CHAPTER IV
MATERIALS AND METHODS

4.1 Chemicals and Reagents

Minimum Essential Medium (MEM), Ham's F-12 Nutrient Mixture medium, TryPLE select, and tryphan blue were obtained from Gibco BRL (GRAND Island, NY, USA). Fetal Bovine Serum (FBS) and Penicillin/Streptomycin were purchased from PAA Laboratories (Pasching, Austria). Sodium pyruvate and Non-essential amino acid were achieved from Hyclone (South Logan, UT, USA). Sodium bicarbonate, Dulbecco's Modified Eagle's Medium-low glucose (DMEM (-) phenol red, 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Methamphetamine (METH), Caffeine, 3-methyladenine (3-MA) were acquired from Sigma (St. Louis, MO, USA).

Protease and phosphatase inhibitor cocktail, rabbit monoclonal anti LC3I,LC3II antibody, Rabbit monoclonal anti caspase-3 antibody, Rabbit monoclonal anti p-4Ebp1, Rabbit monoclonal anti p-MTOR antibody, Mouse monoclonal anti β-actin antibody, biotinylated protein ladder, HRP-goat anti-rabbit IgG (H+L) and HRP-goat anti-mouse (H+L) were purchased from Cell Signaling Technology (Danvers, MA, USA). Pre-stained protein ladder was obtained from GeneDirex (Las Vegas city, Nevada, USA). Skim milk powder was acquired from Criterion (Hardy diagnostics, Santa Monica, CA, USA). Bovine serum albumin (BSA) was purchased from VWR (Radnor, PA). The enhanced chemiluminescense (ECL) detection agent was purchased from Bio-rad (California, USA). Polyvinylidene fluoride (PVDF) membrane was obtained from MERCK Millipore (Darmstadt, Germany).

All other chemicals used in the study were analytical grade and obtained essentially either from Sigma (St. Louis, MO, USA), Bio-rad (California, USA) or Merck Millipore (Darmstadt, Germany).

4.2 Instruments

- Synergy ™ HT multi-detection microplate reader (Bio-Tek instrument, Winooski, VT, USA)
- Phase contrast light microscope (Nikon Eclipse TS100)
- CO_2 incubator (Shellab, Cornelius, OR, USA)
- Biohazard Laminar flow cabinet
- Orbital shaker (Boeco, Humburg, Germany)
- Water bath (Nuve, Ankara, Turkey)
- Dry bath (Labnet International, Wood bridge, NJ, USA)
- Vertical and horizontal gel electrophoresis system and power supply (Bio-Rad, Hercules, CA, USA)

4.3 Cell cultures

SH-SY5Y cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). The cells were routinely grown in Eagle's minimum essential medium (MEM)/ Ham's F-12 (1:1), supplemented with 10 % of fetal bovine serum (FBS), 10 mg/ml of penicillin, 10 U/ml of streptomycin, 1 mM sodium pyruvate and 1 mM non-essential amino acid. Cells were maintained in humidified at 37°C in atmosphere of 5 % CO_2 and 95 % air. The cultures were trypsinised with 1X TryPLE.

4.3.1 Cell viability assay

The MTT (3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay is colorimetric assay used to assess cell viability. It is based on measuring the metabolic activity of viable cells (mitochomdrial dehydrogenase) to convert yellow MTT into a purple formazan product which can be quantified at certain wavelength by a spectrophotometer (Figure 4.1). MTT was dissolved in phosphate buffer saline (PBS) filtered, protected from light and stored at 4°C.

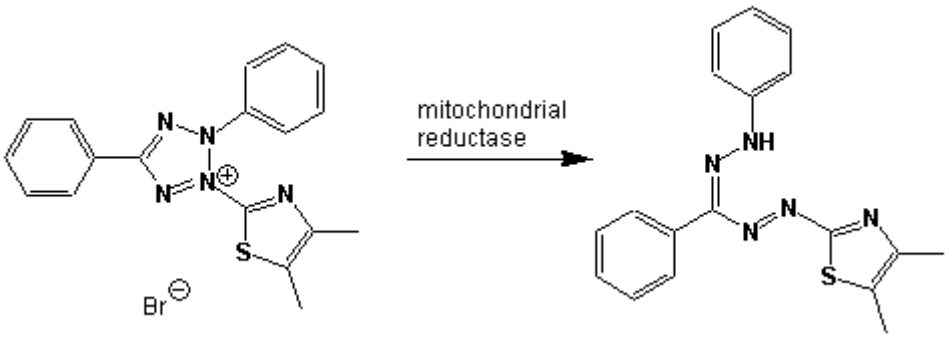

Figure 4.1 MTT reduction in live cells by mitochondrial reductase results in the formation of insoluble formazan, characterized by high absorptivity at 570 nm.

SH-SY5Y cells were seeded on 96 well plates at density of $3x10^4$ cells in each well, and then were grown for 24 hours. Cells were treated with various reagents according to the experiment design. After incubation for 24 hours, 50 ul of MTT reagent (1 mg/ml MTT in PBS) was added in each well. The cultures were incubated for 3 hours. Then MTT was removed and formazan crystal was solubilized with 100 µl DMSO. The reduction of mitochondrial function of living cell was measured at 570/620 nm using a microplate reader (Bio-Tek instrument, Winooski, VT, USA).

4.3.2 Western blot analysis

SH-SY5Y cells were seeded on 6 well plates at density of $9x10^5$ cells in each well. Cells were treated with various reagents according to the experiment design. After incubation, both attached and detached cells were collected. The attached cells were harvested by trypsinization. The sample were wash once with cold PBS before lysed with NP-40 lysis buffer (50 mM Tris-HCl pH7.45, 150 mM NaCl, 0.25% NaDC, 1mM EDTA, and 1% NP-40) containing protease/phosphatase inhibitor cocktail at 4°C for 15 minutes. The lysates were centrifuged at 14,000 rpm for 20 minute at 4°C. The supernatants were collected and the protein concentrations were measured by Lowry method. Bovine serum albumin was used as standard. The proteins were mixed with protein loading buffer and boiled at 95 °C for 5 minutes.

Fourty micrograms of proteins were loaded on to 7.5-13.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 µm PVDF membrane. The transfer efficiency was checked by Ponceau-S red solution. The

membranes were washed with phosphate buffer saline with tween-20 (PBS-T) for 5 minutes, 3 times and blocked with 5% nonfat skim milk in PBS-T for 1 hour. The membranes were incubated with primary rabbit polyclonal antibody to LC3IB, LC3IIB (1:2,000), phosphor-4Ebp1 (1:2,000), phospho-mTOR (1:1,000), caspase3 (1:1,000), and primary mouse monoclonal antibody to β-actin (1:5,000) at 4 $^{\circ}$C overnight. The membranes were washed with PBS-T 3 times followed by horseradish peroxidase conjugated goat anti-rabbit or HRP-conjugated goat anti-mouse secondary antibodies (1: 5,000) for an hour at room temperature. Finally, the protein bands were determined by enhanced chemilunescence using ECL plus^{TM} western blotting detection reagents. The amount of protein, as determined by the intensity of the band, was analyzed by Image J software (NIH, Bethesda, MD, USA). β-actin was used as internal control.

4.3.3 Morphological detection

SH-SY5Y neuroblastoma cells were plate onto 6-well culture plate at density of 9x10^5 cells in each well. Cells were treated with various reagents according to the experiment design. Cell morphology was observed under light microscope (Nikon Eclipse TS100) and was captured the pictures with digital camera (cannon EOS D500)

4.4 Statistical analysis

All values were represented as mean ± S.E.M. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism 5 software (version 5; GraphPad, San Diego, CA, USA). The statistical significance was obtained when the *p* values are 0.05 or less.