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Salen-Manganase Complexes, EUK-8 and EUK-134, Protect Against Protein and Lipid Oxidative in Neuroblastoma Cells and Rat Liver Homogenates *in vitro*

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Abstract: Oxidative damage to proteins and lipids leads to severe failure of their biological functions with subsequent final effects on cells functions in various tissues especially liver and brain. In this study, researchers investigated the protective effects of two salen free radical scavengers namely EUK-8 and -134 and also a standard antioxidant (catechin) in two different models of oxidative stress. In the first study, researchers used Fe^{2+}/Ascorbate Model as a well-validated system for production of ROS in rat liver homogenates and the protective effects of EUK-8, EUK-134 and catechin against this system were evaluated. In the second study, the protective capabilities of these compounds against the free-radical damaging effects of hydrogen peroxide (H_2O_2) on SK-N-MC cell line were evaluated in term of attenuation of intracellular lipofuscin level. Results of the first study indicated that the simultaneous addition of Fe^{2+}/ascorbate and EUK-8 and/or EUK-134 at different concentrations (5, 10, 25 and 50 μM) to the liver homogenate significantly decreased the extent of PCO, LPO and ROS while the level of PB-SH increased relative to the control group. Results of the second study also showed that pretreatment of the cells with EUK-8 and -134 (25 μM) followed by exposure to H_2O_2 restored the viabilities of cells relative to the H_2O_2-treated cells. In addition, each of the compounds significantly and time-dependently reduced the intracellular level of lipofuscin pigments among the H_2O_2-treated cells.

Key words: Lipofuscin, liver, neuron, oxidative stress, ROS level, salen-manganase complexes

INTRODUCTION

Oxidative stress is believed to be involved in the initiation and propagation of a variety of diseases including aging, neurodegeneration, carcinogenesis, coronary heart disease, diabetes and hepatic diseases (Dalle-Donne *et al*., 2003; Bishop *et al*., 2010). These complications usually results due to enhanced level of endogenous Reactive Oxygen Species (ROS) (Farber, 1994; Halliwell, 1999). ROS include hydroxyl (OH), peroxyl (RO_2) and superoxide anion ($O_2^{\bullet -}$) radicals and nonradical species such as hydrogen peroxide (H_2O_2). Despite the beneficial roles as second messengers in some signal transduction pathways at low concentrations, these species become highly toxic to a variety of cells and tissues at evaluated levels (Farber, 1994; Halliwell, 1999). The most pronounced events associated with the ROS-mediated injuries include peroxidation of lipids and oxidation of proteins and DNA. Peroxidation of lipids leads to the production of 4-Hydroxy-2-Nonenal (HNE) and Malondialdehyde (MDA) which act as highly reactive cross-linking agents. One of the recognized outcomes of these oxidative processes is the formation and accumulation of aggregates known as lipofuscin (Brunk and Terman, 2002). Lipofuscin as a histological index of aging is mainly made of oxidized protein (30-60%) and lipids (20-50%) and accumulates mostly in post-mitotic cells such as neurons, cardiac myocytes, skeletal muscle fibers, retinal pigments and epithelial cells (Brunk and Terman, 2002; Jung *et al*., 2007). Many studies have revealed that lipofuscin pigments could induce neurotoxicity through the ROS generation (Brunk and Terman, 2002; Szweda *et al*., 2002). These effects at the low levels are usually counteracted by the endogenous antioxidant defense elements such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, vitamin C, E and uric acid. However under oxidative stress condition, the efficiency of this defense system is declined resulting in ineffective scavenging of free radicals (Fridovich, 1999; Yazdanparast *et al*., 2008). Under these conditions, exogenous natural or synthetic antioxidants are usually prescribed by physicians (Ceriello, 2003). These antioxidants can delay and/or prevent the oxidation processes through simple or complex mechanisms including suppression of oxidation chain reactions, chelation of transitional metal ion catalysts, breakdown of peroxides and scavenging radical (Ames *et al*., 1993). In

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that respect, a number of research groups have synthesized developed low molecular weight scavengers with high antioxidative properties comparable to antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT) (Autzen *et al*., 2003). Among these agents, different derivatives of salen-manganese complexes have shown promising potencies due to their ability to scavenge a wide range of ROS, namely $O_2^{\bullet -}$, H_2O_2, $ONOO^-$ and $RO_2^{\bullet}$ radicals (Baudry *et al*., 1993; Gonzalez *et al*., 1995). The SOD-like and free radical scavenging activities of these compounds have been attributed to the switching capability of the central Mn metal ion between Mn(III) and Mn(II) valance states. These synthetic complexes also have higher stability and bioavailability compared to proteinaceous antioxidant enzymes (Baudry *et al*., 1993; Sharpe *et al*., 2002). EUK-8 and -134 (Fig. 1) are two potent members of this group of compounds whose structures and catalytic activities have been described previously (Rong *et al*., 1999). Both compounds have equivalent SOD activities but EUK-134 possesses a higher CAT activity (Baudry *et al*., 1993; Gonzalez *et al*., 1995). They also decreased the Nitric Oxide (NO) production by accelerating the breakdown of numerous no products to more nonthreatening species. In addition, it has been shown that these compounds possess therapeutic efficacy in several neurological disorders using animal models (Baudry *et al*., 1993; Gonzalez *et al*., 1995; Rong *et al*., 1999; Sharpe *et al*., 2002). In order to expend the knowledge on therapeutic capabilities of these compounds and also to clarify their mechanism of action, the present study was arranged. Primarily, researchers used the Fe^{2+}/Ascorbate Model as a well-validated system for induction of oxidative stress in isolated liver homogenates of rats and then evaluated the protective effects of EUK-8 and -134 against insults to this system. In addition, regarding the role of oxidized lipids and proteins in production of lipofuscin pigments, the probable neuroprotective effects of these compounds against the free-radical damaging effects of H_2O_2 in SK-N-MC neuroblastoma cell line were investigated. Based on the results of this study, EUK-8 and -134 at different concentrations showed inhibitory effects against formation of Protein Carbonyl (PCO), Tiobarbituric Acid Reactive Substances (TBARS) and ROS. In addition, pretreatment of the cells with these compounds led to restoration of cells viabilities and decreased the intracellular lipofuscin level relative to the control (H_2O_2-treated) cells.

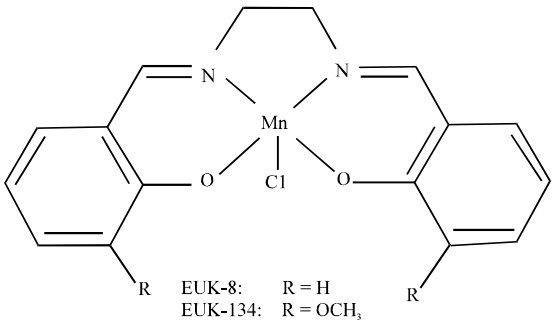

Fig. 1: The chemical structures of the salen-manganese complexes EUK-8 and -134

MATERIALS AND METHODS

Ascorbic acid, catechin, Ferrous Sulphate ($FeSO_4$), Trichloroacetic Acid (TCA), 2, 4-Dinitrophenylhydrazine (DNPH) and Folin-Ciocalteu's Reagent (FCR) were obtained from Sigma (St. Louis, MO, USA). 5, 5'-Dithiobisnitro Benzoic Acid (DTNB), Hydrogen peroxide (H_2O_2) Butylated Hydroxytoluene (BHT) and Thiobarbituric Acid (TBA) and Dimethyl Sulfoxide (DMSO) were obtained from Merck Co (Germany). 2', 7'-Dichlorofluorescein Diacetate (DCFH-DA) was purchased from Molecular probe (Eugene oregon, USA). The cell culture medium (RPMI 1640), penicillin-streptomycin and Fetal Bovine Serum (FBS) were purchased from Gibco BRL (Life technology, Paisley, Scotland). EUK-8 and -134 were prepared by first synthesizing the ligand and then complexing the ligand to manganase using published methods (Boucher and Farrell, 1973). The culture plates were obtained from Nunc (Brand products, Denmark). All other chemicals were analytical grade.

Preparation of liver homogenate: Male Wistar albino rats weighing 200-250 g (purchased from Pasteur Institute, Tehran, Iran) were housed under conventional conditions and were allowed free access to food and water *ad libitum*. The rats were anesthetized using diethyl ether and their abdomens were opened and their livers were quickly removed. All experiments were carried out according to the guidelines for the care and use of experimental animals approved by State Veterinary Administration of University of Tehran. Each liver sample was then cut into small pieces and homogenized in phosphate buffer (50 mM, pH 7.4) with a homogenizer to give a 10% (w/v) liver homogenate and then centrifuged at 5000 g for 15 min at 4°C (Beckman). The supernatant was obtained and the protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

Induction of oxidative stress in liver homogenates: To induce oxidative stress in rat liver homogenates, the oxidant pair Fe^{2+}/ascorbate was used (Ardestani and

Yazdanparast, 2007). The reaction mixture including 0.5 mL of each liver homogenate, 0.9 mL of phosphate buffer (50 mM, pH 7.4), 0.25 mL of $FeSO_4$ (0.01 mM), 0.25 mL of ascorbic acid (0.1 mM) and 0.1 mL of different concentrations of each compound and/or catechin as the standard sample. Each reaction mixture was incubated at 37°C for 30 min.

Measurement of ROS levels: The extent of ROS formation in the reaction mixture was measured following the oxidation of 2', 7'-Dichlorofluorescein Diacetate (DCFH-DA) to the highly fluorescent compound, 2', 7' Dichlorofluorescein (DCF) according to previously published method with slight modification (Ugochukwu and Cobourne, 2003). Each sample composed of 1.85 mL of phosphate buffer (50 mM, pH 7.4) solution, 0.1 mL of liver homogenate and 50 μL of DCFH-DA solution (10 μM). The samples were incubated in an incubator at 37°C for 15 min. The ROS levels were measured via the formation of DCF using a spectrofluorometer (Model Cary Eclipse) with the excitation and emission wavelengths at 488 and 525 nm, respectively.

Determination of Protein Carbonyl (PCO) content: The extent of PCO was measured using the method of Reznick and Packer (1994). Based on this method, about 1 mL of 10 mM DNPH in 2 M HCl was added to the reaction mixture (2 mg protein). Samples were kept for 1 h at room temperature and were vortexed every 15 min. Then, 1 mL of cold Trichloroacetic Acid (TCA) (10%, w/v) was added to each reaction mixture and centrifuged at 3000 g for 10 min. The protein pellet was washed 3 times with 2 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 mL of guanidine hydrochloride (6 M, pH 2.3) and incubated for 10 min at 37°C while mixing. At the end of the incubation time, the absorbance of each sample was recorded at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2\times10^4$ cm M^{-1}) and expressed in terms of percentage inhibition.

Determination of Protein Bound Sulfhydryl groups (PB-SH): The amount of PB-SH groups in each liver homogenate was measured according to the method of Sedlak and Lindsay (1968) using 5, 5'-Dithiobisnitro Benzoic acid (DTNB). For total Thiol (T-SH) measurement, the reaction mixture contained 0.3 mL of the liver homogenate, 1.5 mL of Tris buffer (0.2 M, pH 8.2) plus 0.1 mL of 0.01 M DTNB. The reaction mixture was brought to 5.0 mL using 3.1 mL of absolute methanol. After 15 min, each reaction sample was centrifuged at 3000 g at room temperature for 15 min. The absorbance of each supernatant was read at 412 nm. For non-protein thiol (NP-SH) measurement, an aliquot of 1.7 mL of each homogenate was mixed with 0.3 mL distilled water and 1 mL of 50% TCA. The samples were shaken intermittently for 10-15 min and centrifuged for 15 min at 3000 g. About 2 mL of supernatant was mixed with 2 mL of Tris buffer (0.4 M, pH 8.9) and 0.1 mL DTNB. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no liver homogenate. The experimentally determined molar extinction coefficient at 412 nm was 13,100 in both T-SH and NP-SH procedures. The P-SH groups were calculated by subtracting the NP-SH from T-SH and expressed as nmol mg^{-1} protein.

Determination of lipid peroxidation: The level of lipid peroxidation of each liver homogenate in the presence and absence of different compounds at various concentrations was evaluated by measuring the product of Thiobarbituric Acid Reactive Substances (TBARS) using the method described before (Bahramikia *et al*., 2009). At the completion of the incubation time, each sample was terminated by addition of BHT (2% w/v in 95% v/v ethanol) followed by addition of 1 mL of TCA (20% w/v) to the mixture. After centrifugation at 3000 g for 15 min, the supernatant was incubated with 1 mL of Thiobarbituric Acid (TBA) (0.67%) at 100°C for 15 min. The color intensity of TBARS/TBA complex was measured and the quantity of TBARS formed was determined spectrophotometrically at 532 nm using the absorption coefficient of 1.56×10^5 cm M^{-1}. The data were calculated from a control measurement of the reaction mixture without the test sample and expressed in terms of percentage inhibition.

Cell culture: SK-N-MC cells were cultured at a density of 5×10^4 cells mL^{-1} RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) and incubated at 37°C in a 5% CO_2 humidified atmosphere. Cell numbers and viabilities were assessed using a hemocytometer and the abilities of the cells to exclude trypan blue. Drug treatments were done 24 h after cell seeding. To induce the oxidative stress, H_2O_2 was freshly prepared from 8.4 mM stock solution prior to each experiment. SK-N-MC cells were incubated with each drug for 3 h before exposure to 300 μM H_2O_2.

Cell viability determination: The viabilities of SK-N-MC neuroblastoma cells were determined using the MTT test[24]. The cells (5×10^4) were seeded in 96-well plates for 24 h, they were then treated with different doses (10-100 μM) of EUK-8, -134 and/or catechin for the indicated times. To each cell sample, 10 μL MTT (5 mg mL^{-1} PBS) was added and kept for 4 h. Then, the

plates were centrifuged for 15 min at 2500 rpm and the supernatants were discarded and 200 μL DMSO was added to each cell pellet to dissolve formasan crystals. The absorbance of each sample was recorded at 570 nm with an ELISA reader (Exert 96, Asys Hitch, Ec Austria) after 30 min.

Evaluation of intracellular lipofuscin pigments: Extraction of intracellular lipofuscin was achieved following lysis of each cell sample according to the methods of Emig *et al.* (1995). The cells (5×10^4 cells/well) were seeded in triplicate into 24-well plates for 24 h prior to pretreatments. After pretreatment with each drug derivative (20 μM) for 3 h, each cell sample was treated with 300 μM H_2O_2 for 24, 48 and 72 h. The attached cells in each well were trypsinized with trypsin-EDTA solution followed by cell counting using a hemocytometer. Each plate was then centrifuged and the cell pellet was washed with PBS and the cell content was lysed with lysis buffer containing 1% Triton x-100, 1 mM EDTA and 1 mM PMSF. Each cell lysate was harvested and its fluorescence intensity was monitored on a varian-spectrofluometer (model Cary Eclipse) with an excitation wavelength of 310 nm and emission wavelength of 620 nm. The fluorescence intensities of the samples were then normalized for equal cell numbers.

Statistical analyses: All data are presented as means±SD. The mean values were calculated based on the data taken from at least three independent experiments using freshly prepared reagents. Statistical analyses were performed using Student's t-test. The statistical significances were achieved when $p<0.05$.

RESULTS

The salen derivatives inhibit the ROS formation in the liver homogenate: Figure 2 shows a significant increase in the fluorescence intensity of DCF in the presence of oxidant pairs as compared to the control. However, simultaneous incubation of Fe^{2+}/ascorbate and each of the compounds and/or catechin at different concentrations (5, 10, 25 and 50 μM) reduced the Fe^{2+}/ascorbate-induced DCF fluorescence (Fig. 2). The highest inhibitory effect was observed for EUK-134 and the lowest value was for EUK-8. These data point to the potential of EUK-8 and -134 to reduce basal ROS production in liver homogenate under oxidative condition.

The salen derivatives inhibit the PCO formation in the liver homogenate: The assessment of PCO content is a

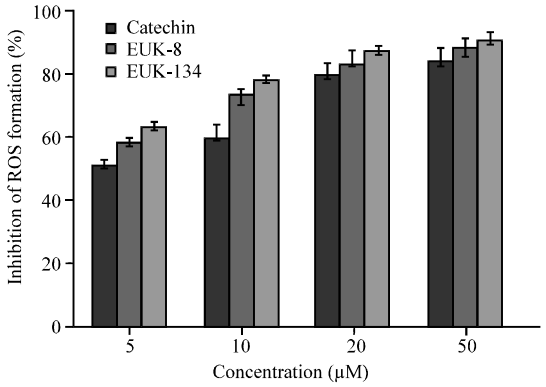

Fig. 2: Inhibitory effects of EUK-8 and -134, relative to catechin, on Fe^{+2}/ascorabate-induced ROS formation. Each value represents the mean±SD (n = 3)

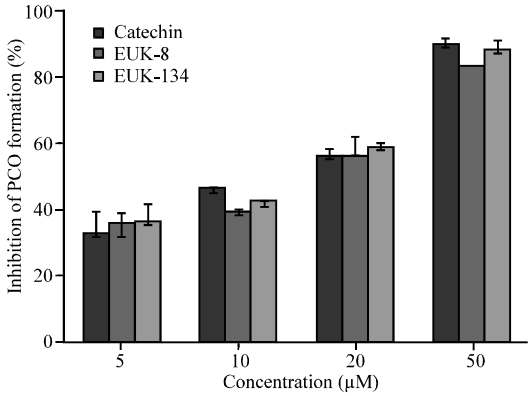

Fig. 3: Protective effects of EUK-8, -134 and catechin against Protein Carbonyl (PCO) formation induced by Fe^{+2}-ascorabate system. Each value represents the mean±SD (n = 3)

widely used marker for oxidative protein modification. As shown in Fig. 3, addition of Fe^{2+}/ascorbate to the liver homogenate significantly increased the extent of PCO formation compared to the control sample. However in the presence of EUK-8 and -134 at variable concentrations (5, 10, 25 and 50 μM), the extent of PCO significantly decreased. The results were compared to catechin (Cat) as the positive control.

Simultaneous addition of Fe^{2+}/ascorbate and catechin resulted in a similar inhibitory effect on PCO formation relative to salen derivatives. Based on Fig. 3, inhibition of PCO formation was found to obey the following order: EUK-134>Catechin>EUK-8.

The salen derivatives protect the P-SH groups from oxidation in the liver homogenate: The measurement of

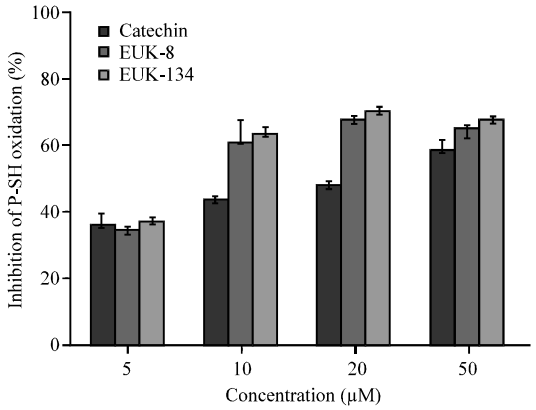

Fig. 4: Inhibitory effects of EUK-8, -134 and catechin on Protein thiol (P-SH) oxidation induced by Fe^{+2}-ascorabate system. Each value represents the mean±SD (n = 3)

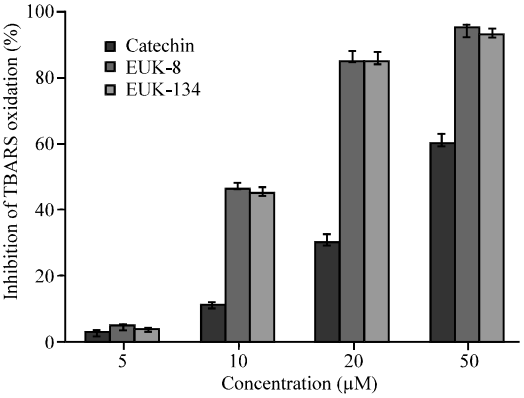

Fig. 5: Protective effects of EUK-8, -134 and Catechin against lipid peroxidation induced by Fe^{+2}-ascorabate system. Each value represents the mean±SD (n = 3)

Protein-Sulfhydryl (P-SH) groups is a useful approach for testing oxidative state of biological systems. As shown in Fig. 4, addition of oxidant pair to the reaction mixture reduced the P-SH contents compared to control sample. However in the presence of each of the compounds at various concentrations (5, 10, 25 and 50 μM), the P-SH content increased by various degrees. The highest protection level was due to EUK-134 and the lowest value observed for EUK-8. A sharp rise in P-SH content in presence of catechin as the positive control was also observed.

The salen derivatives inhibit the level of TBARS in the liver homogenate: Figure 5 shows oxidative damage induced to lipids in terms of TBARS content. The addition of Fe^{2+}/ascorbate to the liver homogenate for 30 min significantly increased the extent of TBARS formation relative to the control sample. However as shown in Fig. 5, simultaneous addition of Fe^{2+}/ascorbate and each of the salens at different concentrations (5, 10, 25 and 50 μM) to the liver homogenates resulted in reduction of TBARS content. The highest percent of inhibition was found for the EUK-134 and the lowest activity was found for EUK-8. As the positive control, catechin showed higher inhibitory effect relative to EUK-8 and -134.

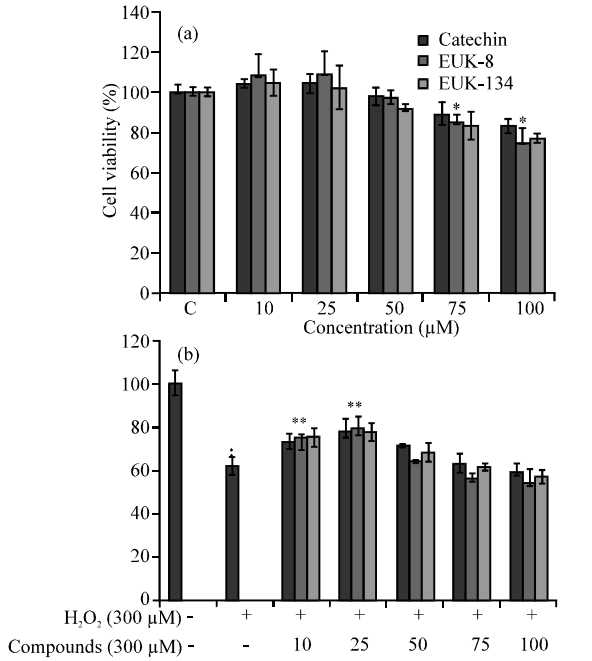

Fig. 6: a) Effects of various concentrations (10, 25, 50, 75 and 100 μM) of EUK-8, EUK-134 and catechin on viabilities of SK-N-MC cells. Cell viability was evaluated by MTT assay; b) Protective effects at 25 μM of EUK-8, -134 and Catechin) on H_2O_2-induced cytotoxicity among SK-N-MC cells. *Significantly different from control ($p<0.05$). **Significantly different from H_2O_2-treated cells ($p<0.05$)

The salen derivatives improve the viabilities of H_2O_2-treated cells: The toxicity of salen derivatives was evaluated based on the viability of cells exposed to variable concentrations of the drugs using MTT assay. According to Fig. 6a, EUK-8 and -134 at low concentrations (10 and 25 μM) showed slight cytotoxicity; however at higher doses (50-100 μM), they showed moderate cytotoxicity. In addition, exposure of the cells to H_2O_2 at 50, 100, 150, 200, 300 and 400 μM caused significant reduction in cells viabilities by almost 8, 18, 27, 36, 48 and 57%, respectively. Based on these

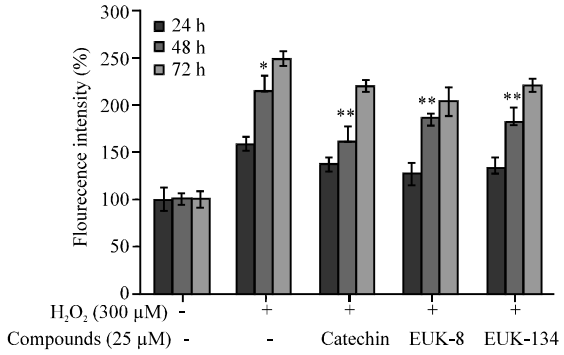

Fig. 7: Inhibitory effects of EUK-8, -134 and catechin on the H_2O_2-induced accumulation of intracellular lipofuscin pigments at various exposure times. SK-N-MC cells were exposed to 25 µM of each derivative for 3 h followed by exposure to 300 µM H_2O_2 for 24, 48 and 72 h. Then, the extent of lipofuscin in each cell lysate was evaluated using a varian-spectrofluometer at excitation and emission wavelengths 488 and 525 nm, respectively. *Significantly different from control cells ($p<0.05$). **Significantly different from H_2O_2-treated cells ($p<0.05$)

data, the entire investigation was carried out at a H_2O_2 concentration of 300 µM which induced a viability loss of about 48%. However, pre-treatment of the cells with various concentrations of EUK-8 and -134 (10-100 µM) significantly reduced the damaging effect of H_2O_2 relative to the control cells treated solely with H_2O_2 as shown in Fig. 6b.

The salen derivatives suppress lipofuscin formation among H_2O_2-treated cells: Exposure of the cells to 300 µM H_2O_2 for 24, 48 and 72 h increased the intracellular level of lipofuscin by 59, 116 and 149% relative to H_2O_2-untreated control cells, respectively. Pretreatment of the cells with 25 µM of EUK-8 and/or EUK-134 for different time intervals (24, 48 and 72 h), diminished the formation of lipofuscin pigments by 32, 29, 46% and 24, 33, 27%, respectively. Treatment with Catechin as the positive control, also resulted in a similar inhibitory effect on the level of lipofuscin as shown in Fig. 7.

DISCUSSION

To get a better understanding on oxidative stress related to disorders, it certainly would be beneficial to rely on different model systems to cover up the majorities of chemical and biological pathways involved (Guillouzo, 1998; Groneberg *et al*., 2002). Regarding this believe in this investigation, researchers used two different model systems to evaluate the antioxidant activity of two salen derivatives namely EUK-8 and -134. In the first approach, we used Fe^{2+}/ascorbate in liver homogenate to induce oxidative stress and in the second approach, H_2O_2 Model in SK-N-MC cell line was used. Fe^{2+}/Ascorbate Model is a well-validated system for production of ROS and induction of oxidative stress status in isolated tissue homogenates mainly liver (Guillouzo, 1998; Bahramikia *et al*., 2009). In this system, addition of ascorbate and iron salt mixture to the tissue homogenate initiates the Fenton-reaction which leads to the production of highly reactive hydroxyl radicals and probably other Reactive Oxygen Species (ROS). The generated free radicals then cause chain-initiation reaction of lipid peroxidation and also trigger complex pathways of protein oxidation (Guillouzo, 1998). Thus, measurement of either the ROS level or the level of oxidation end products of lipids and proteins will provide an index of oxidative damage. Regarding this fact, we measured the extent of ROS in the reaction mixture using DCF. The data shown in Fig. 1 confirmed ROS production in the liver homogenates by the Fe^{2+}/ascorbate system. In addition, the results clearly indicated that in the presence EUK-8 and -134, the DCF fluorescence intensity declined. The free radical neutralization by EUK-8 and -134 might be attributed to the switching capability of the central metal ion (Mn) between Mn^{3+} and Mn^{2+} valence states.

Proteins are the main targets of ROS in the cells and tissues. In the Fe^{2+}/Ascorbate Model, proteins are either directly damaged by ROS or they are modified by the aldehydic products of lipid peroxidation or ascorbate autoxidation. Cumulatively, these processes would lead to carbonylation of some amino acid residues such as lysine, arginine, proline and threonine and/or the peptide backbone of proteins with subsequent formation of PCO products (Dean *et al*., 1997; Stadtman and Levin, 2000; Bahramikia *et al*., 2009).

The results indicated that EUK-8 and -134 were capable of inhibiting PCO production in the Fe^{2+}/Ascorbate Model system as shown in Fig. 2. It has also been shown that both compounds have catalase and superoxide dismutase activities with high scavenging activities against various free radicals such as superoxide and hydroxyl radicals, thereby attenuating the formation of protein oxidation byproducts. The data presented herein provided additional evidences that these compounds were also capable of suppressing the oxidative modification of proteins in liver homogenates. The measurement of Protein Sulfhydryl (P-SH) groups is another useful approach for checking oxidative state of biological system. Regarding the high susceptibility of

P-SH groups to oxidation by free radicals, consumption of antioxidants under the oxidative stress state seems crucial for protecting the functional sulfhydryl groups of proteins (Stadtman and Levin, 2000; Telci *et al*., 2000; Bahramikia *et al*., 2009). The increased P-SH content under the influence of EUK-8 and -134 might indicate chelation of redox active metals and/or the trapping of some ROS. Reactive Oxygen Species (ROS) are not only associated with protein oxidation but also they are capable of effective lipid peroxidation through hydrogen atom abstraction from unsaturated fatty acids (Aruoma, 1998; Dean *et al*., 1997).

In that regard, end products of lipid peroxidation such as MDA and 4-HNE can oxidize protein thiols, incorporate carbonyl groups into polypeptide chains or cause covalent cross linking of different protein molecules leading to impairment of protein functions (Dean *et al*., 1997). In the present study, exposure of rat liver homogenate to the ascorbate and Fe^{2+} oxidation system resulted in enhanced lipid peroxidation. However, treatments with EUK-8 and -134 significantly decreased TBARS contents. Based on these data, it could be concluded that the Mn-salen derivatives might be effective in preventing oxidative damage to proteins.

On the other hand, free radicals have long been recognized as active players in initiation and progression of age-related complications (Stadtman and Levin, 2000; Andersen, 2004). In aging process, the indigenous antioxidants are diminished or become inefficient (Bayati *et al*., 2010). Therefore, lipids and proteins appear to be the most dominant targets to oxidative damage. Thus, the study was extended to the neuroprotective activity of these compounds against the free-radical damaging effects of H_2O_2 among SK-N-MC neuroblastoma cells.

It has been shown that under oxidative stress, H_2O_2 penetrates the lysosomal compartment resulting in the formation of highly reactive hydroxyl and iron-centered radicals which finally leads to lysosomal (Bayati *et al*., 2010; Gonzalez *et al*., 1995). These events consequently lead to oxidative damage to lipids, proteins and carbohydrates and the formation of the intralysosomal aggregates named lipofuscin (Gonzalez *et al*., 1995; Grimm *et al*., 2011).

Lipofuscin or the age pigments are believed to be composed of the oxidized protein and lipid degradation products. The data indicated that EUK-8 and -134 were capable of attenuating the formation of lipofuscin pigments. These results further confirmed the results obtained in the Fe^{2+}/Ascorbate Model system and the results reported by other groups (Baudry *et al*., 1993; Gonzalez *et al*., 1995).

CONCLUSION

The data indicated that EUK-8 and -134 are able to ameliorate oxidative damage to proteins and lipids induced by free radicals. In addition, the results indicated that the anti-oxidant therapy with Mn-salen derivatives lowered the intracellular content of lipofuscin pigments in neuroblastoma cells. This fact opens the probability of using these new compounds against different neurodegenerative disorders. Further global investigations are certainly required to reach this goal.

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