.258	4.5 ± 30.1	9	0.885	0.730
	Tam	291.8 ± 192.8	9	0.169	0.050*	–14.7 ± 17.0	9	0.412	0.258
	4-OH-Tam	196.3 ± 101.1	9	0.088	0.258	–32.2 ± 13.5	9	0.044*	0.050*
	Des	40.1 ± 24.6	9	0.142	0.258	–25.9 ± 21.1	9	0.255	0.050*
	Gen	216.0 ± 156.5	9	0.205	0.258	–56.4 ± 10.9	9	0.001***	0.004**

Note: The cells were also exposed to 0.25 mM H_2O_2 for 5 min and the protein expression was analyzed by Western blot. The results were normalized against actin. SERM/Ctrl, compound-treated control compared to untreated control; H_2O_2 SERM/H_2O_2 Ctrl, compound and H_2O_2 exposed compared to H_2O_2 exposed control; Positive value, increase compared to respective value; Negative value, reduction compared to respective control; SEM, standard error of mean; n, number of repeats; t-test, paired; M–W, Mann–Whitney U-test; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

4. DISCUSSION

We have examined and compared the neuroprotective properties of the estrogenic compounds, E1, E2, Tam and its metabolite 4-OH-Tam, as well as Des and Gen, which have different properties and affinities to ERs. The changes in the neuronal cell morphology, their LDH release and impact on neuroprotective Bcl-family members Bcl-2 and Bcl-xL, as well as BAG-1 were examined. The other interest was to estimate these compounds' impact on expression of amyloidogenic APP and PS-1, known to be affected by OS [32, 60].

E2 is considered as generally neuroprotective, but controversy exists for its benefits in AD. However, it has anti-inflammatory properties and can regulate many cellular defense systems. In our studies, E2 was mainly used as a well-studied reference. E2 belongs to phenolic compounds with an –OH group attached to a carbon of an aromatic ring (**Figure 1**). This reactive –OH group at the C3 position on the A ring of the steroid molecule seems to be crucial for estrogens' anti-oxidative capacity [61]. Although estrogenic compounds have neuroprotective properties [15], they are not recommended to use after the menopause due to their ability to increase the risk breast cancer and play some role in stroke [62]. Nevertheless, we showed that nanomolar concentrations of E2 were neuroprotective in two cell culture models exposed to either H_2O_2 or $A\beta_{1-42}$. The antioxidant mechanisms of E2 may involve the removal of superoxide, the scavenging of ROS and their precur-

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TABLE 7. The effects of 5 nM estrogens and SERMs on amyloid intracellular domain (C99) and Aβ in neuron-like SH-SY5Y cells

	24 h + 24 h (5 nM)	SERM-Ctrl/Ctrl, % Mean% ± SEM	n	t-test	M–W
C99 (11 kD)	E1	124.4 ± 116.7	4	0.430	0.548
	E2	36.1 ± 39.7	5	0.414	0.662
	Tam	106.5 ± 106.5	4	0.254	0.257
	4-OH-Tam	124.0 ± 80.0	4	0.219	0.257
	Des	326.5 ± 157.1	5	0.106	0.004**
	Gen	103.7 ± 76.6	4	0.269	0.257
Aβ (4 kD)	E1	12.9 ± 18.8	4	0.542	1.000
	E2	2.8 ± 15.9	4	0.872	1.000
	Tam	6.1 ± 50.6	4	0.912	1.000
	4-OH-Tam	–17.6 ± 27.4	4	0.568	1.000
	Des	101.0 ± 32.3	4	0.052	0.029*
	Gen	–9.9 ± 16.8	4	0.596	0.343

Note: The APP cleaving products were analyzed by Western blot, and the results were normalized against actin (refer to Figure 3 legend for experimental conditions). SERM Ctrl/Ctrl, compound-treated control compared to untreated control; Positive value, increase compared to respective value; Negative value, reduction compared to respective control; SEM, standard error of mean; n, number of repeats; t-test, paired; M–W, Mann–Whitney U-test; p, p value (statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

sors, or inhibiting their formation. Estrogens likely also up-regulate some endogenous antioxidant and other defenses [63, 64]. Furthermore, their structural resemblance with cholesterol and α-tocopherol suggests distribution to the hydrophobic core of the cell membrane, decreasing its lipid fluidity. The stabilization of the membranes may also affect the antioxidant and protective activity of estrogens, since restrictions on the fluidity of the membrane components can sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions [65]. Estrogens are known transcriptional regulators of antiapoptotic Bcl-2 and Bcl-xL [47, 48, 66], which were also shown in our study. E2 induced also expression of BAG-1 (39 kD) in H_2O_2 unexposed-cells but reduced 26 kD form in H_2O_2-exposed cells.

The relatively weak estrogen, E1, is the least abundant of the three estrogens in menstruating women and the only one present in quantity in postmenopausal women. E1 has only one –OH group, whereas E2 carries two. In comparison to E2, E1 displayed only modest protection against OS, and increased only Bcl-2, whereas reduced Bcl-xL in cells exposed to H_2O_2. Interestingly, E1 increased protein levels of APP and NTF of PS-1, suggesting a possibility to increase Aβ production when conditions are appropriate. The reduction of α-tubulin was also significant when the cells were exposed to H_2O_2, suggesting its leakage from the damaged cells. Estrogens can also enhance the expression of NMDA receptors [67, 68], and the efficacy of different estrogens may be different. H_2O_2 can increase the levels of extracellular glutamate, which can lead to the overstimulation of NMDA receptors and neurotoxicity [69].

Synthetic estrogen Des was selected in this study, since it has high affinity to ERα [70] and it also has two –OH groups (**Figure 1**). Our strain of SH-SY5Y cells expressed mainly ERβ, whereas ERα was hardly detectable. Clinically Des is used in the treatment of advanced prostate cancer. Des can decrease membrane fluidity [71], but it has also been demonstrated that mitochondria and nuclei can metabolize Des to reactive intermediates [72, 73]. Recent studies have indicated that Des can also function as a calcium channel modulator [74]. However, results with Des varied, and it seemed to have some neuroprotective ability in rat hippocampal neuron cultures but not in differentiated SH-SY5Y cultures when H_2O_2 was used. When $A\beta_{1-42}$ was applied in SH-SY5Y cells, Des seemed to be modestly protective against Aβ protein-induced damage. The lack of effect of Des in

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differentiated SH-SY5Y cells suggest that at least part of its neuroprotective effects against H_2O_2 in the hippocampal cells may be mediated by ERα, since these primary neurons express both ER isoforms. In neuron-like SH-SY5Y cells, Des increased protein synthesis of APP as well as production of C99 and Aβ. The NTF of PS-1 instead showed reduction, which may indicate its presence in a high molecular weight active γ-secretase complex. The levels of α-tubulin were also reduced in Des- and H_2O_2-treated cells, suggesting its release. BAG-1 (26 kD), which is mainly localized in the cytoplasm, was reduced in H_2O_2-unexposed cells, unlike its 39 kD form.

The polyphenolic structures of flavonoids and isoflavonoids have been found to scavenge free radicals and to chelate transition metals. Isoflavones are also weak estrogens capable of binding to ERs and exert either estrogenic or antiestrogenic effects. The isoflavonoid Gen is one of the few ERβ agonists with relative high receptor binding affinity, but with modest selectivity for ERβ [75]. Three –OH groups containing Gen, a phytoestrogen, had the ability to reduce LDH release in differentiated SH-SY5Y, as well as in hippocampal neurons. Although hippocampal neurons express ERα, ERβ is the dominant form in the hippocampus, as well as in the cerebral cortex, the cerebellum, the dorsal raphe, and in astrocytes at least in rodents [76, 77]. Therefore, some of the protection afforded by Gen also against Aβ is likely mediated through the ERβ. Gen-mediated neuroprotection seemed to involve Bcl-2 and BAG-1 (39 kD). Nonetheless, although Gen also increased APP protein synthesis, it decreased endoproteolytic NTF of PS-1, suggesting increased processing of APP to neurotrophic soluble APPα. Soluble APPα has also been found to inhibit tau phosphorylation through modulation of the GSK3β signaling pathway [78]. α-Tubulin, interacting with tau through microtubules, was reduced in Gen-treated and H_2O_2-exposed SH-SY5Y cells. Since LDH release indicated neuroprotective property of Gen, increased BAG-1 and the ability to interact with tau may have some role in the reduction of α-tubulin. Gen also increased the levels of α-tubulin in H_2O_2-untreated cells pronouncedly but not in a significant manner, and this might have some neuroprotective implications. Excess of tubulin may, for instance, physically block the damaged cell membrane as well as enhance the neurite recovery by microtubule assembly. However, considering the molecular structure, Gen most likely also functions as a direct antioxidant, but it is a tyrosine kinase inhibitor as well, significantly modulating the NMDA receptor subunit NR2B tyrosine phosphorylation and activity [43].

ER antagonist/partial agonist Tam is used in breast cancer treatment. Tam lacks the –OH group (**Figure 1**), but as a highly lipophilic functional compound, it has an ability to decrease membrane fluidity, which has been suggested to be the mechanism of its antioxidant action [79]. Tam has been shown to be protective against hydroxyl radical generation in the rat striatum [80]. However, some of the influences of Tam can also be mediated by OS [81], and the pro- or antioxidant actions of Tam, as well as its metabolite 4-OH-Tam, likely depend on the specific biochemical environment [82, 83]. 4-OH-Tam has a stronger free radical-scavenger capacity than Tam, likely due to its preferential location in the outer bilayer regions of the membranes where it can donate the hydrogen atom from its –OH group to quench free radicals [84], and inhibit lipid peroxidation.

4-OH-Tam, as well as Tam, can also activate ER-independent pathways [85, 86]. Tam showed no effect in primary neurons, although afforded some protection in differentiated SH-SY5Y cells. 4-OH-Tam instead lowered the LDH release after H_2O_2 exposure in both culture models. These results agree with previous studies suggesting that Tam has no neuroprotective effects at nanomolar concentrations unlike its metabolite 4-OH-Tam [80]. Tam increased expression of Bcl-2, whereas 4-OH-Tam had no effect. Tam also increased Bcl-xL and α-tubulin levels in H_2O_2-unexposed cells, but reduced Bcl-xL, BAG-1 (26 kD) as well as APP levels in H_2O_2-exposed cell. As 4-OH-Tam afforded only modest protection, it had only very minor impact on any examined targets, but reduction of α-tubulin and Bcl-xL levels was apparent in H_2O_2-treated cells. Therefore, the neuroprotective properties of 4-OH-Tam seem to rely on its structure, but other mechanisms seem to also exist [86]. Interestingly, 4-OH-Tam, as well as Tam and Des bind to the estrogen-related receptor-γ (ERRγ), expressed also in SH-SY5Y cells. Among these compounds, 4-OH-Tam has the highest binding affinity to ERRγ, whose binding leads to ERRγ deactivation [87]. Interestingly, ERRγ has been suspected to regulate the dopaminergic neuronal phenotype [88]. Dopaminergic neurons are deteriorating in Parkinson's disease, whereas use of Tam seems to increase the risk of Parkinson's dis-

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ease [89]. Whether this is mediated by inactivation ERRγ by Tam and its metabolites, and/or simultaneous OS with various reasons, remains to be further determined.

OS and estrogenic compounds influence cell membranes and their lipid rafts. Lipid rafts are membrane regions containing higher amounts of glycosphingolipids, saturated phospholipids, and cholesterol than the rest of the membrane, and they play a role in membrane organization and intracellular signaling [90, 91]. Lipid rafts harbor for instance BACE-1 and γ-secretase, and OS seems to increase at least PS-1 in lipid rafts [92–94]. Lipid rafts contain also caveolins (CAVs; isoforms 1–3) [95], and at least CAV-1 regulates γ-secretase-mediated APP processing by modulation of spatial distribution of γ-secretase in the membrane [96]. Further, CAVs seem to regulate ERs trafficking and clustering to caveolae at the plasma membrane. CAV-1 has interactions with ERα, directly influenced by E2 [97], and therefore, CAVs may also modulate efficacy of the SERMs, including during OS.

CAV-1 is also a direct binding partner of the transcription factor nuclear erythroid 2 p45-related factor-2 (Nrf2), which mediates the cytoprotective responses against stress. After OS, CAV-1 limits the movement of Nrf2 from caveolar membranes to the nucleus, and therefore CAV-1 acts as an endogenous inhibitor of Nrf2 [98]. In general, CAVs association partners include also G protein-coupled receptors, tyrosine kinase receptors, various intracellular kinases and second messenger molecules, as well as ion pumps and voltage-gated ion channels. Among these, voltage-dependent anion channel (VDAC) participates in Aβ-induced toxicity [99]. Since VDAC and ERα can associate with CAV-1 at the plasma membrane, variations in this complex may also participate in the modulation of Aβ-induced cell death [100, 101]. E2 also promotes VDAC phosphorylation through the activation of protein kinase A (PKA) and Src-kinase, which may be relevant to keep this channel inactivated. On the contrary, at least Tam can provoke the dephosphorylation of VDAC and eventually its opening, by activating a cascade of phosphatases, including protein phosphatase 2 (PP2A) [102]. As a conclusion of this complexity, the role of other SERMs in regulation of membrane events, influenced by CAVs, ERs, γ-secretase and VDAC, requires further investigation under conditions including OS.

Energy-, but also ROS-producing mitochondria are essential to the cells. Anti-apoptotic Bcl-2 and Bcl-xL localize to mitochondrial membranes, and furthermore, the ERβ seems to be more highly expressed in the brain mitochondria than ERα, having biologically distinct roles. For instance, ERβ seems to be involved in maintenance of mitochondrial membrane potential [103, 104], and therefore, neuroprotection mediated by Gen likely involves also mitochondrial ERβ. Gen, as well as E1, E2, and Tam enhanced Bcl-2 expression, whereas E2, Tam, and Des increased Bcl-xL levels. Both these factors can prevent caspase-1 activation [105]. Bcl-xL also regulates presynaptic plasticity, including neurotransmitter release and recovery, as well as size and number of synaptic vesicle clusters [106, 107]. BAG-1, which itself has anti-apoptotic activity, can also markedly increase the anti-cell death function of Bcl-2 [49].

5. CONCLUSIONS

Our results indicated that E2 was the most potent neuroprotector at nanomolar concentrations with Gen generally having almost equivalent efficacy. The other estrogenic compounds showed variable results. However, E2 was less efficacious in Aβ-induced neuronal damage in comparison to H_2O_2-induced damage. Therefore, the number of –OH groups in estrogenic compounds can be an important determinant of their scavenging capacity, although the results also support the importance of ERβ in neuroprotection, especially in Aβ-induced toxicity. Another aspect of compound-mediated neuroprotection is the expression alterations of the anti-apoptotic protein targets. E2 and Tam increased production of Bcl-2 and Bcl-xL, E1 and Gen only Bcl-2, whereas Des only Bcl-xL. Furthermore, in H_2O_2-exposed cells, Bcl-xL showed reduction with all estrogenic compounds, unlike Bcl-2. The results suggest that Bcl-2 is a more potent protector than Bcl-xL, possibly by its ability to interact with BAG-1. However, our targets represent only minor part of cellular neuroprotective proteins, of which many are regulated by estrogens [108]. Nonetheless, E2 and Gen may also influence tau accumulation, since they increased BAG-1 levels, known to regulate tau protein degradation. E1 may enhance Aβ synthesis by increasing production of APP and NTF of PS-1. Gen also in-

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creased APP, but it reduced NTF of PS-1, and therefore, further studies are needed to determine whether Gen can increase production of neurotrophic soluble APPα, which was found by others to be able to inhibit tau phosphorylation through modulation of the GSK3β signaling pathway.

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