Journal Pre-proof

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PII: S1382-6689(20)30091-0

DOI: https://doi.org/10.1016/j.etap.2020.103415

Reference: ENVTOX 103415

To appear in: *Environmental Toxicology and Pharmacology*

Received Date: 6 February 2020

Revised Date: 5 May 2020

Accepted Date: 19 May 2020

Please cite this article as: Ramaiah MJ, Karthikeyan D, Mathavan S, Yamajala RBRD, Ramachandran S, Vasavi PJaya, Chandana NV, Synthesis, *in vitro* and structural aspects of benzothiazole analogs as anti-oxidants and potential neuroprotective agents, *Environmental Toxicology and Pharmacology* (2020), doi: https://doi.org/10.1016/j.etap.2020.103415

Synthesis, *in vitro* and structural aspects of benzothiazole analogs as anti-oxidants and potential neuroprotective agents

M. Janaki Ramaiah[a]*# Divyapriya Karthikeyan[a#] Sivagami Mathavan[b] Rajesh B.R.D. Yamajala[b]* Srimathy Ramachandran[a,] P. Jaya Vasavi[a], Nuthakki Venkata Chandana[a]

[a]Functional genomics and Disease Biology Laboratory, School of Chemical and Biotechnology, SASTRA Deemed University, Thanjavur-613401, Tamil Nadu, India,

[b]Organic Synthesis and catalysis Laboratory, School of Chemical and Biotechnology, SASTRA Deemed University, Thanjavur-613401, Tamil Nadu, India

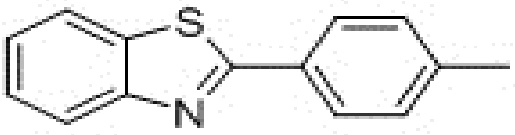
* Corresponding authors (**Main**): M. Janaki Ramaiah, E-mail: janakiramaiah@scbt.sastra.edu,

Equal first authorship

phone No.: +91-8870948256

Graphical abstract

6b



6c

Catalase activity

Neuroprotection

Highlights

- Benzothiazole analogs modulate catalase activity and cause neuroprotection.
- Benzothiazole analogs bind to catalase enzyme and have shown drug-likeliness.
- Neuroprotection in H_2O_2 exposed U-87 MG cells occurs *Via* inhibition of ROS levels.

Abstract

Catalase, an important antioxidant enzyme, is known to have a neuroprotective role against neurodegenerative disorder. Earlier study has focussed on benzothiazole-triazole hybrid molecules that are larger in size and molecular weight and inhibit the amyloid β (Aβ)-catalase interaction thus aid in neuroprotection. Here we have synthesized the novel benzothiazole molecules with low molecular weight using One-pot methodology and assayed the neuroprotective effects of the synthesized compounds in the U87 MG cell line under H_2O_2 induced stressed condition and compared with other cell lines such as breast cancer (MCF-7) and macrophage (RAW-264.7) using cell viability assay. These analogs were found to enhance the neuronal cell viability and protect neuronal cells from the ROS mediated neuronal damage induced by H_2O_2. Furthermore, compounds **6a, 6b, 6c, 6d, and 7a** modulate catalase and enhanced the catalase activity up to 90% during the H_2O_2 exposure in the U87MG cell line. These analogs **(6a, 6b, 6c and 6d)** have exhibited strong binding energies of -7.39, -7.52, -6.5 and -7.1 as observed by molecular modeling studies using AutoDockTool-1.5.6. Lig Plot + program using potent analogs **6b** and **6c** and catalase enzyme indicated the presence of hydrophobic interactions in the catalytic site of catalase enzyme. Furthermore, a simulation study was conducted between ligand and catalase protein by DESMOND software that further strengthens these ligand and enzyme interactions. *In silico* ADMET study was conducted by

the Swiss ADME program revealed the drug-likeliness of these analogs. The present study has identified benzothiazole analogs such as **6b, 6c and 6d** have potential catalase modulating activity and is comparable with that of known drug Valproic acid, thus help in neuroprotection. This study can be further taken up for the *In vivo* animal model study for the possible therapy.

KEYWORDS

Alzheimer's disease; neuroprotection; catalase; benzothiazole; hydrogen peroxide

1. Introduction

Alzheimer's disease (AD) as well as other neuronal disorders such as Parkinson's, needs urgent medical attention. The treatment options in AD include small molecules that can block amyloid-beta (Aβ) interactions, reduction of the caspase protein expression, inhibition of the reactive oxygen species (ROS) dependent enzyme activity (Longo & Massa, 2004). ROS enhances the β-Amyloid protein level which in turn causes direct injury to the central nervous system (CNS) and induce neuronal cell death (Guo, Sun, Chen, & Zhang, 2013). In order to protect from the ill effects of ROS, cells can employ various antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and non-enzymatic antioxidant factors such as All-trans retinol 2, ascorbic acid, α-tocopherol, α-carotene, and glutathione, etc. (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012).

Antioxidants are compounds inhibit the formation of peroxides or quench the peroxide or scavenge the species that can generate peroxides. The production of antioxidant enzymes takes place in glial cells and helps in the protection of the neuronal cells from oxidative stress (Wilson, 1997; Peuchen et al., 1997). The high amount of ROS was generated when neuronal cells were exposed to radiation or oxidative stress in SH-SY5Y neuronal cells. But the amount of ROS was drastically reduced when SH-SY5Y cells co-cultured with U-87 MG glial cells.

This indicates that co-culturing of SH-SY5Y with U-87 MG glial cells can enhance the mitochondrial membrane potential by upregulating the production of antioxidant enzymes superoxide dismutase and antioxidant glutathione in SH-SY5Y cells (Saeed et al., 2015).

Catalase (CAT) is a common enzyme found in nearly all living organisms that are exposed to oxygen, comprising of four protein subunits and four iron ions and has one of the highest turnover numbers of all the enzymes (Nisticò et al., 1992). The catalase enzyme binds with high affinity with the amyloid β plaques (Aβ plaques) and thereby making the catalase enzyme inactive (Chilumuri, Odell, & Milton, 2013). H_2O_2-induced oxidative stress and cellular injury is a well-established and useful model for studying The anti-oxidative, and cytoprotective nature of compounds against neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Studies have indicated that Xanthohumol (Xn) analogs and Dithiolethiones were found to have neuroprotection nature against H_2O_2 induced reactive oxygens species and apoptosis in PC12 cells (a neuronlike rat pheochromocytoma cell line) and N2a cells (mouse neuroblastoma N2a cells) respectively (**Bai et al., 2019; Song et al., 2020**). Thus, we have taken H_2O as a neurotoxin in our experiments to mimic the condition of AD-like cell death. Studies have indicated that inactivated catalase enzyme cannot breakdown hydrogen peroxide (H_2O_2) which is generated in response to Aβ (Zhang et al., 1996).

Non-conventional compounds are exhibiting promising beneficial effects on Alzheimer´s disease (AD). These include methylene blue, natural products, organic phosphorous compounds, and Chinese medicine (de Castro et al., 2019). The symptomatic AD can be treated up to one year with acetylcholinesterase (AChE) inhibitors such as tacrine, donepezil, rivastigmine, and galanthamine. Thus, repositioning of available drugs can be considered as an alternative strategy (de Castro et al., 2018). In addition metal-based drugs and several chelating agents can also help in decreasing the symptomatic effects of several neurological disorders (Sales et al., 2019). Interestingly, benzothiazole compounds such as phenyl benzothiazoles and

Manganese-benzothiazole can also be used as spectroscopic and MRI probes respectively (Pereira, Goncalves, Mancini, Kuca, and Ramalho, 2019; Rosa, Silva Giacoppo, de Cunho, Fernandes, and Horvath, 2019).

Natural compounds are found to possesses anti-oxidant compounds and regulate anti-oxidant genes by various mechanisms such as direct radical scavenging and activation of antioxidant enzymes (Fraunberger, Scola, Laliberté, Duong, & Andreazza, 2016). Benzothiazole aniline (BTA) has been reported to inhibit beta-amyloid protein aggregations. A tetra (ethylene glycol) derivative of benzothiazole induces the spinogenesis *via* a Ras-dependent pathway (Megill et al., 2013). Compounds that are heterocyclic (aromatic) in nature have a good therapeutic role in the field of drug discovery (Goyal, Kaur, & Goyal, 2018; Jouha et al. 2017; Sucheta, Tahlan, & Verma, 2017; Hiremathad, & Piemontese, 2017). Benzothiazole (BT) and its derivatives are found to have heterocyclic structures (Hutchinson, Jennings, Vishnuvajjala, Westwell, & Stevens, 2002; Hutchinson et al., 2002) 6-trifluoromethoxy-2-benzothiazolamine, was able to block excitatory amino acid-mediated neurotransmission (Evindar, & Batey, 2006; Sharma, Sinhmar, Sharma, Rajak, & Pathak, 2013; Malik, Manvi, Nanjwade, Singh, & Purohit, 2010). Compounds such as benzothiazole aniline tetra (ethylene glycol) (BTA-EG_4) and BTA-EG_6 have been known to specifically disrupt the interaction of catalase with Aβ (1-42) by binding to multiple regions and eventually inhibiting the binding of catalase and also has the ability to penetrate the blood-brain barrier. These compounds were also found to work better in the AD animal model (Megill et al., 2013). Tacrine-allyl/propargylcysteine–benzothiazole trihybrids cause the anti-ROS activity, acetylcholinesterase enzyme activity inhibition, and 5-fold decrease in amyloid-beta (Aβ) peptide production (Hiremathad et al., 2016). The methods for selecting docking studies are chemi-informatics techniques, AutoDock and QM calculations (De Lima. Francisco, da Cunha, Radic, Taylor, and Franco, et al.2017). Also, AutoDock version 3.0.5 was used for the prediction of binding conformations of flexible ligands.

Earlier studies by Kumbhare et al., 2012 have studied the anti-cancer activity of benzothiazoles on breast and other cancer cell lines. Here we have synthesized various analogs of benzimidazoles, benzothiazoles, and benzoxazole compounds. All of the benzimidazole, benzothiazole, and benzoxazole derivatives (5a-5c, 6a-6d, 7a) were produced in pure form without any column chromatography, characterized by 1H NMR spectra, and the known compounds were confirmed by comparing their spectral data and melting points with those reported in the literature (Xue, & Long, 2014; Yang et al. 2014; Ye et al. 2017; Gopalaiah, & Chandrudu, 2015). The 1H NMR data were provided in the **supplementary figures S1 and Supplementary file S2** Here we have studied the possible role of these analogs on neuronal protection by reducing ROS level and enhancing the catalase activity.

2. Materials and Methods

2.1. Chemistry

Heterocyclic compounds are part of many natural products and are vital in the pharmaceutical industry, due to the presence of the structure of these heterocycles in different drugs consumed worldwide. Among them, benzimidazoles, benzothiazoles, and benzoxazoles are of great interest, since they are present as the core motif in many of the biologically active molecules. A wide variety of methods have been developed for the synthesis of benzimidazoles, benzothiazoles, and benzoxazoles. Usually, these compounds are prepared through the condensation of 1, 2-diamines /2-aminothiols / 2-aminophenols with aldehydes or acyl chlorides. These are also prepared by the condensation of 1, 2-diamines/2-aminothiols/2-aminophenols with α-phenyl glyoxylic acid.

As part of our continuing efforts to develop green synthetic methodologies, we report herein the synthesis of these heterocycles by an efficient protocol under eco-friendly conditions. This

method involves the use of graphene oxide as a catalyst and water as a green solvent under room temperature and heating conditions **(Figure 1)**.

The condensation reaction of *o*-phenylenediamine / 2-aminothiols (1 mmol) with α-phenyl glyoxylic acid (1 mmol) was carried out in the presence of GO catalyst in the water at room temperature. The catalyst loading of 5, 10, 20 mg was tested. Reactions with 10 mg and 20 mg catalyst gave similar yields in 1h. In the absence of catalysts, the reaction did not proceed at all even after a long reaction time. In the case of benzothiazoles, the condensation of 2-aminophenols with α-phenyl glyoxylic acid resulted in the product formation only in heating condition and the reaction took 10h to get completion. All of the benzimidazole, benzothiazole, and benzoxazole derivatives were produced in pure form without any column chromatography, characterized by ^{1}H NMR spectra, and the known compounds were confirmed by comparing their spectral data and melting points with those reported in the literature. All reagents and solvents were of analysis or synthesis grade and were used without further purification unless otherwise indicated. ^{1}H NMR was recorded on a Bruker AVANCE III 400 MHz NMR at 400 or on a Varian 300 instrument at 300 in CDCl3 or DMSO-d6. Chemical shift is reported in ppm with the solvent residual peak as reference; CDCl3 (dH 7.26), DMSO-d6 (dH 2.50) and CD3OD (dH 4.87). The reactions were monitored by thin-layer chromatography (TLC), on silica plated (Silica gel 60 F254, E. Merck) aluminum sheets, detecting spots by UV (254 and 365 nm). All the analogs used in the study were represented in **Figure 2** with the corresponding codes.

2.2. Cell lines

Human malignant glioma (U-87 MG), human breast cancer cell line (MCF-7), human neuronal cell line (SH-SY5Y) and mouse macrophage cell line (RAW 264.7) were obtained from NCCS, INDIA. All the cell lines were cultured in DMEM supplemented with 10% fetal bovine serum

(FBS), 4 mM glutamine, 100 units/ml penicillin, and 100µg /ml streptomycin. All the cell lines were maintained at 37 °C in the CO_2 incubator.

2.3. Effects of compounds on neuronal cell viability

In order to choose the concentration of neurotoxin that is needed to produce adequate neurotoxicity, various concentrations of H_2O_2 (425 µM - 9450 µM) were tested on U-87 MG cell line. 1 x 10^4 cells / well of 96 well plate was incubated for 12 h with the H_2O_2 (i.e. neurotoxin) and cell viability was determined by using MTT dye. Here the compounds were incubated 24h before H_2O_2 addition. The 50 µL MTT solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well, the plates were incubated at 37°C for 4 h, the culture medium containing MTT was removed and 100 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The absorbance was measured at 570 nm using a Multiplate reader. Results were expressed as a percentage of the untreated cells.

$$\text{Cell Viability \%} = (OD_{570}\text{ nm treated} / OD_{570}\text{ nm untreated}) \times 100\%$$

2.4. Combinatorial effect of benzothiazole analogs and H_2O_2 toxicity

Stock solutions of benzothiazole analogs were prepared in DMSO prior to dilution to the required concentration in the culture medium. Synthesized compounds stock solutions were dissolved in DMSO and varying concentrations ranging from 1µM to 64 µM were employed. To produce oxidative stress, H_2O_2 of varying concentrations was freshly prepared from 30% (w/v) H_2O_2 stock solution before each experiment. To study the neuroprotective effect of benzothiazole analogs against H_2O_2 toxicity, 1 x 10^4 cells were seeded in a 96-well flat-bottom plate and allowed to adhere for 24 h at 37 °C in the CO_2 incubator. After 24 h of incubation, the culture medium was replaced with a fresh medium. For testing the effects of these compounds, the cells were pre-treated with test compounds for 12 h before treatment with H_2O_2. For testing the effects of H_2O_2 toxicity, cells were incubated for 4 h before the determination of cell viability. Then MTT assay was performed.

2.5. Intracellular ROS detection using DCFH-DA assay

The degree of ROS generation in cells was measured using fluorescence assay with 2', 7'-dichlorodihydrofluorescein diacetates (H_2DCFDA, Sigma). DCFH-DA is the most widely used probe for detecting intracellular H_2O_2 and oxidative stress. After diffusion into the cell, DCFDA / H2DCFDA / DCFH-DA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). Briefly after treatment, the cells were scraped and washed in PBS and the pellet re-suspended in 200 µl of PBS loaded with 10 µM H_2DCFH-DA at 37°C for 30 min and then the cells were washed twice with PBS and centrifuged at 1000 rpm for 5 min. Finally, the pellet was re-suspended in 200 µl PBS and the fluorescence intensity of DCF was measured with Fluorescence Spectroscopy. The measurement of ROS is calculated Excitation (Ex) / Emission (Em) at 470nm / 545nm.

2.6. Catalase activity assay

Catalase activity was measured using the manufacturer's instructions (Catalase, ab83464) (Abcam, USA). Here, 2×10^6 was treated with the potential compounds (**6a-6d, 7a**) for 24 h. Then lysates were isolated using cold lysis buffer, and the samples were subjected to catalase activity assay. Here the compound treated cell lysates were allowed to interact with 12 µL of H_2O_2 (1 mM solution) and followed by the addition of stop solution (25ºC for 5 minutes), to stop the reaction between catalase present in the lysate and H_2O_2 (**represented as -H**). Similarly, the same amount of lysate was subjected to inhibition with stop solution (25ºC for 5 minutes) followed by the addition of H_2O_2 and is **represented as +H**. A master mix of developer consists of 46 µL of assay buffer, 2 µL of OxiRed Probe, and 2 µL of HRP Solution was prepared and added into each well. The plates were incubated at 25ºC for 10 min. The catalase present in the sample reacts with hydrogen peroxide (H_2O_2) to produce water and oxygen. The unconverted H_2O_2 reacts with the probe to produce a product that can be measured

calorimetrically at OD_{570} nm using a microplate reader. Therefore, the catalase activity present in the sample is inversely proportional to the signal obtained.

2.7. *In Silico* docking studies

Docking studies will be employed at different stages of drug discovery to predict a ligand-receptor interaction and also to rank the compounds based on their binding energies. The three-dimensional structure of human erythrocyte catalase (ID: 1QQW), were obtained from the protein data bank. All the non-protein molecules were removed. AutoDock 4.0 (Morris et al., 2009) software was used for docking study. AutoDock tools 1.5.6 (Huey, Morris, Olson, & Goodsell, 2007) were used to prepare, run, and analyze the docking simulations. Kollman united atom charges and polar hydrogen was added to the protein. The grid box size was set at 65X65X65 Å (x, y, and z) centered on the protein with the spacing between grid points at 0.375 Å. Docking results were analyzed and visualized by AutoDock tools and PyMOL (DeLano, 2002).

3. Results and Discussion

3.1. Effect of hydrogen peroxide (H_2O_2) on the viability of neuroblastoma and glioblastoma cancer cell lines (SH-SY5Y and U-87 MG)

Hydrogen peroxide (H_2O_2) is considered as one of the major neurotoxic compounds that produce high amounts of free oxide radicals in the cells, especially in the neuronal cells. It is associated with the genesis of neurodegenerative diseases such as Alzheimer's, Multiple sclerosis and Parkinson's disease (Bayani, 2009). To understand the neurotoxic effect of H_2O_2 on cells that are of glial origin, we have taken U-87 MG cells. Various concentrations (425-9500 μM) of H_2O_2 were added to the U-87 MG cells for a period of 12h. The concentration of 425 μM resulted in a 20% reduction in cell viability, whereas 950 μM resulted in 40% reduction in cell viability. Thus, we considered 950 μM as an optimal concentration for inducing oxidative stress (hypoxia condition) for further experiments on neuroprotective studies of

Benzothiazole analogs. Surprisingly, above 4750 μM concentration, the cell started a protective mechanism against H_2O_2 to a certain extent which clearly indicates the cell adaption to H_2O_2 mediated toxic or hypoxic effects (**Figure 3a**).

A similar experiment was conducted in neuroblastoma (SH-SY5Y) cells with H_2O_2 ranging from (0-256 μM). Here we observed 2-8 μM 30% reduction in cell viability whereas 40% cell reduction was observed at concentrations ranging from 16-256 μM (**Figure 3b**). It is evident from the studies of (**Fan et al., 2012**) that glial cells i.e. U-87 MG cells protect neuronal cells from indirect effects of radiation and oxidative stress in the brain. Maybe due to these reasons, we need to use higher concentration (950 μM) to understand H_2O_2 mediated cell death in U-87 MG (**Saeed et al., 2015**).

3.2. Neuroprotective effect of benzothiazole analogs on H_2O_2 induced neurotoxicity in U-87 MG

The neuroprotective effect of benzothiazole analogs (5b, 5c, 6a-6d, and 7a) was studied on U-87 MG cell lines exposed to oxidative stress (H_2O_2). Here 2 μM concentration of compound produced effective cytotoxicity up to 30% whereas 4 μM concentration of compound had a drastic change in the cell viability. The cells were incubated with benzothiazole analogs for 12 h and were followed by addition of H_2O_2 (950 μM as deduced from the above studies) for 4 h. Surprisingly the combination of both compound and H_2O_2 did not cause any considerable reduction in the cell death probably due to compounds may directly chelate H_2O_2 and nullify its effects or compounds might modulate the catalase activity **(Figure 4a)**. The standard molecule such as Valproic acid (VA) also has shown neuroprotection when cells were treated with H_2O_2. Similar experiments were conducted using Breast cancer cell line (MCF-7) **(Figure 4b)** and normal mouse macrophage cell line (RAW 264.7) **(Figure 4c)** and a similar trend was observed.

3.3. Effect of benzothiazole analogs on H_2O_2 induced ROS in glioblastoma cells

ROS is modulated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Min et al., 2015). H_2O_2 generates highly reactive oxygen species (ROS) and cause apoptosis of neuronal cells (Lee et al., 2008; Wang et al., 2008), leading to various neurodegenerative diseases such as Alzheimer's disease **(**Zhu, Su, Wang, Smith, & Perry, 2007), Parkinson's disease (Thomas, 2009), Huntington's disease (Chen, 2011) and amyotrophic lateral sclerosis (Barber, Mead, & Shaw, 2006). So, to understand the neuroprotective effect of benzothiazoles, we have treated U-87 MG cells with compound alone (2 μM) as well as the combination of both compound and H_2O_2 (950 μM as deduced from the cell viability experiment). The amount of ROS generated was measured. Compounds induced ROS generation in U-87 MG cells, but when compounds encountered H_2O_2 they have decreased the amounts of ROS in U-87 MG cells, this shows that compounds binding to H_2O_2 and inhibiting the H_2O_2 mediated ROS generation **(Figure 5)**.

3.4. Effect of benzothiazole analogs on catalase activity

Catalase is the ubiquitous enzyme that prevents cell oxidative damage (Alfonso-Prieto, Biarnés, Vidossich, & Rovira, 2009). Catalase degrades the H_2O_2 to give H_2O and O_2 with high efficiency (George, 1947). Catalase binds to Aβ with high affinity and catalase-Aβ interaction in Alzheimer's disease deactivates catalase enzyme function that leads to the high amounts of H_2O_2 and high cytotoxicity in neuronal cells. A small molecule inhibitor of catalase amyloid interaction protects the neuronal cells from the evil effects of amyloid induced cytotoxicity (Habib, Lee, & Yang, 2010). Thus, we were interested in understanding the catalase activity in cells treated with compounds and H_2O_2. Lysates were allowed to interact with H_2O_2 (12 mM) and measured the changes in optical density (OD) values at 570 nm. The less OD indicates more catalytic activity. Compounds **6b, 6c, and 6d** have exhibited **88%, 90%,** and **85%** of catalase activity respectively. The positive control has shown 94% of catalase activity (**Figure**

6). The catalytic activity of these compounds was found to be almost equal to the standard neuroprotective compounds such as VA. This indicates that these compounds have promising neuroprotective effects.

3.5. *In silico* docking studies with Human Erythrocyte Catalase (HEC)

We observed an enhanced catalase activity in cells treated with a combination of H_2O_2 and benzothiazole analogs. This may be due to 2 possibilities. Either compound might chelate H_2O_2 action and also enhance the catalase activity during the H_2O_2 treatment in U-87 MG cells. Thus to understand the interaction between benzothiazole analogs and catalase was conducted using template 3D structure PDB ID: 1QQW retrieved from the RCSB PDB database. The incomplete, modified, missing, and cris-cross residues were studied by using AutoDockTools-1.5.6. 3D docking interactions for benzothiazole analogs and VA inside the active site cavity of the Human Erythrocyte Catalase (HEC) was performed. The compounds such as **5b, 5c, 6a, 6d, 7a,** have exhibited efficient binding ability with catalase as shown in **Figure 7.** From the molecular modeling studies, it was found compounds have exhibited higher binding energy when compared with the standard neuroprotective agent [HDAC inhibitor (i.e.) Valproic acid (VA)]. Among all, **5b** (△G = -7.88), **5c** (△G = -7.68), **6a** (△G = -7.69), **6d** (△G=-7.1), **7a** (△=-7.65) whereas VA has exhibited binding energy of -4.82 **(Table 1)**. The above data indicates these potential compounds need to be further studied in Alzheimer's model [Tg2576 mouse model – Aβ model or APPK670/671L].

Furthermore, 2-D representation of the ligands **6b, 6c** in the active site of catalase generated using LigPlot+. Ligplot + program designs for representing ligand-protein, ligand nucleic acid interactions and generates hydrophobic contacts as represented by red arcs with radiating lines. We could observe that the major interaction force for the ligands in hydrophobic. The chlorine and methyl groups of **6b** and **6c** also contribute for the hydrophobic interactions with active

site residues, Val 73 and Val74 (**Figure 8**). Also, simulation study was conducted for the protein ligand complex using DESMOND simulation package for duration of 1 ns. The RMSD profile of the ligands does not deviate much and indicate a stable interaction of ligands with the catalase docking during simulations **(Supplementary figure 3 or Figure S3**). Furthermore, the druglikeliness of these compounds was tested using in silico ADMET analysis using SwissADME prediction program. All these compounds have exhibited moderate solubility and followed Lipinski rule (**Table-2**).

4. Conclusions

Drug development has been a principal driving force in the rapid maturation of the field of medicinal chemistry during the past several decades (Khan, Ibrar, Abbas, & Saeed, 2014). Here, we have synthesized a broad range of novel and efficient Benzothiazole analogs to inhibit the Catalase-amyloid β interaction and thereby activating the efficiency of the antioxidant enzyme, catalase. This article is also focused on reducing the loss or death of neuronal cells. The structure-activity relationship of the reported parent compound (i.e.) azole, revealed that the choice of a suitable substitution pattern on the basic skeleton plays a key role in regulating the biological potential of the synthesized compounds.

Antioxidants are therapeutic drugs against neuronal loss, as they have the capability to combat by inhibiting and chelating free radicals. Interestingly, antioxidants have a wide scope to sequester metal ions involved in neuronal plaque formation and prevent oxidative stress (ROS). In this context, antioxidant therapy is an urgent requirement for scavenging free radicals and for protection against the neuronal degeneration in post-oxidative stress (Poulsen, Prieme, & Loft, 1998). O_2^- may cause damage to neuronal cells ultimately resulting in loss of cell structure and function of the brain and this is one of the main characteristics of neurological disorder. In AD, the neuronal cells are filled with hyperphosphorylated Tau tangles and insoluble Aβ

proteins such as Aβ (1-42) resulting in the loss of neuronal function. Apart from Aβ (1-42), the other most important problems include loss of function of anti-oxidant enzymes such as catalase and SOD. Thus, modulating these enzymes will help in neuroprotection.

Benzothiazoles were found to be anti-cancerous, anti-diabetic, anti-leishmanial, etc. Chilumuri et.al has found that the compound BTA-EG4 blocks the binding interaction of catalase to Aβ, thereby helping in the neuroprotection. The main drawback of the compounds was found to have higher molecular weight, which might hinder the passage of compounds through the blood-brain barrier (BBB). Thus, we tried to synthesis low molecular weight compounds namely benzothiazole analogs with various substitutions such as methyl, methoxy, phenyl, chloro, and bromo in the benzothiazole skeleton (i.e. parent compound).

We initially initiated our work by creating a ROS condition using various concentrations of H_2O_2, using SH-SY5Y and U-87 MG cell lines. To our surprise, the SH-SY5Y cells responded at a concentration of 256μM whereas U-87 MG cells responded at a higher concentration of 1900 μM, this may be due to the glial cells have the potential to tolerate a high amount of stress such as H_2O_2 and the function of glial cells is to protect the neuronal cells. Keeping this as an optimal concentration, we tested the neuroprotective effect of the benzothiazole analogs and found that the compounds **6a, 6b, and 6c** are resistant to H_2O_2 mediated hypoxia and thus helps in cell viability.

Some of the anti-inflammatory and antioxidant drugs such as coumarin derivative drugs possess the capability to attenuate elevated levels of ROS in cells (Kontogiorgis, Xu, Hadjipavlou-Litina, & Luo, 2007). Compounds that nullify the H_2O_2 mediated effects can have the inhibitor activity on ROS. So, we tested the effect of the compound on ROS in U-87 MG cells, in which we observed **6a, 6b, and 6c** has caused the reduction in ROS level as indicated by DCF-DA fluorometric assay.

Further, the activity of catalase was measured using Catalase Activity Colorimetric Assay Kit, as catalase is the most important key enzyme that helps in cell viability during hypoxia, stress at peroxisomes and mitochondria junction, in which the benzothiazole compounds **6a, 6b, and 6c** showed high catalytic activity when compared with other analogs. Thus, these analogs have found to have potent neuroprotective activity. To strengthen our results, we performed a molecular docking study to predict the binding-conformation of small-molecule ligands to the appropriate target protein, as these computational methodologies have become a crucial component of many drug discovery events (Kitchen, Decornez, Furr, & Bajorath, 2004). The molecular docking was performed with the help of AutoDockTools-1.5.6, in which the compounds **6a, 6b, and 6c** showed high binding energy. The $\triangle G$ values, simulations obtained from desmond software, lig plot+ analysis revealed that the correlation between binding energies as well as interaction of 6b, 6c compounds with catalase. Root mean square deviation (RMSD) profile revealed the ligands does not deviate much and binding is specific between 6b, 6c with catalase. These theoretical findings as well as experimental catalase activity indicated that benzothiazole based compounds especially 6b and 6c are effective in modulating catalase. Therefore, the compounds need to be tested in animal models for further confirmation as a potent drug for the treatment of Alzheimer's disease by protecting the neuronal cells from oxidative damage.

Statistical analysis

Statistical analysis was performed using the graph-pad software to evaluate the significant difference between the control and treated samples. All variables were tested in three independent experiments. The results were reported as mean ± SD. * represents p-value < 0.05, ** represents p-value < 0.01 and *** represents p-value < 0.001

Acknowledgments

Thanks to DST –SERB (EMR/2017/001201) for financial support and thanks to SASTRA management for the infrastructure and constant encouragement.

Disclosure of statement

No potential conflict of interest was reported by the authors

ORCID ID: https://orcid.org/0000-0003-1814-2819 Janaki Ramaiah

http://orcid.org/0000-0002-2446-9503 Divyapriya Karthikeyan

https://orcid.org/0000-0002-2914-3617 Rajesh YBRD

NO ORCID for Srimathy Ramachandran,

NO ORCID P. Jaya vasavi

NO ORCID for Nuthakki Chandana

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Figure 1. Schematic representation of synthesis procedure of various Benzothiazole analogs used in the study.

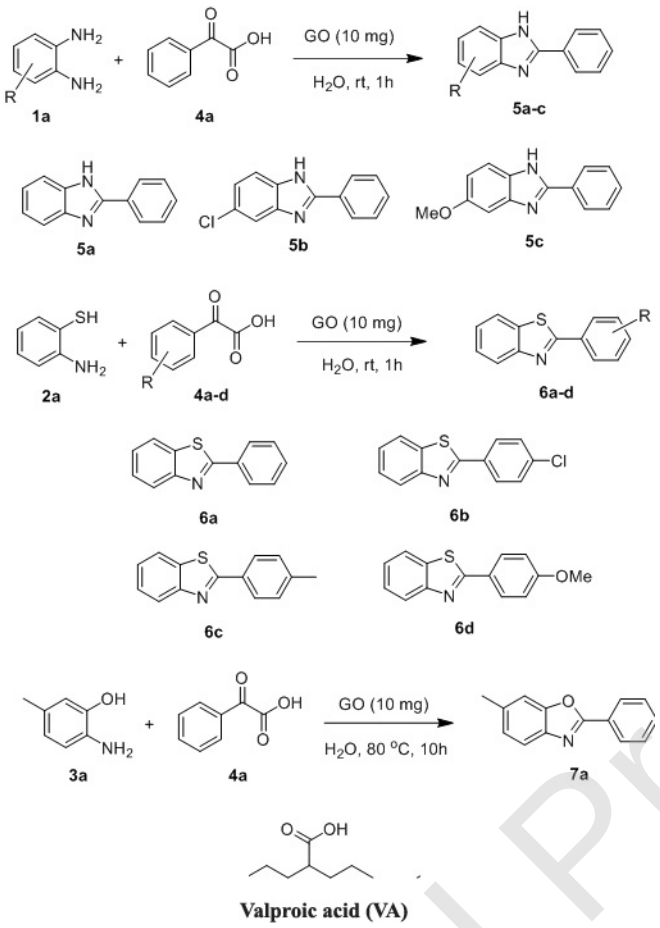


Figure 2. The chemical structures of various Benzothiazole analogs used.

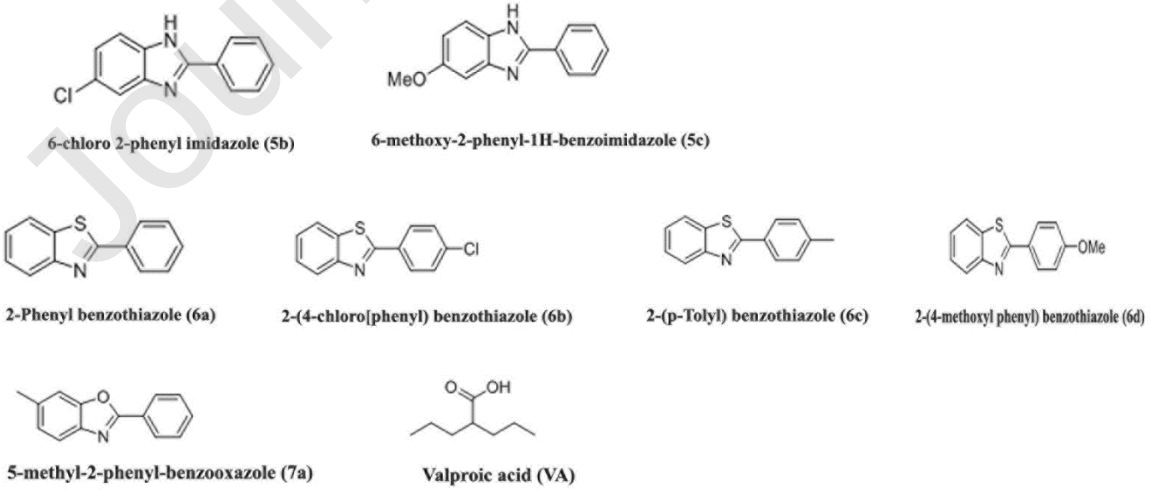


Figure 3a. Effect of H_2O_2 on the viability of U87MG cells: Various concentrations of H_2O_2 (425 – 9500µM) was added to human glioblastoma cancer cells (U87MG) and cells were incubated for 12h. The concentration of 950 µM H_2O_2 resulted in 40% reduction in the cell viability. This was followed by MTT based cell viability assay. The OD570 nm value was represented as percentage of cell viability. Three independent experiments were conducted and GraphPad software was used to test statistical significance. ** represents p-value < 0.01 and *** represents p-value < 0.001

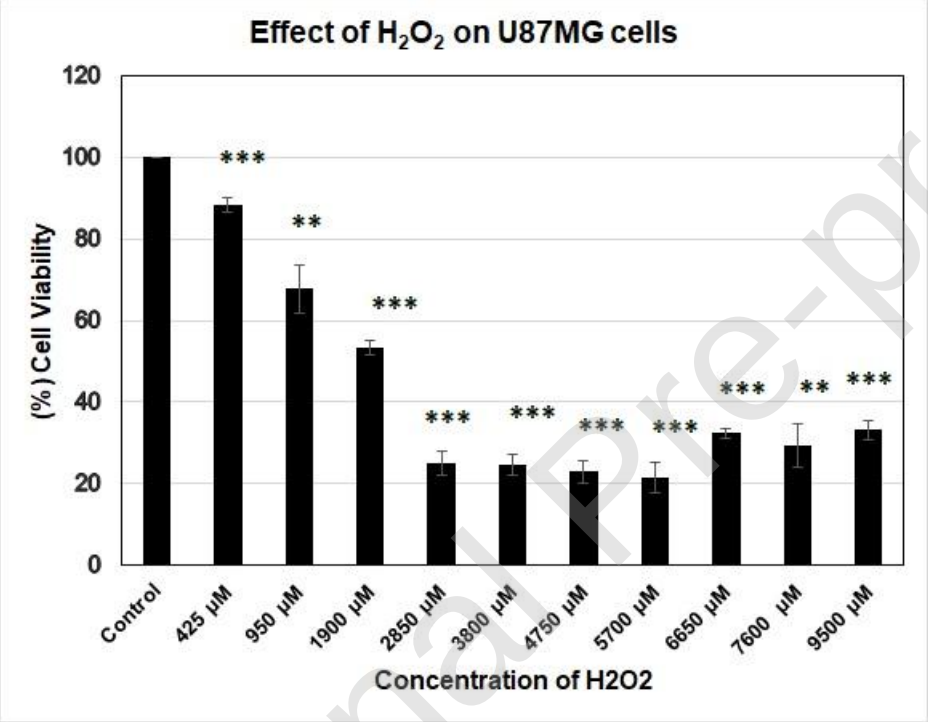


Figure 3b. Effect of H_2O_2 on neuroblastoma SH-SY5Y cells.

The SH-SY5Y cells were treated with H_2O_2 at a concentration ranging from 0 – 256 µM and incubated for 12 h. Forty percent reduction was observed from 16-256 µM concentration. This is followed by MTT based cell viability assay. The OD_{570} value was converted to % values. Three independent experiments were conducted and GraphPad software was used to test statistical significance. ** represents p-value < 0.01 and *** represents p-value < 0.001

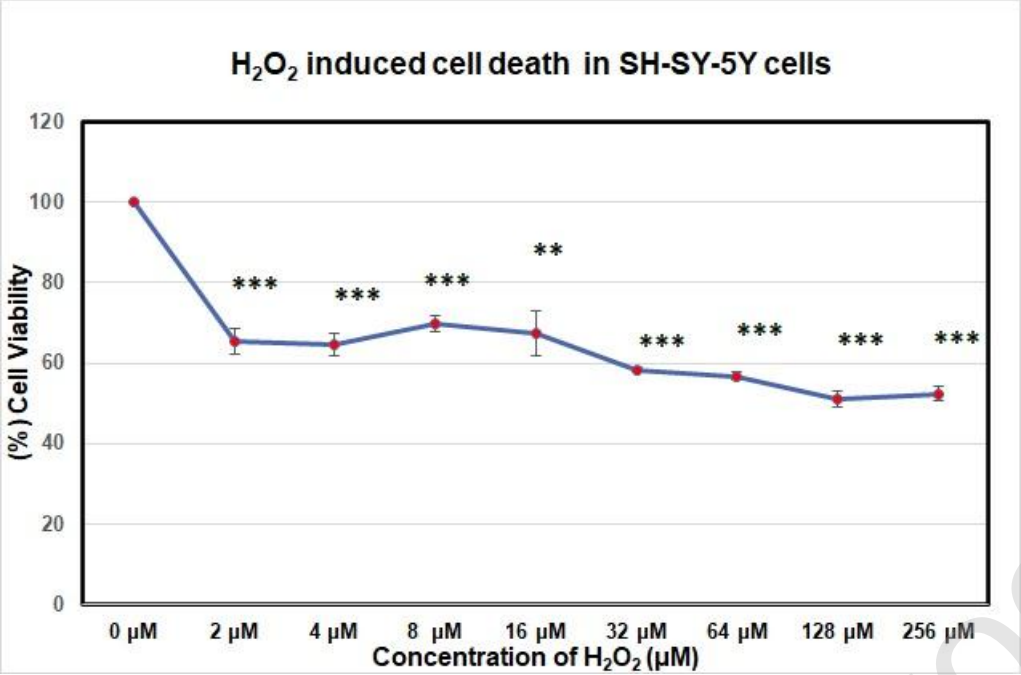


Figure 4a. Effect of Benzothiazole analogs on H_2O_2 induced neurotoxicity in U-87 MG cells. U-87 MG cells were pre-treated with compounds (5b, 5c, 6a-6d, 7a, and VA) of concentration 2 and 4 µM for 12h followed by 4h of H_2O_2 treatment (950 µM) as obtained in the above studies. Then the cell viability was determined. Here, Valproic acid (VA) is the standard neuro protective drug employed. Here 2 µMc indicates 2 µM of compound and 4 µMc indicates 4 µM of BT compounds used in the study. Three independent experiments were conducted and GraphPad software was used to test statistical significance. *** represents p-value < 0.001

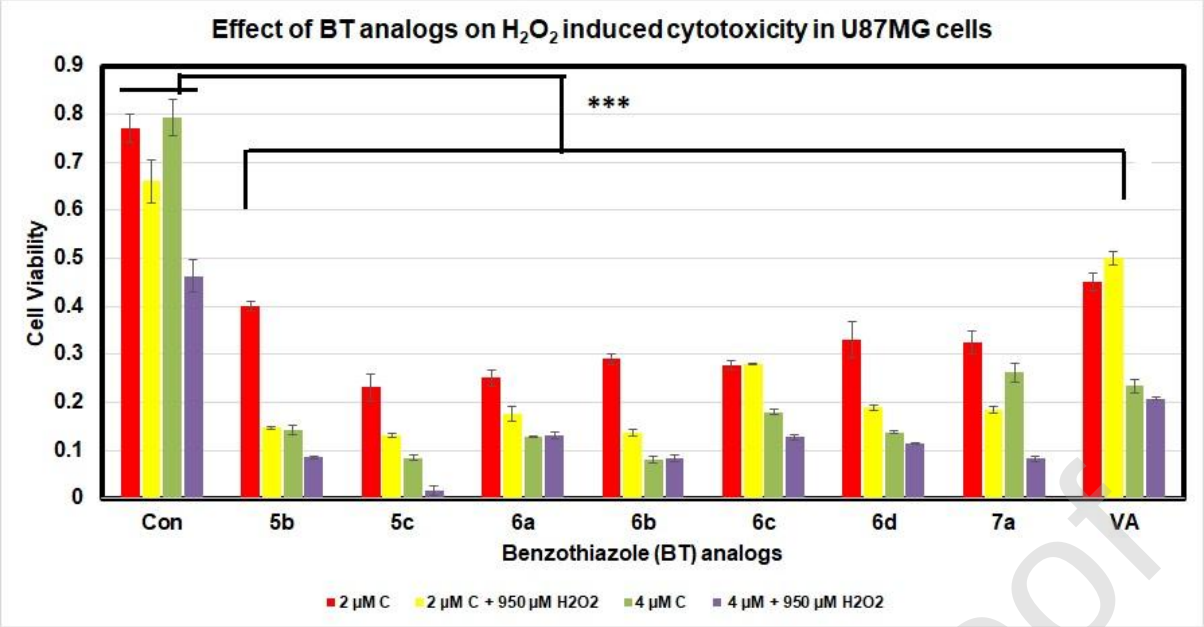


Figure 4b. Effect of Benzothiazole analogs on H_2O_2 induced cytotoxicity in human breast cancer cells (MCF-7). MCF-7 cells were pre-treated with compounds (5b, 5c, 6a-6d, 7a, and VA) of concentration 0.5, 1, 2 µM for 12h followed by 4h of H_2O_2 treatment (128 µM as obtained from previous studies in literature). Then the cell viability was determined. Here Valproic acid (VA) is the standard neuro protective drug employed. Here 0.5 µMc indicates 0.5µM of compound, 1µMc indicates 1µM of BT compound and 2 µMc indicates 2 µM of BT compound used in the study. Three independent experiments were conducted and GraphPad software was used. ** represents p-value < 0.01 and *** represents p-value < 0.001

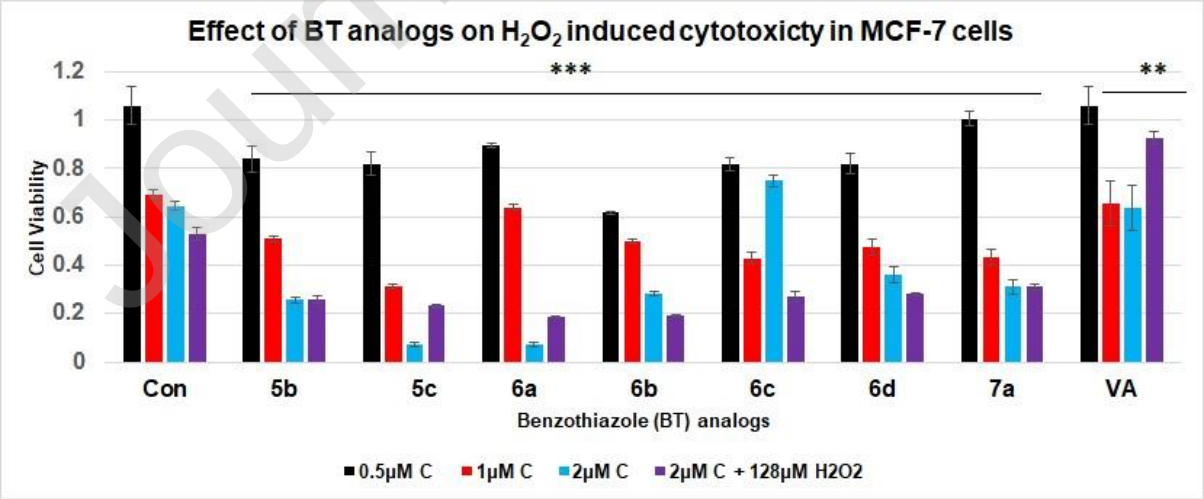


Figure 4c. Effect of Benzothiazole analogs on H_2O_2 induced cytotoxicity in mouse macrophage cells (RAW 264.7 cells). Mouse macrophage cells RAW 264.7 cells (normal cells) were treated with compounds of concentration 2 µM for 12h followed by 4h of H_2O_2 treatment (128 µM). Then the cell viability was determined. Here 2 µMc indicates 2 µM of BT compound used in the study. Valproic acid (VA) is the standard neuroprotective drug employed. Three independent experiments were conducted and GraphPad software was used to test statistical significance. *** represents p-value < 0.001

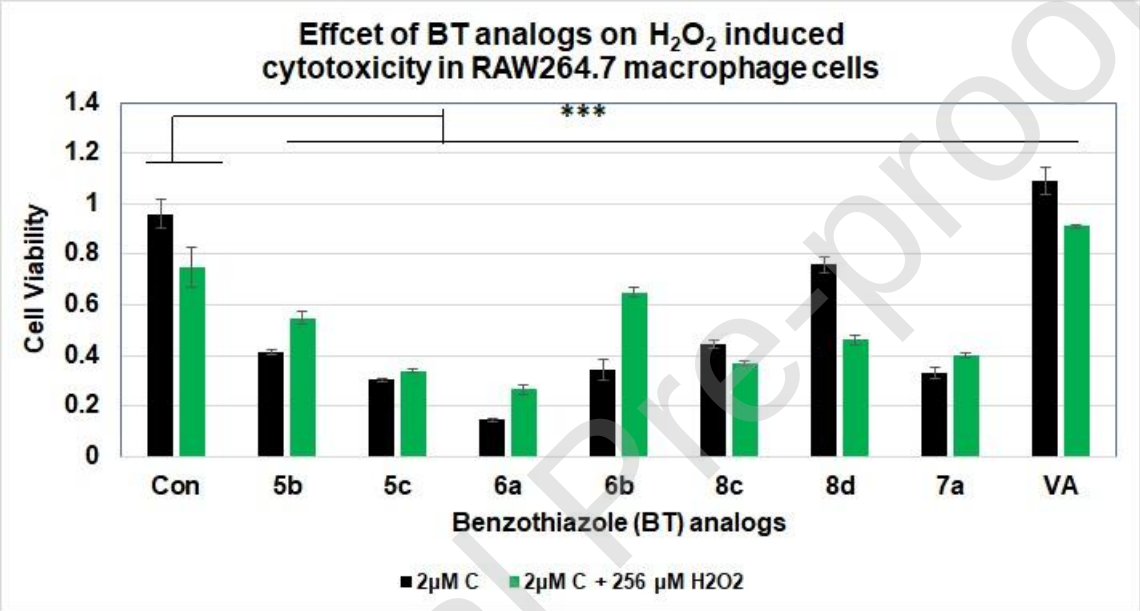


Figure 5. Benzothiazole analogs modulation of ROS in U87MG cells. U-87 MG cells were treated with compounds of concentration 2 µM for 12 h followed by 4 h of H_2O_2 treatment. The cells were then pelleted out and resuspended in PBS containing 10mM H_2DCFHDA dye. Here compound alone or combination of compound + H_2O_2 was employed. The cells were then incubated at 37°C for 30 min. The fluorescence intensity of DCF Ex/Em 470 / 545nm. We observed a decrease in ROS level when compound encounters H_2O_2. Results indicated that compounds by themselves cause cytotoxicity, but the combination of H_2O_2 and compound have exhibited the cell-protective activity. Here, 6d was found to be more protective in comparison with standard compound VA. Here compound mediated inhibition of ROS was compared with

compound alone. Three independent experiments were conducted and GraphPad software was used to test statistical significance. ** represents p-value < 0.01 and *** represents p-value < 0.001

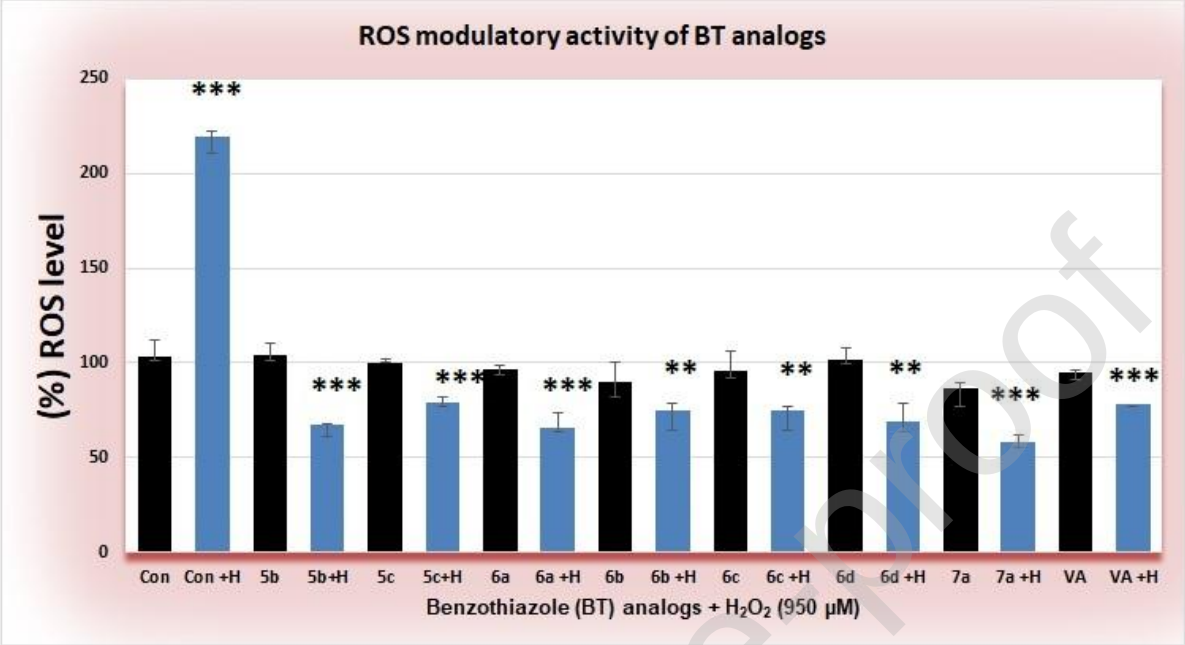


Figure 6. Effect of Benzothiazole analogs on catalase activity. U-87 MG cells were seeded and drug treatment (2 µM) with potential compounds [6a-6d, 7a] was conducted for a period of 24 h. Here, Valproic acid (VA) was used as a standard. The lysates were subjected to catalase activity assay. Here (-H) indicates the lysates obtained after drug treatment were not allowed to react with catalase. (+H) indicates the lysates were allowed to react with H_2O_2 (12 µL of 1 mM) followed by the addition of stop solution. BT analogs 6b, 6c and 6d have shown catalase activity of 88%, 90% and 85%. Positive control has shown 95% of catalase activity. Three independent experiments were conducted and GraphPad software was used to test statistical significance. ** represents p-value < 0.01 and *** represents p-value < 0.001

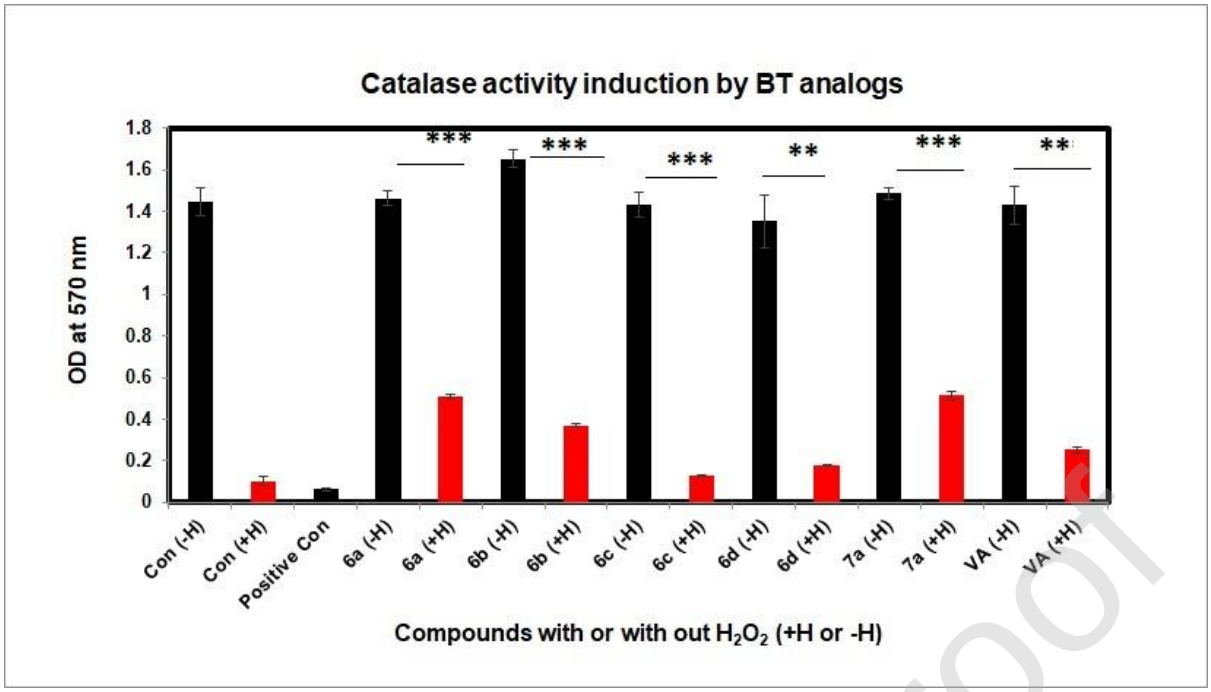


Figure 7. Protein docking studies of Benzothiazole analogs with human erythrocyte catalase protein.

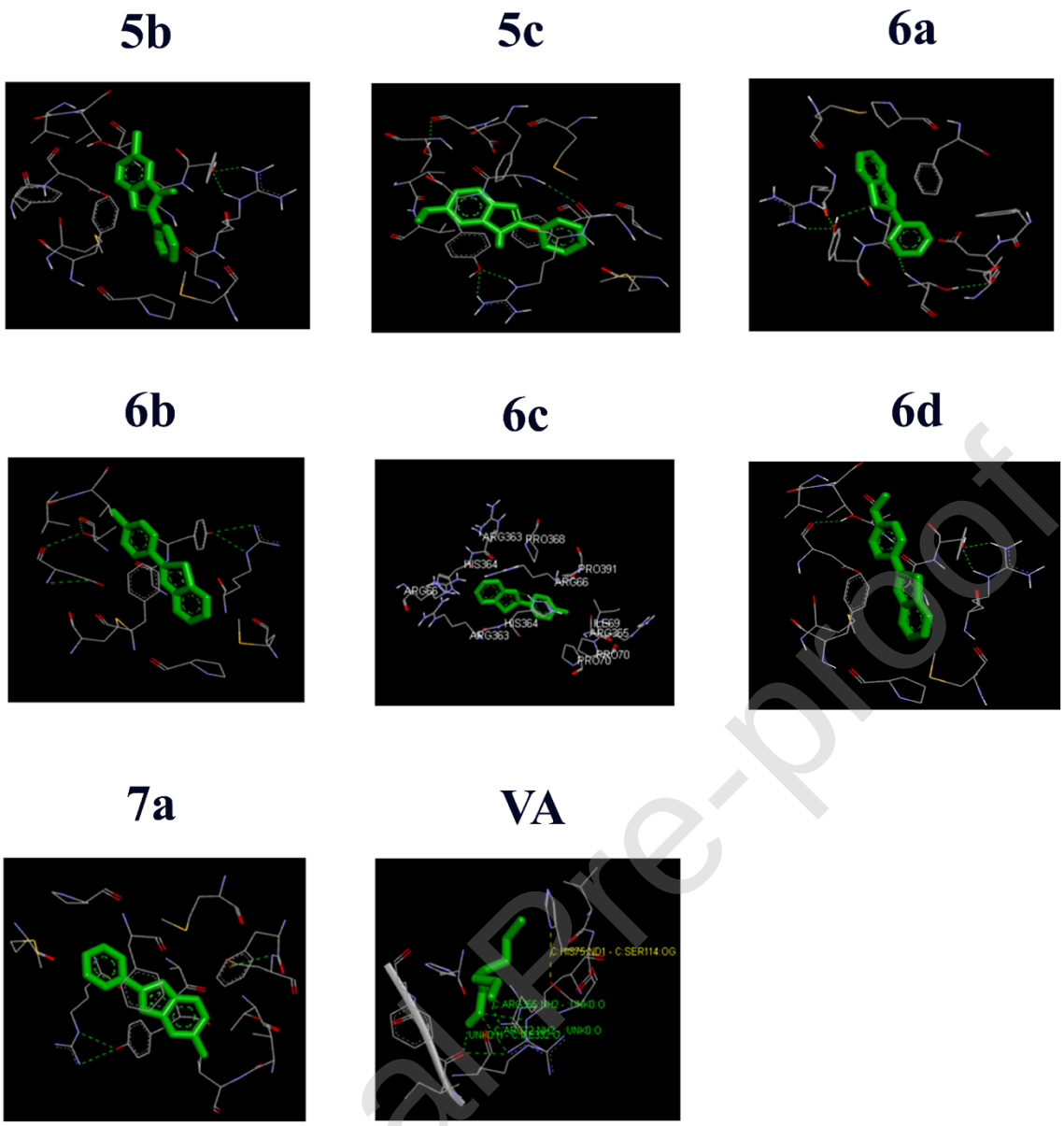
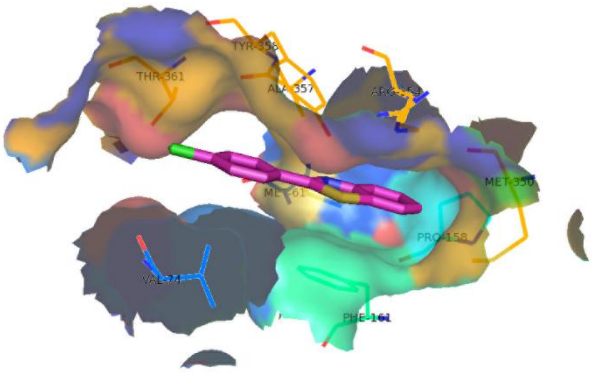
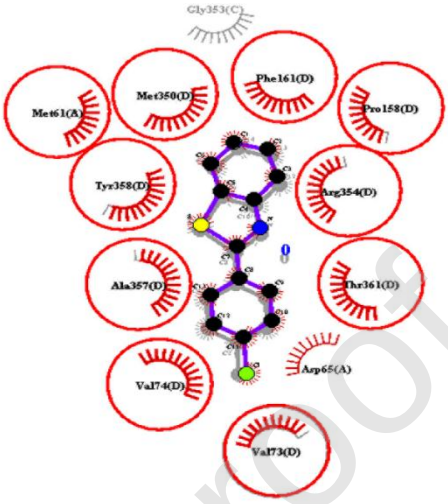


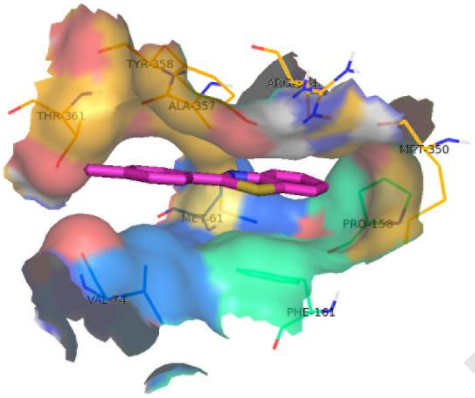
Figure 8. 2D representing of the ligand interactions with the catalase generated for compounds 6b and 6c using LigPlot+

6b





6c



6c
6b

Table 1: Compound ligand interaction study with Benzothiazole analog and catalase protein

Cpd	Conf.	Binding energy	Ligand efficiency	Inhib constant (μM)	Electrostatic energy	Intermol energy	Torsional energy	Unbound energy	Interacted residue	Protein and Ligand interaction	No. of H-bonds
5b	con 9	-5.35	-0.33	120.17	-0.08	-5.65	0.3	-0.24	ARG320 TYR379	ARG320:HH21	2
	con 1	-7.58	-0.47	2.79	-0.02	-7.88	0.3	-0.24	---	TYR379:OH ---	0
5c	con 1	-7.08	-0.42	6.47	-0.02	-7.68	0.6	-0.3	---	---	0
	con 6	-6.22	-0.37	27.67	-0.09	-6.81	0.6	-0.31	ARG72	ARG72:HE	1
6a	con 1	-7.39	-0.49	3.82	0.00	-7.69	0.3	-0.24	ALA345	ALA345:HN	1
	con 4	-6.65	-0.44	13.32	-0.02	-6.95	0.3	-0.24	---	---	
6b	con 7	-5.88	-0.37	48.75	-0.01	-6.18	0.3	-0.24	ALA213	ALA213:HN	1
	con 1	-7.52	-0.47	0.08	-0.01	-7.82	0.3	-0.24	---	---	0
6c	con 1	-6.50	-0.41	17.11	-0.01	-6.80	0.3	-0.18	HIS364	HIS364:HD1	1
6d	con1	-7.1	-0.47	2.91	-0.03	-7.85	0.3	-0.24	-----	-------	0
7a	con 1	-7.35	-0.46	4.09	0.00	-7.65	0.3	-0.26	---	---	0
	con 6	-6.63	-0.41	13.82	0.01	-6.93	0.3	-0.26	ALA345	ALA345:HN	1
VA	con 1	-4.82	-0.48	295.18	-1.32	-6.61	1.79	-0.23	ARG72	ARG72:HE ARG72:HH22	2

Table 2

***Insilico* ADMET prediction for the compounds used in the study by Swiss ADMET prediction program.**

Compound	Smiles	Druglikeness					Pharmacokinetics						Water Solubility	
		Lipinski	Ghose	Veber	Egan	Bioavilability Score	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2D6 inhibitor	Log K_p	Log *S* (ESOL)	Class
5a)	C12=CC=CC=C1NC(C3=CC=CC=C3)=N2	Yes	Yes	Yes	Yes	0.55	High	Yes	Yes	Yes	No	-5.18 cm/s	-3.76	Soluble
5b)	ClC1=CC=C2C(N=C(C3=CC=CC=C3)N2)=C1	Yes	Yes	Yes	Yes	0.55	High	Yes	Yes	Yes	No	-4.95 cm/s	-4.32	Moderately soluble
5c)	COC1=CC=C2C(N=C(C3=CC=CC=C3)N2)=C1	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	Yes	-5.39 cm/s	-3.77	Soluble
6a)	C12=CC=CC=C1SC(C3=CC=CC=C3)=N2	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	No	-4.56 cm/s	-4.51	Moderately soluble
6b)	ClC(C=C1)=CC=C1C2=NC3=CC=CC=C3S2	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	No	-4.33 cm/s	-5.07	Moderately soluble
6c)	CC(C=C1)=CC=C1C2=NC3=CC=CC=C3S2	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	No	-4.39 cm/s	-4.78	Moderately soluble

6d)	COC(C=C1)=CC=C1C2=NC3=CC=CC=C3S2	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	No	-4.77 cm/s	-4.52	Moderately soluble
7a)	CC1=CC=C(N=C(C2=CC=CC=C2)O3)C3=C1	Yes	Yes	Yes	Yes	0.55	High	Yes	No	Yes	No	-4.72 cm/s	-4.3	Moderately soluble