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Title Page

Title:

Perturbation of Dopamine Metabolism by 3-Amino-2-(4'-Halophenyl)propenes Leads to Increased Oxidative Stress and Apoptotic SH-SY5Y Cell Death

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Running Title Page

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Dopamine metabolism perturbation and oxidative stress

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Abbreviations: APP – 3-amino-2-phenylpropene, VMAT - vesicular monoamine transporter, DβM – dopamine-β-monooxygenase, MAO – monoamine oxidase, b_{561} – cytochrome b_{561}, DA – dopamine, NE – norepinephrine, Asc - ascorbic acid , DOPAC – 3,4-dihydroxyphenylacetic acid, HVA – homovanillic acid, ROS – reactive oxygen species, GSH – reduced glutathione, NAC – *N*-acetyl-L-cysteine

Abstract

We have recently characterized a series of 3-amino-2-phenylpropene (APP) derivatives as reversible inhibitors for the bovine adrenal chromaffin granule vesicular monoamine transporter (VMAT) which have been previously characterized as potent irreversible dopamine-β-monooxygenase (DβM) and monoamine oxidase (MAO) inhibitors. Halogen substitution on the 4'-position of the aromatic ring gradually increases VMAT inhibition potency from 4'-F to 4'-I, parallel to the hydrophobicity of the halogen. We show that these derivatives are taken up into both neuronal and non-neuronal cells, and into resealed chromaffin granule ghosts efficiently through passive diffusion. Uptake rates increased according to the hydrophobicity of the 4'-substituent. More importantly, these derivatives are highly toxic to human neuroblastoma SH-SY5Y, but not toxic to M-1, Hep G2 or HEK-293 non-neuronal cells at similar concentrations. They drastically perturb DA uptake and metabolism in SH-SY5Y cells under sub-lethal conditions, and are able to deplete both vesicular and cytosolic catecholamines similar to amphetamines. Additionally, 4'-IAPP treatment significantly increases intracellular ROS and decreases GSH levels in SH-SY5Y cells, and cell death is significantly attenuated by the common antioxidants α-tocopherol, *N*-acetyl-L-cysteine (NAC) and glutathione (GSH), but not by the non-specific caspase inhibitor Z-VAD-FMK. DNA fragmentation analysis further supports that cell death is likely due to a caspase-independent ROS-mediated apoptotic pathway. Based on these and other findings, we propose that drastic perturbation of DA metabolism in SH-SY5Y cells by 4'-halo APP derivatives causes increased oxidative stress leading to apoptotic cell death.

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Introduction

Oxidative stress in the central and peripheral nervous systems plays a significant role in neurodegenerative disorders, aging and the toxicity of a large number of neurotoxins (Cadet and Brannock, 1998; Halliwell, 2006). Auto-oxidizable catecholamines DA, NE, and E and their metabolites are known to generate H_2O_2, reactive oxygen species (ROS) and organic radicals under aerobic conditions due to their intrinsic redox properties (Hald and Lotharius, 2005; Ogawa et al., 2005). Therefore, catecholaminergic neurons are inherently subjected to higher oxidative stress and more free radical damage than other types of neurons (Adams Jr. et al., 2001; Graham, 1978). Although most reactive radical species are effectively scavenged by enzymatic defense mechanisms and cellular antioxidants *in vivo*, excessive generation may lead to extensive cellular damage (Frei et al., 1989; Fridivich, 1986).

Numerous studies indicate that efficient uptake, biosynthetic conversion, and storage of catecholamines in vesicles are mandatory for proper functioning of catecholaminergic neurons. The vital proteins responsible, including vesicular H^+-ATPase (V-H^+ATPase), cytochrome b_{561} (b_{561}), dopamine-β-monooxygenase (DβM), and vesicular monoamine transporter (VMAT), have been well characterized (Beers et al., 1986; Wakefield et al., 1986; Wimalasena and Wimalasena, 2004; Wimalasena and Wimalasena, 1995). V-H^+-ATPase generated transmembrane pH gradient is mandatory for the granular accumulation of catecholamines, as well as the regeneration of intragranular Asc from semidehydroascorbate, through b_{561}. In addition to providing reducing equivalents to DβM-reaction, a high concentration of intragranular Asc ensures a reductive environment, preserving catecholamines from auto-oxidation. Therefore, tightly coordinated, well-integrated functions of V-H^+-ATPase, VMAT, DβM, and b_{561} are not only necessary for efficient granular accumulation, storage, and biotransformation of catecholamines, but also essential for relieving catecholamine-induced oxidative stress. Perturbation of catecholamine metabolism through impaired intra-granular accumulation and bio-transformation could lead to increased oxidative stress and eventual neurodegeneration.

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In agreement with the above proposals, recent studies show that the neuropharmacological and neurotoxic effects of a large number of illicit drugs and neurotoxins are closely associated with interference of catecholamine storage and metabolism. For example, increasing evidence suggests that amphetamine-related drugs exert their effects by increasing the non-exocytotic release of dopamine in some regions of the brain (DiChiara and Imperato, 1988; Sabol and Seiden, 1992) through direct interaction with plasma membrane dopamine transporter (DAT) and VMAT (Riddle et al., 2005; Rudnick and Wall, 1992; Schuldiner et al., 1993). Additionally, the neurotoxicity of the Parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP^+) is believed to be at least partially due to its ability to interfere with vesicular uptake/storage of dopamine (DA) in dopaminergic neurons (Daniels and Reinhard, 1988; Liu et al., 1992; Lotharius and O'Malley, 2000).

We have recently characterized a series of 3-amino-2-phenylpropene (APP) derivatives as novel reversible inhibitors for the bovine adrenal chromaffin granule VMAT (Perera et al., 2003) which have been previously characterized as irreversible DβM (May et al., 1983; Padgette et al., 1985) and MAO (McDonald et al., 1985) inhibitors. We have reported that VMAT inhibition potencies of 4'-halogenated APP derivatives were dependent on the 4'-subtituent and increased in the order 4'-F<4'-Cl<4'-Br<4'-I. In the present study, we demonstrate that while 4'-halogen substituted APP derivatives are highly toxic to SH-SY5Y cells; they are not toxic to non-neuronal cell lines at similar concentrations, and cell toxicities of these derivatives parallel their relative VMAT inhibition potencies and hydrophobicities. These compounds are efficiently accumulated into both neuronal and non-neuronal cells and resealed chromaffin granule ghosts through simple diffusion, and uptake rates follow the same 4'-subtituent dependency as SH-SY5Y toxicity. They drastically reduce DA uptake, storage and metabolism, and dissipate the catecholamine content in SH-SY5Y cells at low concentrations and short incubation times. Furthermore, 4'-IAPP treatment significantly increases intracellular ROS specifically in SH-SY5Y cells and also lowers reduced glutathione (GSH) levels. Additionally, toxicity is attenuated by commonly used antioxidants. DNA fragmentation analysis and caspase inhibition studies suggest that cell death may involve a caspase-independent apoptotic pathway. Based on these and other findings, we propose that

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perturbation of DA metabolism by 4'-halo APP derivatives through VMAT, DβM, and/or MAO inhibition and granular accumulation in SH-SY5Y cells leads to increased oxidative stress and cell death. Our data support the proposal that proper storage of catecholamines in storage vesicles is not only vital for normal physiological functioning, but also necessary to protect catecholaminergic neurons from self-induced oxidative stress.

Materials and Methods

Reagents

Synthesis and characterization of all APP derivatives have been previously reported (Padgette et al., 1985; Perera et al., 2003). MPP^+I was synthesized according to the literature method of Das (Das et al., 1993). APP derivatives (10 mM), GBR 12909 dihydrochloride (1 mM) and desipramine hydrochloride (1 mM) stock solutions were prepared in water, and rotenone (4 mM) stock solution was prepared in ethanol. Commercial α-tocopherol was diluted with EtOH to a 50 mM stock solution. The non-specific caspase inhibitor Z-VAD-FMK (R&D Systems) was diluted to a 20mM stock solution in DMSO. All cell culture media and reagents were purchased from Sigma-Aldrich except FBS which was purchased from Cambrex. All other reagents (highest purity available) and laboratory supplies were purchased from either Fisher Scientific or Sigma-Aldrich.

Instrumentation

UV-visible spectra were recorded on a Cary Bio 300 UV-visible spectrophotometer (Varian, Inc.). Fluorescence emission spectra were recorded using a Jobin Yvon-Spex Tau-3 spectrofluorimeter (ISA Instruments, Inc.). Analyses of catecholamines were carried out using reversed-phase HPLC with electrochemical detection (HPLC-EC) on a C_{18} reversed-phase column as previously described (Wimalasena and Wimalasena, 2004; Wimalasena and Wimalasena, 1995). Reversed-phase HPLC with ultraviolet detection (HPLC-UV) analyses were performed on a Spectra System P4000 gradient pump equipped with a SCM 1000 vacuum degasser coupled to an LDC Analytical SM 4000 UV detector using

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a C_{18} reversed-phase column (Supelco). Elution buffer consisted of a 50:15:35 ratio of 50 mM NaOAc, pH 4.5: CH_3CN: CH_3OH. Flow rate was 0.8 mL/minute. Cell sonications were performed using two 2-second pulses using a Fisher Scientific Sonic Dismembrator Model 100.

Cell Culture

SH-SY5Y cells were purchased from ATCC (Manassas, VA); M-1 cells were obtained from Dr. Karyn Turla (Friends University), Hep G2 and HEK-293 cells were from Dr. Tom Wiese (Fort Hays State University). *S*H-SY5Y human neuroblastoma cells (CRL-2266), HEK-293 human embryonic kidney (CRL-1573) and Hep G2 liver carcinoma cells (HB-8065) were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37 °C and 5% CO_2. M-1 murine renal tubule cells (CRL-2038) were cultured in 1:1 DMEM:Ham's F-12 media supplemented with 5% FBS, 5μM dexamethasone and 2.5 mM L-glutamine at 37 °C and 5% CO_2. All cells were cultured in 100 mm^2 tissue culture plates until about 80% confluence, and then were seeded into multi-well plates depending on the nature of the experiment. When EtOH or DMSO was used as a co-solvent, final concentrations were kept at ≤ 0.1% or 1.0%, respectively.

Preparation of Chromaffin Granule Ghosts

Chromaffin granules and lysed granule membranes were prepared from fresh bovine adrenal medullae as previously described (Wimalasena and Wimalasena, 2004; Wimalasena and Wimalasena, 1995). Granule membranes were washed and resealed to contain 20 mM Tris-phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, 4.0 μM copper, 100 μg/mL catalase and 20 mM Asc. The resealed ghosts were purified by a 15% Ficoll, 0.3 M sucrose, 10 mM HEPES, pH 7.0 discontinuous density gradient as previously described (Wimalasena and Wimalasena, 2004; Wimalasena and Wimalasena, 1995).

Cell Viability

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Cell viability was measured using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay (Denizot and Lang, 1986). Briefly, cells were seeded into 96-well plates at $5x10^4$ cells/well and grown 1-2 days to achieve ~80% confluence. Cells were treated with the desired concentration of 4'-halo APP derivatives (0-1000 μM) in a total volume of 50 μL of phenol red-free complete DMEM for 24 h. After the incubation period, 20 μL of 5 mg/mL MTT solution was added to each well and was incubated for 2 h at 37 °C. The resulting formazan was solubilized by addition of 200 μL detergent solution (50% DMF, 20% SDS) followed by incubation for 12 h at 37 °C. Cell viability was determined by quantifying the formazan, based on the difference in the absorbance at 570 nm and 650 nm. Results are expressed as % of APP untreated controls.

Neuroprotective Studies

SH-SY5Y cells were grown in 96-well plates as described above and were incubated with various concentrations of either α-tocopherol (0-25 μM), *N*-acetyl-cysteine (NAC; 0-25 μM), or glutathione (GSH; 0-25 μM) in DMEM for 2 h. Then, 200 μM 4'-IAPP (final concentration) was added and cells were incubated for 24 h, and cell viability was determined by the MTT assay as described above. Results are expressed as % of APP untreated controls. The effects of the inhibition of caspases on cell viability was evaluated utilizing the non-specific caspase inhibitor Z-VAD-FMK (0-25 μM in DMEM) using the same procedure described above for antioxidants. Results are expressed as % of APP untreated controls.

Cellular Uptake

Uptake of 4'-halo APP derivatives into SH-SY5Y or HEK-293 cells was determined by reversed-phase HPLC-UV. Cells were seeded into 6-well plates at $1x10^6$ cells/well and grown 1-2 days to achieve ~80% confluence. The media was removed and a solution containing 50 μM 4'-halo APP derivative in warm Krebs-Ringer buffer (KRB – 125 mM NaCl, 2 mM KCl, 1.4 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 20 mM HEPES, 5 mM glucose, pH 7.4) was added to each well and incubated for 0 and 30 minutes at 37°C, at which time the media was removed and cells were washed three times with ice-cold

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KRB. Washed cells were transferred into a 1.5 mL microcentrifuge tube and centrifuged at 500 x *g* for 5 minutes. The supernatant was discarded and the cell pellet was treated with 100 μL of 0.1M $HClO_4$. The coagulated proteins were pelleted by centrifugation at 15,000 x *g* for 5 minutes at 4 ^oC and the concentration of each 4'-halo APP in acidic supernatants was determined by reversed-phase HPLC-UV analysis as detailed above. All 4'-halo APP levels were normalized to the respective protein concentrations and the readings were corrected for non-specific binding by subtracting the corresponding time zero point readings from the 30 minute readings.

Granular Uptake

The ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM $MgSO_4$, 100 μg/mL catalase, 5 mM ATP, 5 mM Asc in a total volume of 1.75 mL. This mixture was pre-incubated for 10 min at 30 °C and reactions were initiated by the addition of 100 μM of the desired 4'-halo APP derivative. Aliquots (400 μL) of the incubate were withdrawn at 0, 15, 30, and 45 time intervals, diluted into 5.0 mL of ice-cold 0.4 M sucrose and stored on ice until the end of the experiment. Finally, ghosts were re-isolated from these samples washed as previously reported (Wimalasena and Wimalasena, 2004; Wimalasena and Wimalasena, 1995), lysed with 0.1 M $HClO_4$, and intragranular 4'-halo APP concentrations were determined by HPLC-UV as detailed above. All 4'-halo APP levels were normalized to the respective granule protein concentrations and the readings were corrected for non-specific binding by subtracting the corresponding time zero point readings from the other time point readings.

Catecholamine Perturbation

SH-SY5Y cells were seeded into 12-well plates at $0.5x10^6$ cells/well and grown to near confluence prior to experimentation. Cells were incubated with 40 μM of each 4'-halo APP derivative in KRB for 10 min at 37 °C. DA was then added to the medium to a final concentration of 50 μM, and samples were

incubated an additional 30 min at 37 °C. Cells were washed three times with ice-cold KRB and harvested as detailed above and intracellular DA, NE, DOPAC and HVA levels were quantified by reversed-phase HPLC-EC analysis as detailed above. In a second set of experiments, SH-SY5Y cells were initially incubated with 50 μM DA for 30 minutes, washed with KRB followed by incubation with 40 μM 4'-IAPP in KRB for 30 min. Cells were washed, harvested and catecholamine levels were quantified. Catecholamine levels were normalized to the total protein content of each sample and compared to parallel untreated control samples.

Reactive Oxygen Species (ROS)

Intracellular ROS levels were quantified by using the 2',7'-dichlorofluorescin diacetate (DCF-DA) method (Oubrahim et al., 2001). Briefly, SH-SY5Y or HEK-293 cells were seeded into 6-well plates at $1x10^6$ cells/well and grown for 1-2 days in complete DMEM to near-confluence. Cells were treated with desired concentrations of 4'-IAPP for 1 h followed by addition of 50 μM DCF-DA for 1 h. Cells were washed, harvested, and lysed with 0.1 M Tris buffer (pH 7.5) containing 1% Triton X-100, and the DCF fluorescence of supernatants were measured (ex: 504 nm/em: 526 nm) following removal of cellular debris by centrifugation at 15,000 x *g* for 5 minutes at 37°C. Fluorescence readings were normalized to the total protein content of respective samples using the bicinchoninic acid method (Smith et al., 1985).

Intracellular Reduced Glutathione (GSH)

Intracellular reduced glutathione levels were measured using the monochlorobimane (MCB) based fluorescence assay (Nair et al., 1991). SH-SY5Y cells were grown in 12-well plates and treated with various concentrations of 4'-IAPP in complete DMEM for 24 h. MCB was then added to a final concentration of 40 μM in each well and further incubated for 30 min. Cells were washed and suspended in 1 mL Ca^{2+}/Mg^{2+} free PBS, transferred into 1.5 mL microcentrifuge tubes and sonicated. The characteristic fluorescence of the reduced glutathione adduct of bimane was measured and quantified (Ex:

390 nm/ Em: 478 nm). Fluorescence readings were normalized to the total protein content of each sample.

Apoptotic DNA Laddering

SH-SY5Y cells were cultured in 60 mm^2 plates ($5x10^6$ cells/plate) for 24 h and treated with 0, 100 or 300 μM 4'-IAPP or 4 μM rotenone for 24 hours. Total cellular DNA was extracted using the Qiagen DNeasy DNA Isolation Kit according to the manufacturer's instructions, including treatment with Proteinase K and RNase A. DNA samples were loaded onto a 1.2% agarose gel and electrophoresed at 70 V for 2-3 hours until the bromophenol blue band had migrated approximately 2/3 down the gel. DNA bands were stained with ethidium bromide, visualized on a UV trans-illuminator and photographed using a Kodak Gel Logic 100 system. Cells treated with 4 μM rotenone were included as a positive control for ROS induced apoptotic cell death (Watabe and Nakaki, 2004).

Protein Quantification

Protein quantifications were generally carried out by the Bradford method (Bradford, 1976) except in ROS analysis experiments where the protein was determined by the bicinchoninic acid method due to the presence of 1% Triton X-100 in the samples (Smith et al., 1985).

Data Analysis

All results represent the mean of at least three experimental trials. Error bars represent the sample SD and significance was tested by either two-tailed Student's t-test or one-way ANOVA. Values of $p < 0.05$ were considered statistically significant. When applicable, quantitative experimental data were normalized to the protein content of each sample.

Technical Statement

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Due to the potentially unclear mode of action and seemingly nonspecific mode of cellular entry, extreme caution was used when utilizing the compounds discussed in this paper in accordance with published precautions of related neurotoxins (Przedborski et al., 2001).

Results

As summarized in Table 1, we have previously shown that APP derivatives are novel inhibitors for bovine chromaffin granule VMAT (Perera et al., 2003). These studies have also shown that, 4'-OHAPP, a structural analog of VMAT substrate tyramine, behaves as a competitive inhibitor for VMAT having a similar affinity to that of DA. Additionally, data in Table 1 shows that 4'-halogen substitution increases the VMAT inhibition potency from –F, to –I, parallel to their overall hydrophobicities. Previous studies have shown that APP derivatives possess potent irreversible inhibition properties toward MAO (McDonald et al., 1985) and DβM (Padgette et al., 1985). The structural similarities, gradually increasing VMAT inhibition potencies and hydrophobicities within the series of 4'-halogen substitutes APP derivatives make them a unique series of probes that can be used to examine the effects of the perturbation of DA metabolism in catecholaminergic neurons.

Cellular uptake of 4'-halo APP derivatives was examined using a series of short-time incubations employing low concentrations. In these experiments, SH-SH5Y cells were incubated with each 4'-halo APP derivative and the intracellular APP concentrations were quantified by HPLC-UV as detailed in "Materials and Methods". The zero time points were used to determine the degree of non-specific binding of each derivative and were subtracted from the 30 min readings to accurately determine the intracellular concentrations. As shown in Fig. 1, 4'-ClAPP, 4'-BrAPP and 4'-IAPP were effectively taken up into SH-SY5Y cells, while uptake of 4'-FAPP considerably lower. Furthermore, the relative uptake of these derivatives were both time- and concentration-dependent, and not significantly altered by the presence of 1 μM DAT or NET inhibitors - GBR 12909 or desipramine, respectively (data not shown). These derivatives were also taken up into non-neuronal HEK-293 cells in a concentration- and time- dependent manner with the rates similar to that of SH-SY5Y cells (data not shown).

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We previously reported that 3'- and 4'-OHAPP derivatives are not taken into resealed chromaffin granule ghosts at a detectable rate (Padgette et al., 1985; Perera et al., 2003). However, as 4'-halo APP derivatives are significantly more hydrophobic, and are freely taken up by SH-SY5Y and other cells; we expected that these derivatives may also be taken up into chromaffin granule ghosts through simple diffusion. In order to test this possibility, a series of standard uptake experiments were carried out with resealed chromaffin granule ghosts and the above APP derivatives. The data in Fig. 2 show that while 4'-I and 4'-BrAPP derivatives were taken up effectively, 4'-ClAPP was taken up relatively slowly. Similar to cellular uptake, 4'-FAPP uptake was low and could not be accurately quantified under the experimental conditions.

Initial screening experiments have revealed that only certain APP derivatives are highly toxic to human neuroblastoma SH-SY5Y cells. Interestingly, while the 4'-halogenated derivatives were found to be the most toxic, the more polar 3'- and 4'-OH derivatives, with comparable VMAT inhibition potencies to that of 4'-halo derivatives, were found to have no apparent toxicity to these cells (data not shown). Based on these observations, 4'-halo APP derivatives were chosen as the best candidates for detailed toxicological and structure-activity studies. As seen in Fig. 3A, these derivatives induce marked concentration-dependent cellular death on SH-SY5Y cells over a 24 h incubation period. The estimated LC_{50} values range from 867 μM for 4'-FAPP to 204 μM for 4'-IAPP. Comparative toxicity experiments with MPP^+ (1-methyl-4-phenylpyridinium), which has been extensively used as a model dopaminergic neurotoxin (Burns et al., 1983; Lotharius and O'Malley, 2000; Song et al., 1997), have shown that the 4'-halo APP derivatives are significantly more toxic than MPP^+ to SH-SY5Y cells, under similar experimental conditions. Exposure of SH-SY5Y cells to 1000 μM MPP^+ for 24 h reduces cell viability to ~75% which is equivalent to the effect of 100 μM 4'-IAPP treatment for the same time period. Data presented in Fig. 3B further demonstrates that 4'-IAPP mediated SH-SY5Y cell death is time-dependent. At 400 μM, 4'-IAPP induced cell death was quite rapid with greater than 50% cell death occurring within the initial 3 h and after 24 h no viable cells could be detected.

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To determine the specificity of the observed toxicity of 4'-halo APP derivatives, 4'-IAPP was evaluated against several commonly used non-neuronal cell lines, (Hep G2, HEK-293 and M-1). As shown in Fig. 3C, 4'-IAPP is not toxic to any of these cell lines in the concentration range tested (0 to 400 μM). While both Hep G2 and HEK-293 cells maintain 100% viability throughout this concentration range, interestingly, M-1 cells appear to show a concentration dependent stimulation of cell growth with respect to a parallel control. However, at 4'-IAPP concentrations above 500 μM, some modest cellular toxicity has been observed in HEK-293 and Hep G2 cells.

As shown in Fig. 3B, SH-SY5Y cell death induced by 4'-IAPP was quite rapid. In addition, the cellular toxicities of these derivatives are also associated with rapid morphological changes. Morphological changes associated with 45 min 4'-IAPP (100 or 300 μM) treatment is shown in Fig. 4. The normal oblong, extended appearance of SH-SY5Y cells (Panel A) was altered to a shrunken spherical appearance following 300 μM 4'-IAPP treatment (Panel C). Cells treated with 100 μM 4'-IAPP show only modest morphological changes at 45 min (Panel B), but became increasingly apparent at longer exposure times (data not shown). On the other hand, similar treatments of HEK-293 and Hep G2 cells with 4'-IAPP show no significant morphological changes, consistent with observed resistance of these cells to 4'-IAPP toxicity.

We have previously reported that APP derivatives effectively interfere with the accumulation of DA in resealed chromaffin granule ghosts (Perera et al., 2003). Current studies using resealed granule ghosts (Fig. 2) show that that 4'-halo APP derivatives may effectively accumulate in storage vesicles, further perturbing the DA metabolism. Thus, a series of experiments was performed to evaluate the ability of 4'-halo APP derivatives to interfere with DA uptake and storage. SH-SY5Y cells were initially incubated with 40 μM of the desired 4'-halo APP derivative for 10 min followed by 50 μM DA for 30 min as described in "Materials and Methods" in order to evaluate the degree of DA uptake impairment. Data in Fig. 5A demonstrate that 4'-halo APP pretreatment drastically reduces intracellular catecholamine levels (DA, NE, DOPAC and HVA) and the extent of these effects were in the order 4'-IAPP > 4'-BrAPP

> 4'-ClAPP > 4'-FAPP. Similarly, catecholamine levels in cells pre-loaded with exogenous DA by incubating SH-SY5Y cells with 50 μM DA for 30 minutes are also significantly depleted by the incubation with 100 μM 4'-IAPP in KRB for 50 min (Fig. 5B). However, catecholamine depletion under these conditions was somewhat slower, requiring higher concentrations of 4'-haloAPP and longer incubation times to obtain comparable reductions. Although all four 4'-halo APP derivatives were investigated, only the results of 4'FAPP and 4'IAPP are displayed in Fig. 5 for purposes of clarity. In almost all experimental trials, the degree of catecholamine depletion induced by 4'ClAPP and 4'BrAPP was between that of 4'FAPP and 4'IAPP.

The ROS levels of 4'I-APP treated SH-SY5Y and HEK-293 cells were measured using standard techniques as detailed in "Materials and Methods". As shown in Fig. 6, treatment of SH-SY5Y cells with 4'-IAPP (50 and 150 μM) for 1 h results in a rapid increase of intracellular ROS levels by about 20% and 40% respectively, compared to an untreated control. On the other hand, treatment of HEK-293 cells under the same conditions does not result in increased intracellular ROS levels, suggesting 4'-IAPP induced increased ROS production is specific to SH-SY5Y cells.

Data presented Fig. 7 show that commonly used antioxidant *N*-acetyl-L-cysteine (NAC) significantly attenuates cell death in a concentration-dependent manner. For example, preincubation of cells with 25 μM NAC for 2 h followed by 200 μM 4'-IAPP treatment for 24 h increased viability by ~20%, compared to an untreated control. Similar experiments with GSH showed that it is somewhat less effective, but still showed a statistically significant increase in cell viability by ~15% (data not shown). Interestingly, α-tocopherol pretreatment provides the most protection of the antioxidants studied, increasing cell viability by ~30% at 25 μM concentration, under similar conditions, compared to an untreated control (data not shown). The effect of the non-specific caspase inhibitor Z-VAD-FMK was used to evaluate whether a caspase-mediated apoptotic cell death pathway is involved in 4'-IAPP induced SH-SY5Y cell death. These experiments have revealed that Z-VAD-FMK up to 25 μM concentration was not effective in protecting cells from 200 μM 4'-IAPP induced SH-SY5Y cell death (Data not shown).

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To determine the effect of increased ROS production on the overall antioxidant balance of SH-SY5Y cells, intracellular GSH levels were fluorometrically determined as described in "Materials and Methods". Upon 24 h exposure of SH-SY5Y cells to 25, 50 and 100 μM 4'-IAPP, intracellular GSH levels were found to be significantly decreased in a concentration-dependent manner compared to untreated control (Fig. 8).

4'-IAPP treated SH-SY5Y cells were examined for internucleosomal DNA cleavage – a marker of apoptotic activity. As seen in Fig. 9, agarose gel electrophoresis of nuclear DNA isolated from 300 μM 4'-IAPP treated SH-SY5Y cells shows a distinct fragmentation pattern (Lane 3) in multiples of 200 bp, similar to that induced by the standard apoptotic agent rotenone (4 μM, Lane 4). Background smearing visible in the control sample (Lane 1) and other lanes is due to a population of dead cells present in the samples prior to the beginning of the experiment. However, the apoptotic laddering pattern is distinctly present in Lanes 3 and 4 compared to the control, indicative of apoptotic activity.

Discussion

Cellular uptake of 4'-halo APP derivatives is dependent on the nature of the 4'-subtituent, and increases parallel to substituent hydrophobicity (Fig. 1). The observation that uptake into SH-SY5Y cells is not inhibited by the presence of DAT or NET inhibitors, and comparable rates of accumulation into non-neuronal and SH-SY5Y cells suggests that cellular uptake is likely through simple diffusion. Parallel studies have shown that 4'-halo APP derivatives are also efficiently accumulated into resealed granule ghosts in a time- (Fig. 2) and concentration- (data not shown) dependent manner. The efficiency of granular accumulation follows a trend similar to that of cellular uptake where rates increased according to the hydrophobicity of the molecule (4'-F<4'-Cl<<4'-Br~4'-I). In contrast to the behavior of 4'-halo APP derivatives, more polar 4'-OHAPP and 3'-OHAPP were not taken up into cells or granule ghosts in detectable amounts as previously reported (Perera et al., 2003). This evidence further confirms that both

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cellular and granular uptake of 4'-halo APP derivatives must occur through simple diffusion due to their high hydrophobicities.

Among APP derivatives tested, 4'-halogen substituted derivatives are the most toxic to SH-SY5Y cells, and toxicity parallels both cellular and granular uptake efficiencies (Fig. 3A). Additionally, 4'-IAPP is significantly more toxic to SH-SY5Y cells than the well-characterized dopaminergic neurotoxin MPP^+ under identical experimental conditions. Toxicities are concentration- and time-dependent, and gradually increasing from 4'-F (EC_{50} = 867 μM) to 4'-I (EC_{50} = 204 μM) (Fig. 3B). In contrast, they are not significantly toxic to non-neuronal cells. Similarly, 4'-OH and 3'-OHAPP derivatives are non-toxic to both SH-SY5Y and non-neuronal cells under similar experimental conditions.

We have previously shown that ring-substituted APP derivatives are good competitive inhibitors for chromaffin granule VMAT with respect to DA. Among APP derivatives tested, 4'-OH, 3'-OH and 4'-halo derivatives are the most potent (Perera et al., 2003). Previous studies have shown that APP derivatives are also potent turnover-dependent, irreversible inhibitors for DβM (May et al., 1983; Padgette et al., 1985) and MAO (McDonald et al., 1985). Thus, the specific toxicity of 4'-halo derivatives to SH-SY5Y cells could be associated with a combination of factors that are not present in non-neuronal cells including efficient and non-specific passage into cells and storage vesicles, reversible inhibition of VMAT and/or irreversible inhibition of DβM and MAO leading to perturbation of catecholamine metabolism. Strong further support for this proposal could be derived from the observation that pretreatment of SH-SY5Y cells with sub-lethal concentrations of 4'-halo APP derivatives for short time periods drastically reduces DA uptake, conversion, and metabolism (Fig. 5A). Similarly, experiments in which SH-SY5Y cells were first loaded with exogenous DA [SH-SY5Y cells contain low levels of endogenous DA (unpublished observation)] further show that 4'-halo APP derivatives are capable of depleting pre-accumulated intracellular catecholamines (Fig. 5B) similar to that reported following reserpine treatment (Rudnick, 1997). In addition, both experiments show the catecholamine-depleting potency of 4'-halo APP derivatives parallel their cellular toxicities, cellular/granular uptake efficiencies

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and VMAT inhibition potencies. However, we note that the effects of 4' halo APP on intracellular catecholamine levels under uptake conditions (Fig. 5A) were more drastic than under DA pre-loaded conditions (Fig. 5B). A potential explanation for this observation could be that these compounds, in addition to the perturbation of intracellular catecholamines, may also inhibit the uptake of extracellular DA, similar to that was observed with amphetamine and related compounds (Kahlig et al., 2006; Schuldiner et al., 1993). However, additional studies are certainly necessary to confirm this proposal firmly.

The toxicities of 4'-halo APP derivatives are associated with rapid morphological changes (Fig. 4) which are commonly associated with apoptotic cell death (Mattson, 2006). Thus, the rapid onset of effects on SH-SY5Y cells observed in time course studies is consistent with the loss of cell viability through an apoptotic pathway. A strong support for this proposal is provided by the DNA laddering experiments where characteristic 200 bp DNA fragmentation patterns were observed in 4'-IAPP treated SH-SY5Y cells (Fig. 9). Such apoptotic processes may be initiated by a catecholamine-induced oxidative stress, since cellular toxicity of 4'-IAPP appears to be associated with the perturbation of catecholamine metabolism, as argued above. This notion is further supported the observation that 4'-IAPP induced rapid morphological changes correlate well with time frames of catecholamine perturbation as well as generation of excessive intracellular ROS. The exposure of SH-SY5Y cells to moderate 4'-IAPP concentrations show significant depletion of intracellular catecholamine levels and a simultaneous substantial increase in ROS levels (Fig. 6). Additionally, treatment of HEK-293 cells under similar conditions resulted in no ROS increase, further suggesting that these effects are associated with 4'-IAPP-mediated perturbation of catecholamine metabolism in SH-SY5Y cells. Consistent with prolonged, excessive ROS production, 4'-IAPP induced a significant depletion in GSH levels in SH-SY5Y cells at longer incubation times (Fig. 8). These findings were further confirmed by the observation that commonly used antioxidants NAC, GSH and α-tocopherol (25 μM) were found be effective in protecting SH-SY5Y cells from 4'-IAPP induced cell death (Fig. 7). However, studies with the non-specific caspase inhibitor Z-VAD-FMK suggest that the cell death is not due to a caspase-mediated pathway. These findings

support the conclusion that 4'-halo APP-mediated SH-SY5Y cell toxicity may be due oxidative stress induced caspase-independent apoptotic cell death caused by excessive perturbation of catecholamine metabolism in SH-SY5Y cells.

Taken together, the above findings that 4'-halo APP derivatives are accumulated into both neuronal and non-neuronal cells, but are specifically toxic to SH-SY5Y cells suggest that specific characteristic(s) present in these, but absent in non-neuronal cells, is responsible for 4'-haloAPP-induced toxicity. In addition, as mentioned above, these derivatives act as potent reversible VMAT and irreversible DβM and MAO inhibitors, and are also efficiently accumulated into catecholamine storage vesicles. Thus, they should perturb DA uptake, storage and metabolism similar to that of amphetamines (Kahlig et al., 2005). This notion is strongly supported by the observation that treatment with 4'-IAPP under sub-lethal conditions drastically reduced DA uptake, storage, and metabolism, and the extent of these effects parallel their toxicities. Thus, drastic, prolonged perturbation of DA storage and metabolism resulting high oxidative stress is the most likely cause of 4'-halo APP toxicity specifically to SH-SY5Y cells. However, additional experimental data is certainly necessary to firmly establish the precise mechanism(s) of 4'-halo APP toxicity to these cells, as it is not yet clear whether exclusive VMAT, DβM or MAO inhibition, or a combination thereof, is primarily responsible for the observed catecholamine perturbation and neurotoxicity.

The intragranular environment is equipped to protect catecholamines from auto-oxidation by employing an acidic milieu, a high concentration of Asc and low levels of redox-active materials, including transition metals. In contrast, cytosolic or extracellular conditions may favor the auto-oxidation of catecholamines due to neutral pH (7.4), low Asc concentration and the presence of various redox active materials. Auto-oxidation of catecholamines is known to produce reactive harmful radicals and increased oxidative stress (Ogawa et al., 2005; Sulzer et al., 2000; Tse et al., 1976). Thus, proper storage and metabolism of catecholamines in storage vesicles is not only vital for normal physiological functioning, but to protect cells from self-induced oxidative stress.

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Legends for Figures

Figure 1. Uptake of 4'-halo APP into SH-SY5Y cells. Cells were grown in 6-well plates and were incubated with the desired 4'-halo APP derivative (50 μM) in KRB for 30 min. Media was removed and cells were washed three times with ice-cold KRB. The intracellular 4'-halo APP concentrations were determined by reversed-phase HPLC-UV and were normalized to respective cellular protein concentrations. All readings were corrected for non-specific binding by subtracting the corresponding t = 0 readings. Data represent mean ± S.D. of triplicate samples and are statistically different based on one-way ANOVA analysis ($p < 0.001$).

Figure 2. Uptake of 4'-halo APP into resealed chromaffin granule ghosts. Resealed chromaffin granule ghosts prepared as detailed in "Materials and Methods" were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM ATP, 5 mM $MgSO_4$, 100 μg/mL catalase, 5 mM Asc. This mixture was pre-incubated for 10 min at 30 °C and reactions were initiated by addition of 100 μM of the desired 4'-halo APP derivative. Aliquots (400 μL) of the incubate were withdrawn at 0, 15, 30, and 45 min time intervals and intragranular levels of 4'-halo APP derivatives were quantified by HPLC-UV and were normalized to respective granule protein concentrations. The intragranular concentration of 4'-FAPP was low and could not be quantified accurately due to sensitivity limitations of HPLC-UV. All readings were corrected for non-specific binding by subtracting the corresponding t = 0 readings. Data represent mean ± S.D. of triplicate samples. (●), 4'-ClAPP; (○), 4'-BrAPP; (▼), 4'-IAPP.

Figure 3. 4'-Halo APP Toxicity is both concentration- and time-dependent, and specific for SH-SY5Y cells. *Panel A.* SH-SY5Y cells grown in 96-well plates were treated with 0-1000 μM of 4'-halo APP derivatives in phenol-red free complete DMEM for 24 h and cell viability was determined by the MTT assay as detailed in "Materials and Methods". The classical neurotoxin MPP^+ was included for

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comparative purposes. Data represent mean ± S.D. of at least six separate experiments carried out in triplicate sets. (●), MPP^+; (○), 4'-FAPP; (▼), 4'-ClAPP; (▽), 4'-BrAPP; (■), 4'-IAPP. *Panel B.* SH-SY5Y cells grown as above were treated with 400 μM 4'-IAPP in phenol-red free complete DMEM for the various time intervals (0-12 h) and cell viability was measured using the MTT assay. Data represent mean ± S.D. of at least six separate experiments carried out in triplicate sets. *Panel C.* SH-SY5Y, HEK-293, Hep G2 and M-1 cells were grown in 96-well plates and treated with various concentrations of 4'-IAPP (0-400 μM) in complete medium for 24 h cell viabilities were determined by MTT assay. At higher 4'-IAPP concentrations (> 500 μM) some nonspecific cell death was observed in HEK-293 and Hep G2 cells but not in M-1 (data not shown). Data represent mean ± S.D. of at least six separate experiments carried out in triplicate sets. (●), SH-SY5Y cells; (○), HEK-293 cells; (▼), Hep G2 cells; (▽), M-1 cells

Figure. 4. Morphological changes of 4'-IAPP treated SH-SY5Y cells. SH-SY5Y cells were grown in 60 mm^2 culture plates for 24 h and treated with 0, 100, or 300 μM 4'-IAPP. After 45 min incubation, cells were photographed under phase-contrast microscopy at 100 x magnification. *Panel A.* untreated cells; *Panel B.* cells treated with 100 μM 4'-IAPP; *Panel C.* cells treated with 300 μM 4'-IAPP. Scale bar, 50 μm.

Figure 5. Effect of 4'-IAPP on catecholamine metabolism in SH-SY5Y cells. *Panel A.* Cells were grown in 12-well plates and incubated with 40 μM concentrations of each 4'-halo APP derivative in KRB for 10 min at 37°C. DA was then added to a final concentration of 50 μM and samples were further incubated for 30 min at 37°C. Cells were washed, harvested, and intracellular DA, NE, DOPAC and HVA levels were quantified by reversed-phase HPLC-EC analysis and normalized to respective cellular protein concentrations. Catecholamine levels are expressed as % of control DA levels (100%). Data represent mean ± S.D. of four samples. *Panel B.* Cells were grown in 12-well plates and incubated with 50 μM DA for 30 minutes, washed once with ice-cold KRB, then incubated with 100 μM 4'-IAPP in

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KRB for 50 min. Following incubation, cells were washed, harvested and catecholamine levels were quantified by reversed-phase HPLC-EC analysis and normalized to respective cellular protein concentrations. Catecholamine levels are expressed as % of control DA levels (100%). Data represent ± S.D. of four samples. *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Effect of 4'-IAPP on intracellular ROS levels. Intracellular ROS levels were quantified by the 2',7'-dichlorofluorescin diacetate method (Oubrahim et al., 2001). SH-SY5Y and HEK-293 cells were incubated with various concentrations of 4'-IAPP in complete DMEM for 1 h, then the media was removed and 50 μM DCF-DA was added and cells incubated an additional 1 h. Cells were washed, lysed and DCF fluorescence was measured (Ex: 504nm/Em: 526nm) and normalized to respective cellular protein concentrations. Data represent mean ± S.D. of at least triplicate samples. *p<0.05, **p<0.01 compared to untreated control.

Figure 7. Neuroprotective effect of *N*-acetyl-L-cysteine (NAC). SH-SY5Y cells were seeded into 96-well plates as described in "Materials and Methods" and incubated for 2 h with 0 – 25 μM NAC in phenol-red free DMEM at which time 4'-IAPP was added to a final concentration of 200 μM to each well. Cells were incubated for 24 h and viabilities were measured by MTT assay. We note that toxicity of 200 μM 4'-IAPP was slightly higher in this experiment in comparison to the data in Fig 3, probably due to slight differences in experimental conditions and number of cell passages. Data represent mean ± of five samples. *p<0.05, **p<0.01 compared to unprotected control.

Figure 8. Effect of 4'-IAPP on intracellular GSH levels. Intracellular reduced glutathione levels were measured using the monochlorobimane based fluorometric assay (Nair et al., 1991). SH-SY5Y cells were grown in 12 well plates and treated with various concentrations of 4'-IAPP in complete DMEM for 24 h. Then, 40 μM monochlorobimane (MCB) was added to each well and cells were incubated an additional

30 min. Cells were washed, lysed, and the characteristic fluorescence of the GSH-bimane adduct was quantified (Ex: 390 nm/ Em: 478 nm) and normalized to respective cellular protein concentrations. Data represent mean ± S.D. of at least triplicate samples. *$p<0.05$, **$p<0.01$ compared to untreated control.

Figure 9. 4'-IAPP induces apoptotic DNA laddering in SH-SY5Y cells. Cells were incubated for 24 h with either 4'I-APP [0 μM (Lane 1), 100 μM (Lane 2), 300 μM (Lane 3)] or rotenone [4 μM (Lane 4)]. DNA from treated cells was recovered using the Qiagen DNeasy DNA Isolation Kit and was electrophoresed on a 1.2% agarose gel. Image was cropped and inverted from original photo, with DNA marker bands representing 1,517 bp, 1,200 bp, 1,000 bp, 900 bp, 800 bp, and 700bp. Both 300 μM 4'-IAPP and 4 μM rotenone show characteristic apoptotic 200 bp laddering while control and 100 μM 4'-IAPP lanes do not.

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Tables

Table 1. DA uptake inhibition in resealed bovine chromaffin granules. Data from Perera et al., (2003).

	K_i (μM) ± S.D.	$K_{m,DA}/K_i$[a]
4'-OHAPP	15.5 ±0.9	1.52
3'-OHAPP	16.7 ± 1.1	1.40
4'-FAPP	42.3 ± 3.1	0.80
4'-ClAPP	18.0 ± 0.9	1.76
4'-BrAPP	17.7 ± 2.0	1.79
4'-IAPP	12.9 ± 2.3	2.96

[a]The non-standard $K_{m,DA}/K_i$ ratios were calculated from the respective K_i and $K_{m,DA}$ parameters determined for each compound to normalize the variation of K_m for DA for different ghost preparations. These values also provide a measure of their relative abilities to compete with DA under the same experimental conditions.

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Figure 1

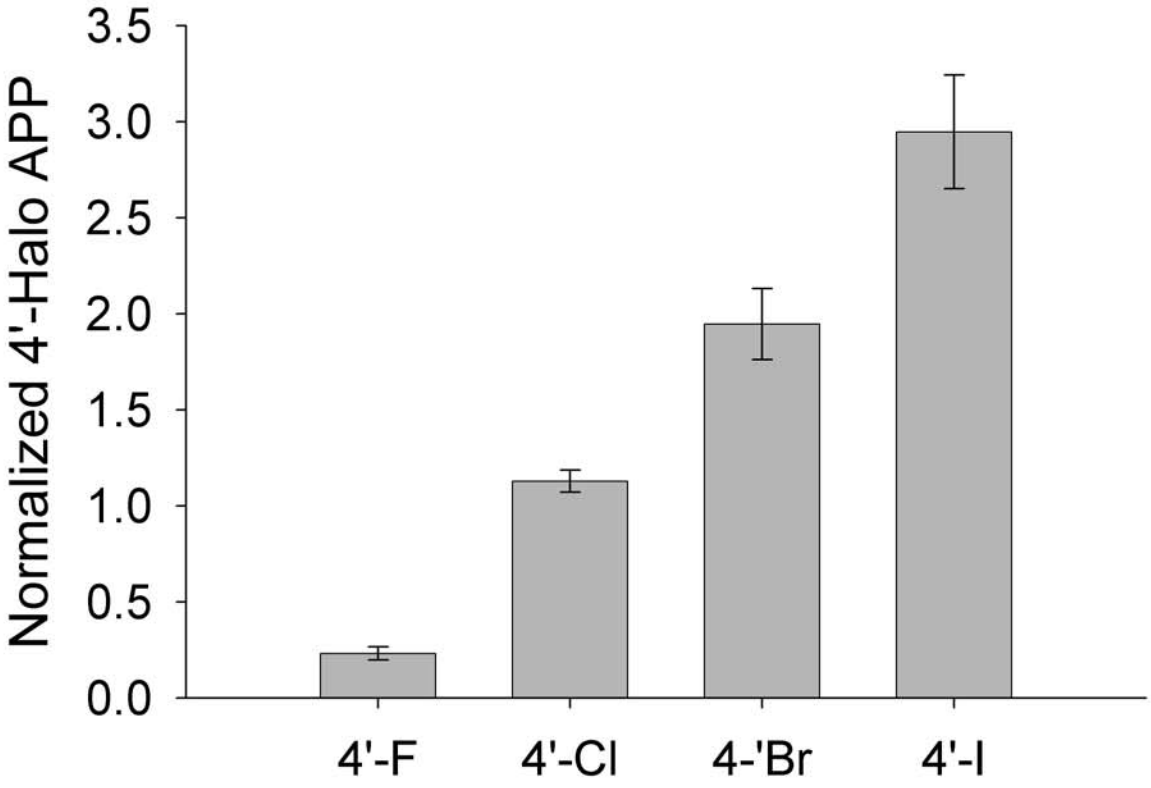

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Figure 2

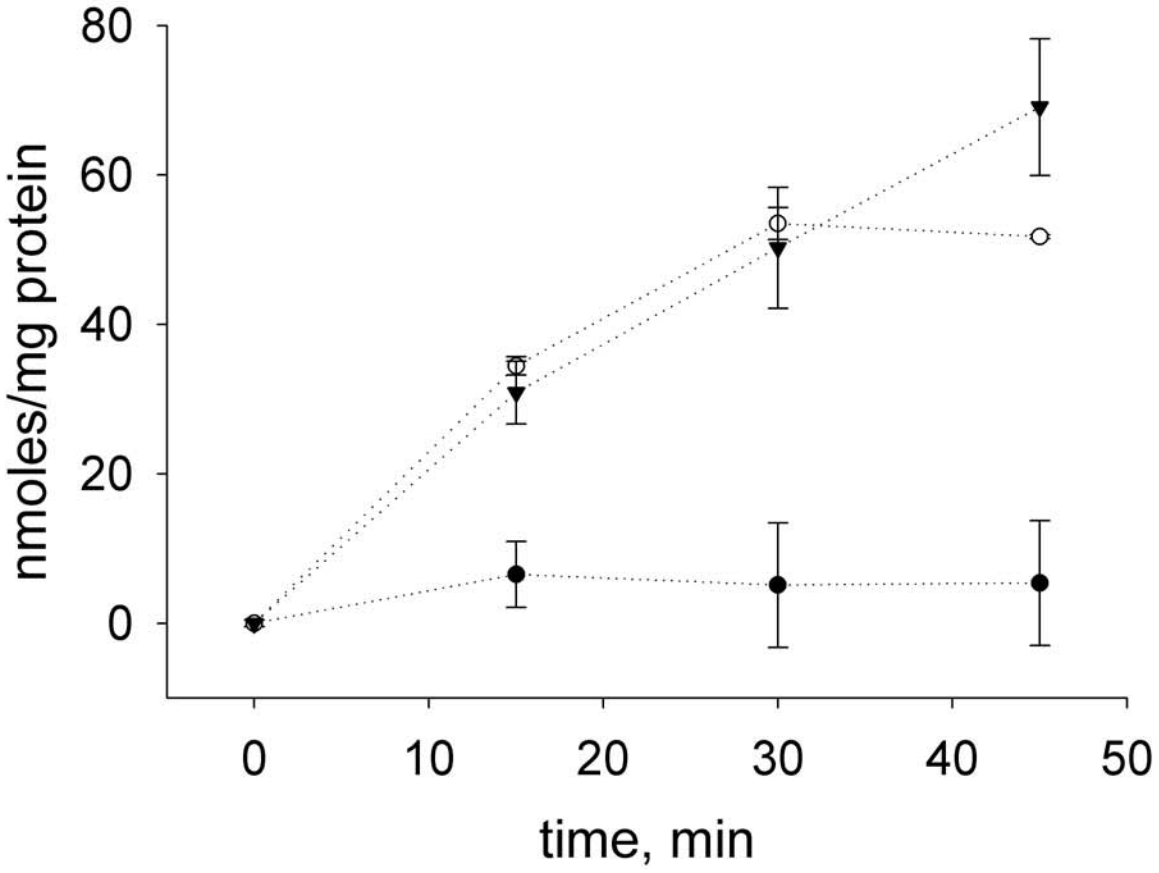

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Figure 3

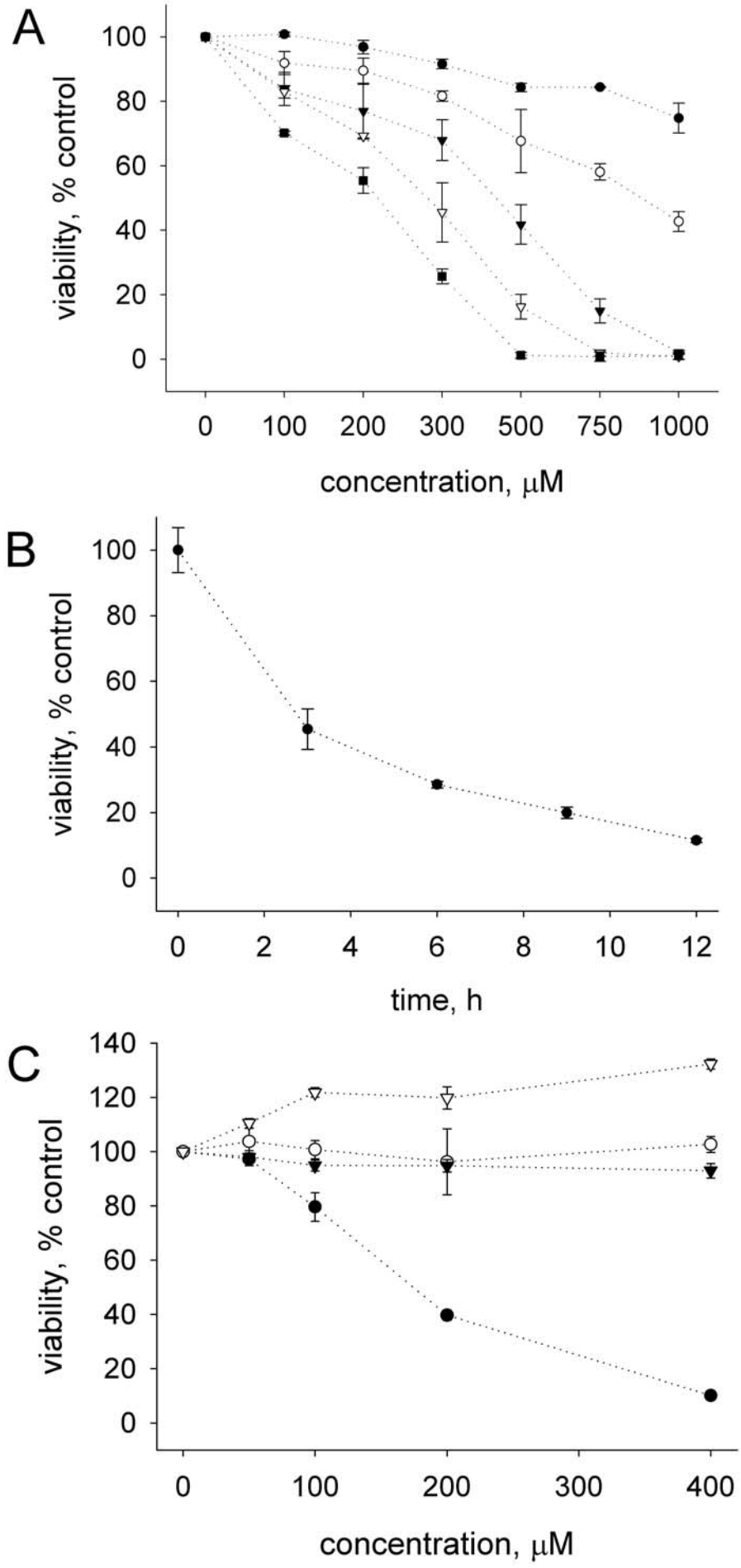

Figure 4

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Figure 5

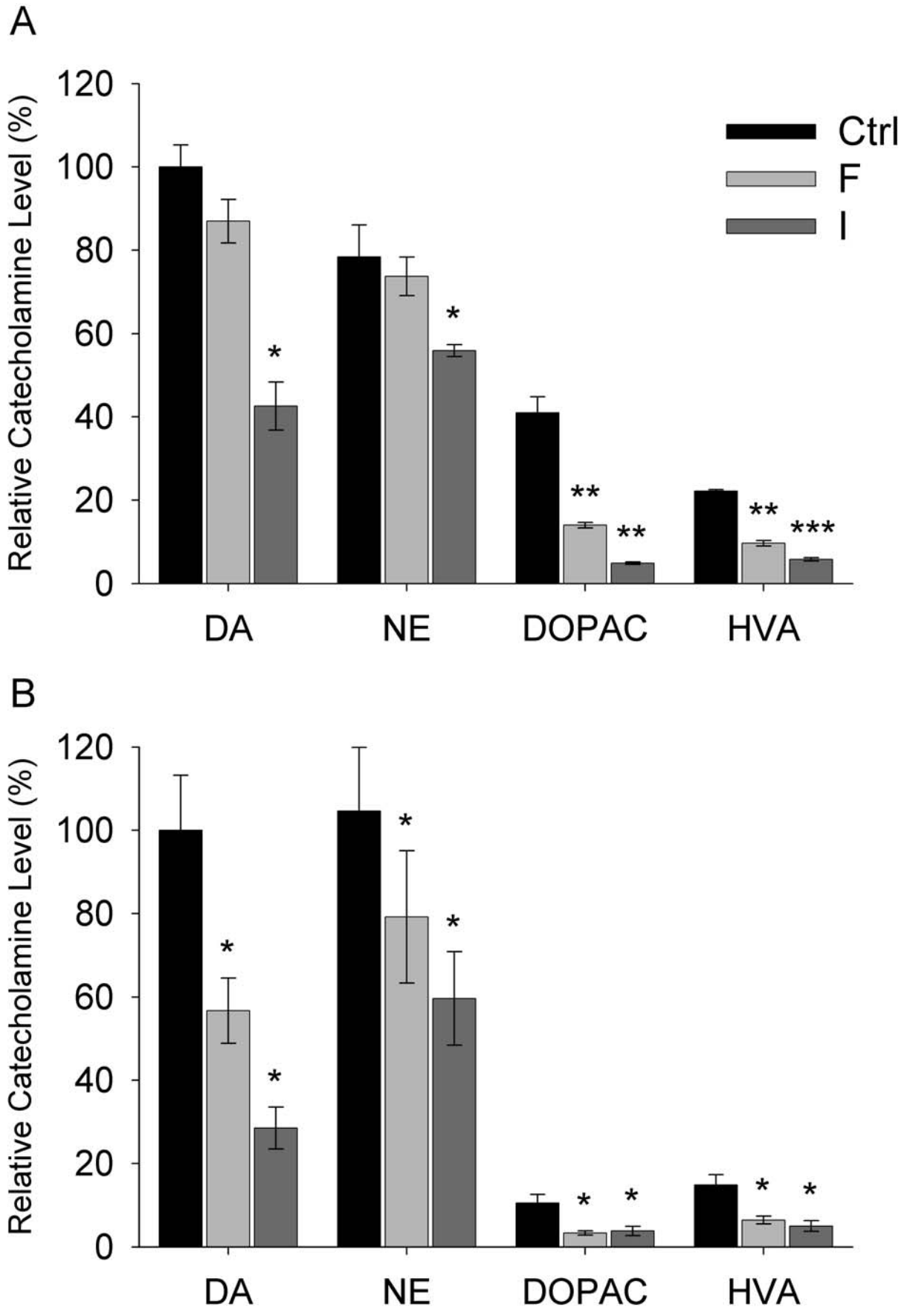

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Figure 6

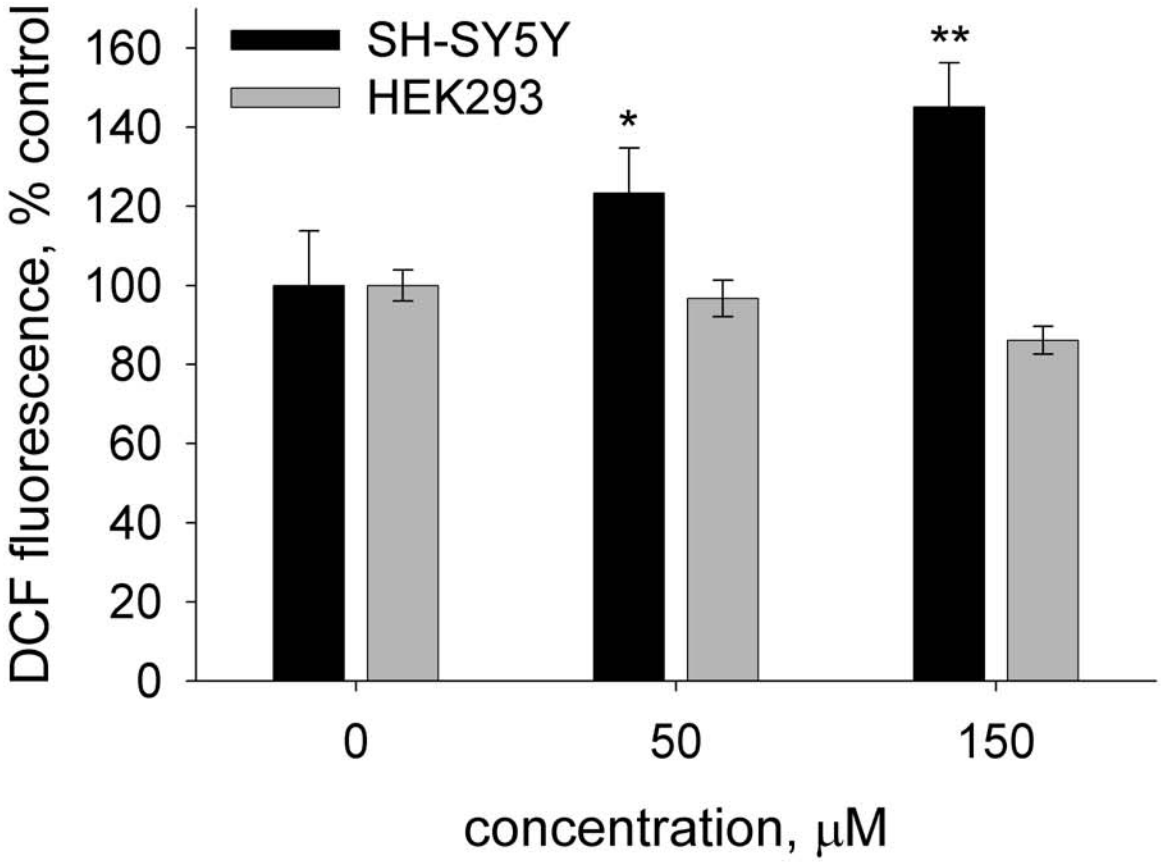

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Figure 7

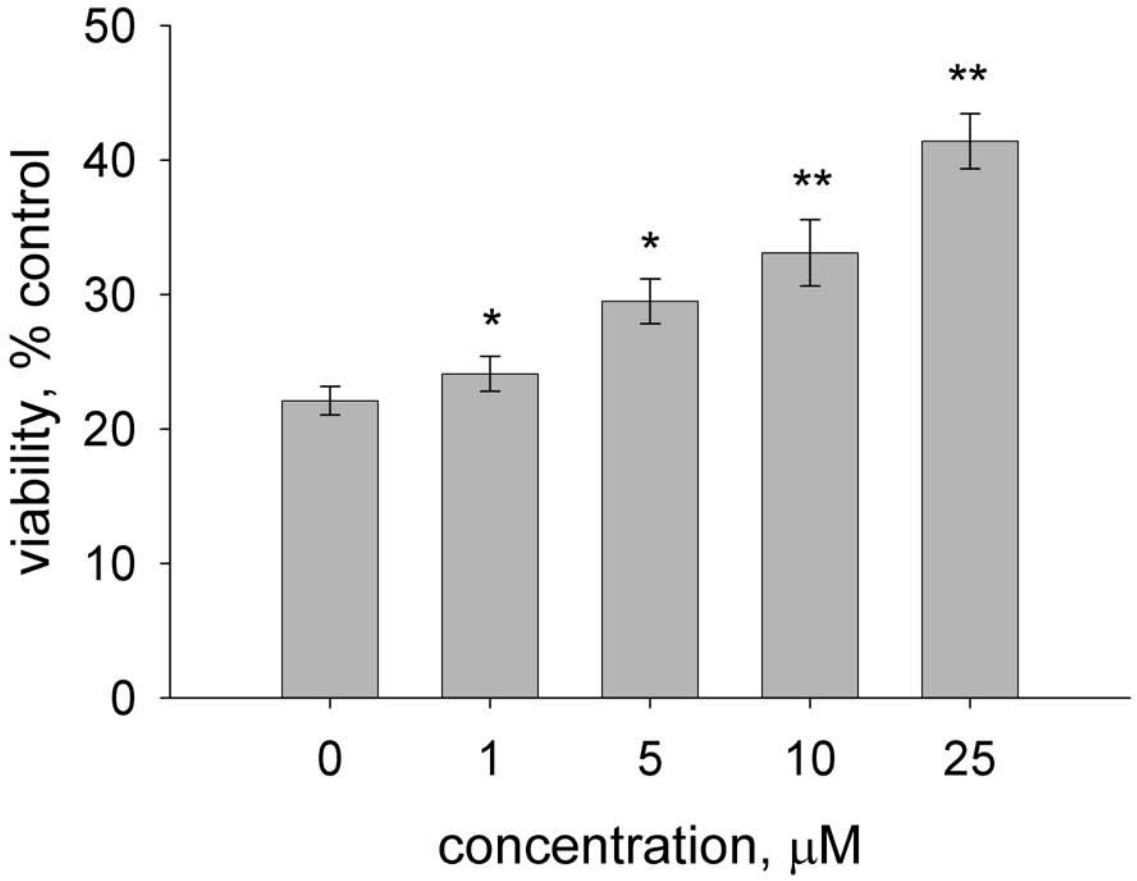

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Figure 8

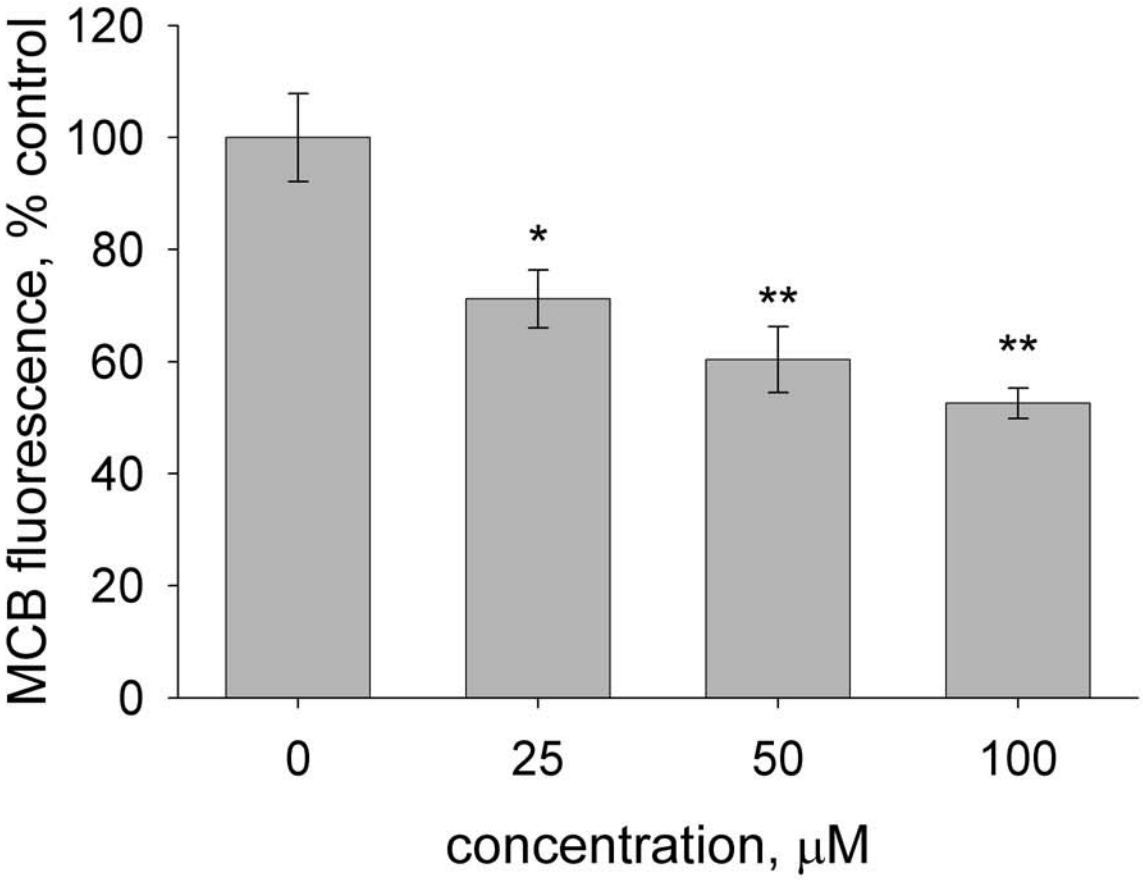

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Figure 9