Chapter-1

CORM-A1 mediated improvement in t-BHP induced oxidative stress in hepatocyte via Nrf2 activation.

Introduction

Liver is a vital organ that detoxifies metabolites/xenobiotics generated during drug exposure (Yan *et al.*, 2009) and hence is liable to damages caused by accumulation of free radicals or reactive oxygen species (ROS) (Muriel *et al.*, 2016). Role of antioxidant defense system in regulating pathophysiology of liver diseases is therefore greatly emphasized. Hepatoprotective agents that protect cellular antioxidant defense system, scavenge free radicals and reduce the risk of ROS induced damages. Such hepatoprotective agents are the key players in management of various kind of liver injuries (Singal *et al.*, 2011).

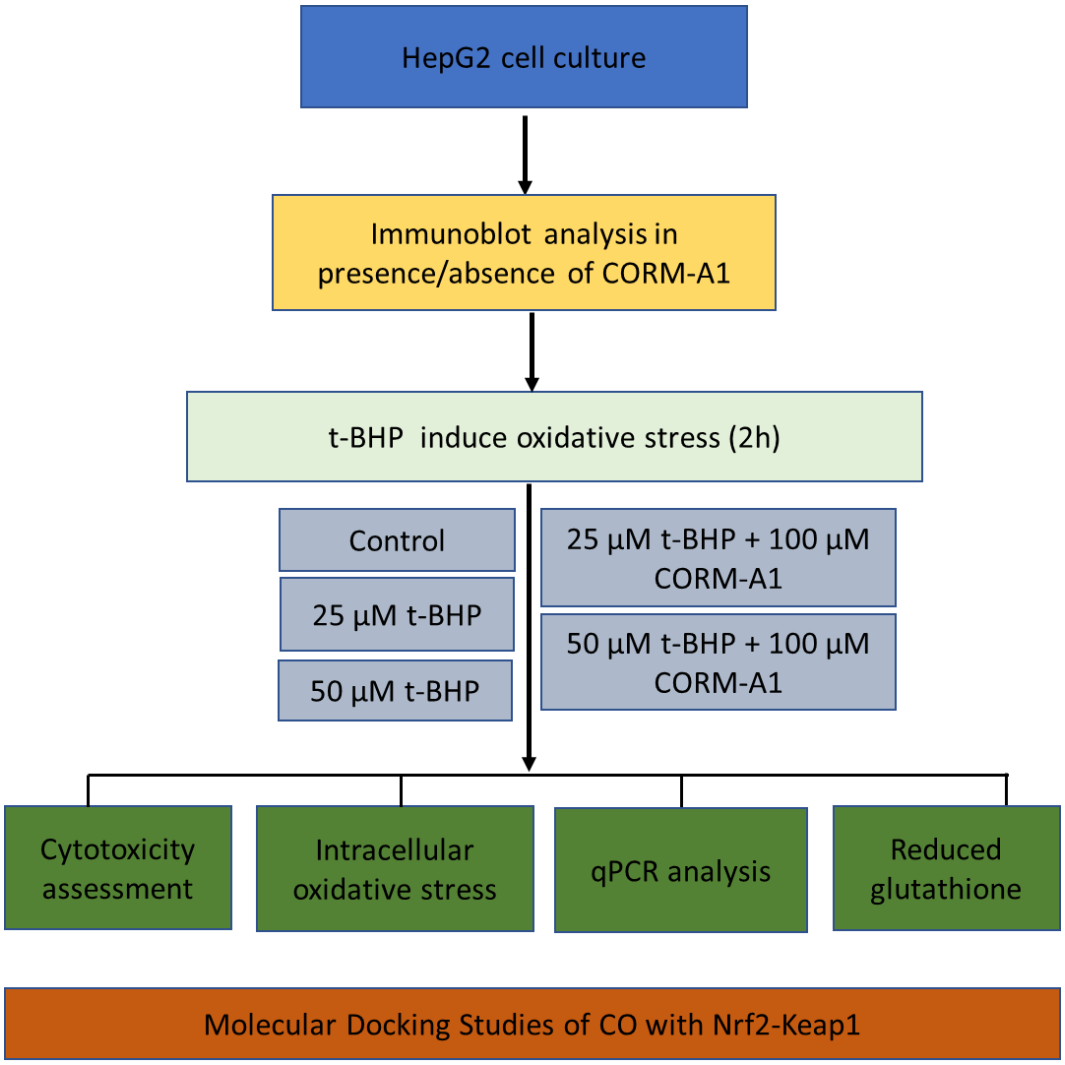
Oxidative stress widely contributes in initiation and progression of hepatotoxicity. Hepatic oxidative stress is induced by several factors such as alcohol, drugs, heavy metals, high-fat diet, etc. Hepatocytes, the key parenchymal cells, suffer the most from oxidative stress in case of liver injury. Oxidative stress activates Kupffer cells that produces a range of cytokines (Tsukamoto, 2002). These cytokines contribute in recruitment of several immune cells to the damage site. Persistent stress condition for a prolonged period of time results in activating apoptotic pathways in hepatocytes. Proliferation of stellate cells and collagen synthesis is also a result of oxidative stress that eventually culminate in promoting fibrosis and cirrhosis (Svegliati Baroni *et al.*, 1998). In response to high oxidative stress, hepatocytes use a sophisticated antioxidant system comprising antioxidant proteins, enzymes and transcription factors to maintain cellular redox homeostasis. Hence, regulation of hepatic ROS can possibly play a critical role in the treatment of various liver diseases. A range of test compounds have been reportedly screened for their

potential to improve intracellular enzymatic and nonenzymatic antioxidant status via targeting different pathways (Jadeja *et al.,* 2016, Vitaglione *et al.,* 2005). In this regard, current work focus on upregulation of Nrf2-Keap1 pathway with cytoprotective genes in the epicenter of our investigation.

Nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor of the Cap-n-collar basic leucine zipper family (Moi *et al.,* 1994) has been recognized as a key regulator of oxidative stress in different tissue and cell types including hepatocytes. In reaction to oxidative stress, Nrf2 binds to the promoter region of ARE sequences and induce expression of antioxidant and cytoprotective genes (Espinosa-Diez *et al.,* 2015b). Nrf2 interacts with cytoplasmic Keap1 and is targeted to proteasomal degradation in basal conditions. Keap1, a substrate adaptor for a Cul3-containing E3 ubiquitin ligase is a primary regulator of Nrf2. Keap1 has three functional domains, namely broad complex, tramtrack and bric-a-brac (BTB). However, under oxidative stress, phosphorylation of Nrf2 results in its dissociation from Keap1 and subsequent translocation to the nucleus where it heterodimerizes with one of the small Maf proteins (Jaramillo *et al.,* 2013, Lau *et al.,* 2013). Further, it binds to ARE sequences and functions in partnership with other nuclear proteins. The resultant transcriptional activation of ARE-responsive genes viz. hemeoxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), glutathione peroxidase (GPX), and glutamate-cysteine ligase catalytic subunit (GCLC) mount a strong antioxidant response (Espinosa-Diez *et al.,* 2015b, Reddy, 2008b).

In recent years, Nrf2 activators of natural and synthetic origin have extensively been reported for their antioxidant potential in a variety of experimental systems (Jadeja *et al.*, 2016). The approach of utilizing Nrf2 activators for improving cellular efficacy in combating ROS can be very beneficial. CO is known to induce upregulation of HO-1 and related antioxidant genes and improve pathophysiology of liver (Slebos *et al.*, 2003). Further, beneficial role of CO has recently been reported in vascular dysfunction (Zimmermann *et al.*, 2007), inflammation (Almeida *et al.*, 2016), tissue ischemia (Motterlini, 2007) and organ rejection (Nakao *et al.*, 2006). This study showcases efficacy of novel CO releasing molecule "CORM-A1" as a possible up regulator of HO-1 via Nrf2 activation and providing cytoprotection in oxidatively stressed hepatocytes.

Experimental Design



Results

CORM-A1 prevents t-BHP induced oxidative stress and hepatocyte injury in HepG2 cells

Tert-Butyl hydroperoxide (t-BHP) induced oxidative stress, GSH depletion and hepatotoxicity in HepG2 cells serves as an ideal model to study improved cell viability and functionality induced by a test compound. HepG2 cells were treated with t-BHP (5, 10, 25, 50 µM) alone or in combination with CORM-A1 and cell viability was studied by MTT assay. Results showed a dose dependent decrement in cell viability following t-BHP treatment, with a highest dose (50µM) recording minimum number of viable cells at 2h (30%). CORM-A1 co-supplementation (100 µM) accounted for significantly higher viable cells at 2h stage (Fig.1.1). Owing to the results obtained herein, cells were checked for oxidative stress. Control, t-BHP exposed (25 and 50 µM) or t-BHP + CORM-A1 (100 µM) treated cells were stained with DCFDA. Prominent green fluorescence in t-BHP (25 and 50 µM) treated cells indicated heightened intracellular oxidative stress (Fig.1.2). However, t-BHP + CORM-A1 group showed relatively weaker fluorescence (2 fold at 25 µM and 1.5-fold at 50 µM) as compare to t-BHP treated groups (Fig.1.2) indicating comparatively reduced intracellular oxidative stress.

CORM-A1 induced transcriptional activation of Nrf2-ARE genes in t-BHP treated HepG2 cells.

Nrf2 is a key transcriptional factor that regulates intracellular antioxidant status of cells. We assessed the effect of CORM-A1 on t-BHP treated HepG2 cells. t-BHP

treatment showed moderate to significant decrement in mRNA of Nrf2 and HO-1 genes as compared to control. Co-treatment with CORM-A1 witnessed reciprocal improvement in expression levels of the said genes. Keap1 mRNA did not show much alteration in its expression levels in all the treatment groups (Fig.1.3). mRNA levels of NQO-1 and total content of GSH showed significant decrement following t-BHP but t-BHP+CORM-A1 treated group recorded a significant increment in the said parameters (Fig.1.4-A).

CORM-A1 facilities Nrf2 translocation in HepG2 cells

Translocation of Nrf2 was studied in the nuclear fraction of HepG2 cells. Immunoblot analysis of Nrf2 reveled a steady increment at 30, 60- and 120-min following CORM-A1 (100 μM) treatment. HO-1 is a key antioxidant protein that showed consistent upregulation at 60- and 120-min following CORM-A1 exposure (Fig. 1.4-B).

CORM-A1 docks at Kelch domain of keap1 protein.

CORM-A1 releases CO in the medium that diffuses through the cellular membrane to showcase its effects. Herein, it was hypothesized that CO bind to Keap1 and dissociated Nrf2-Keap1 heterodimer. This freed Nrf2 translocate to nucleus to further display it anti-oxidative potential. Hence, docking interactions of CO with Keap1 protein was studied (Fig.1.5) and the results were compared with a small molecule (3S)-1-[4-[(2,3,5,6-tetramethylphenyl) sulfonylamino]-1-naphthyl] pyrrolidine-3-carboxylic acid (RA839). Both, CO or RA839 are known to dock at

kelch domain of Keap1(5CGJ) (Winkel *et al.*, 2015) thus inhibiting protein-protein interactions between Nrf2 and Keap1 as evidenced by its crystal structure (Protein Data Bank - code 5CGJ). The binding energies of CO in a best pose was-1.6kcal/mol. The ligand interaction analysis with Discovery studio Client demonstrated conventional hydrogen bond of CO with VAL A: 606, GLY A: 367 and C-H bond with VAL A: 604, LEU A: 365 amino acids of Keap1 protein. Comparative analysis of binding pattern with RA839 showed involvement of VAL A: 606 only. 3D visualization of CO binding on Keap1 protein revealed that its binding location was at the base of Nrf2 binding pocket. CO binding induced changes in 3D configuration of Nrf2 binding pocket on Keap1 protein leading to disassociation of Nrf2 and its possible activation.

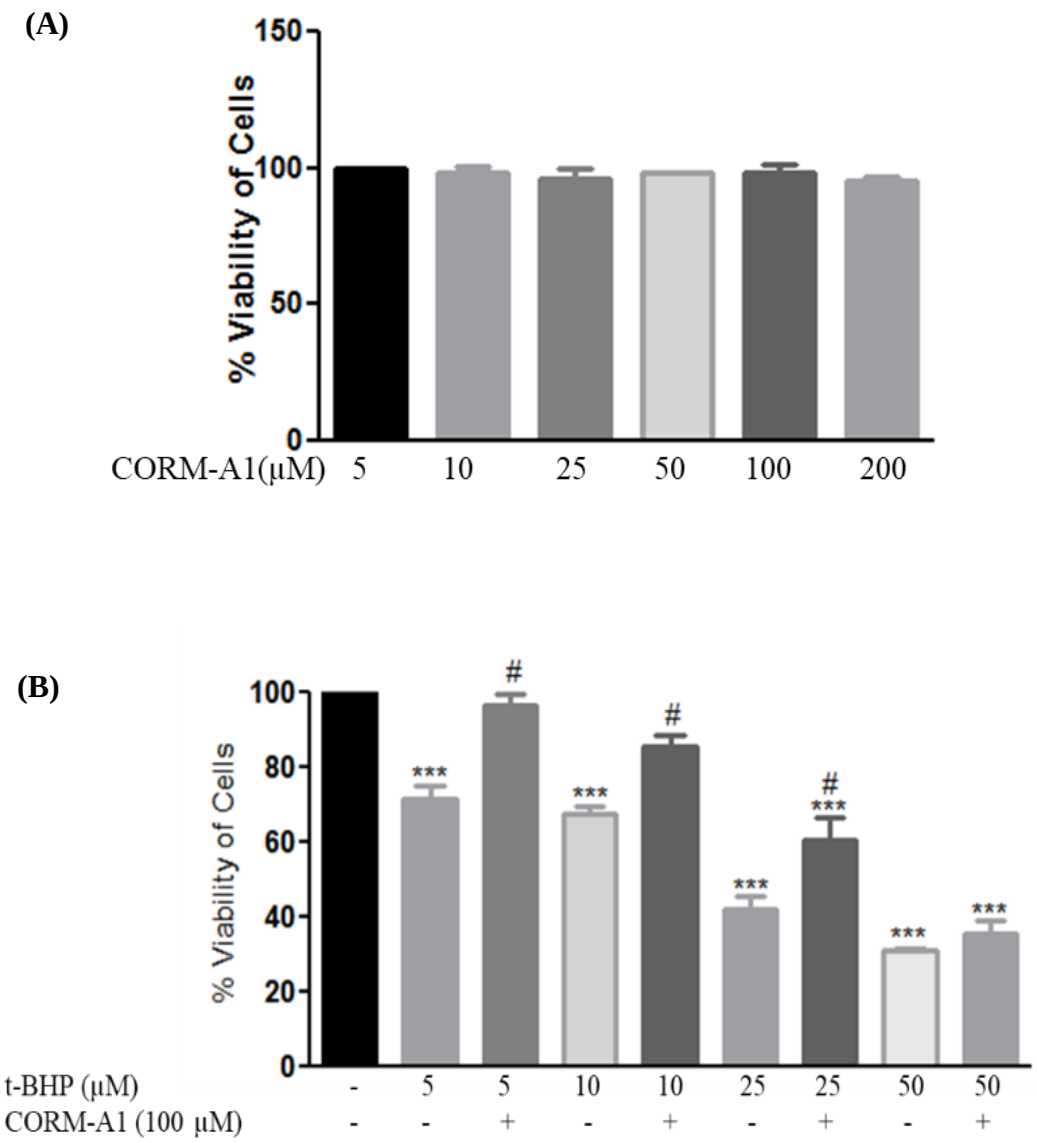
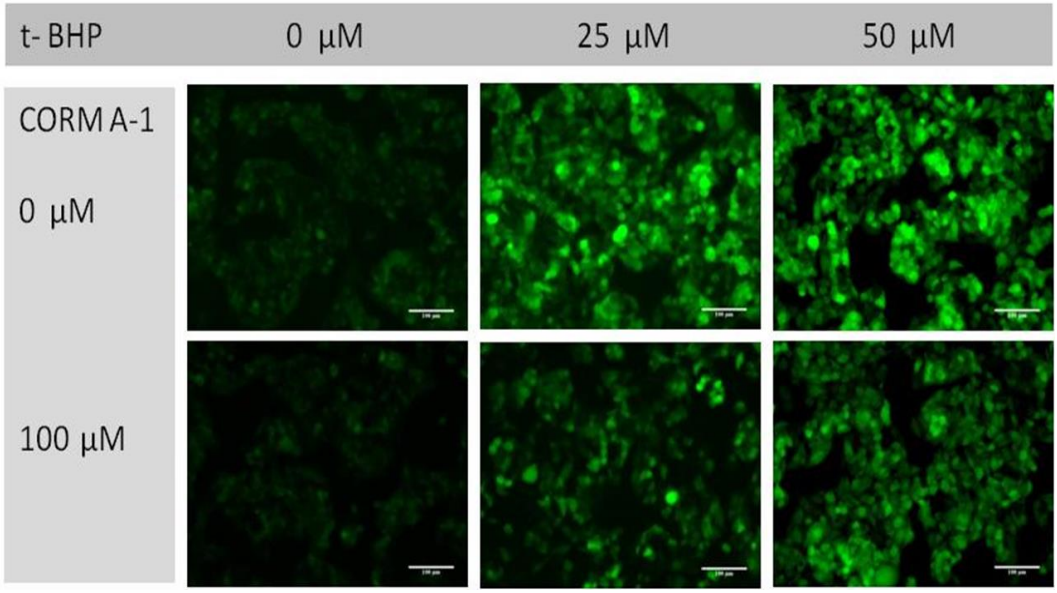


Fig.1.1 (A) Cell viability assessment of CORM-A1 for 24h. (B) Cytoprotective potential of CORM-A1 (100 µm), against t-BHP induced oxidative stress in HepG2 cells for 2h. MTT assay results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001is when compared with respective t-BHP treated group (n=3).

(A)



(B)

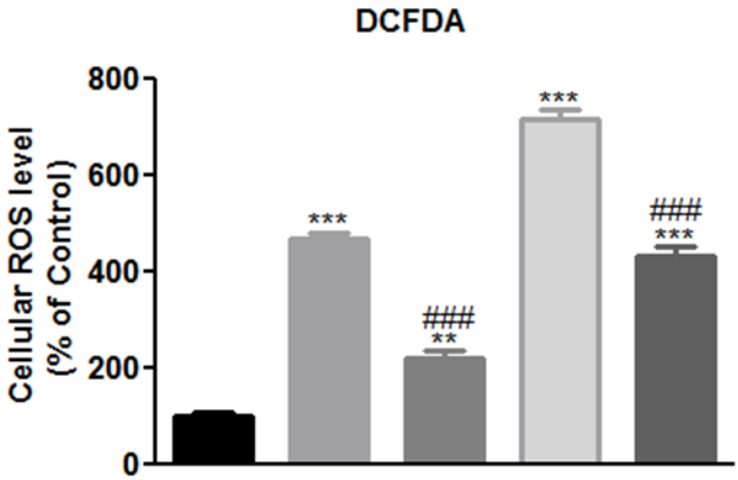


Fig.1.2. (A) Intracellular ROS measurement using DCFDA staining (B) Quantification of the same images. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001is when compared with respective t-BHP treated group (n=3).

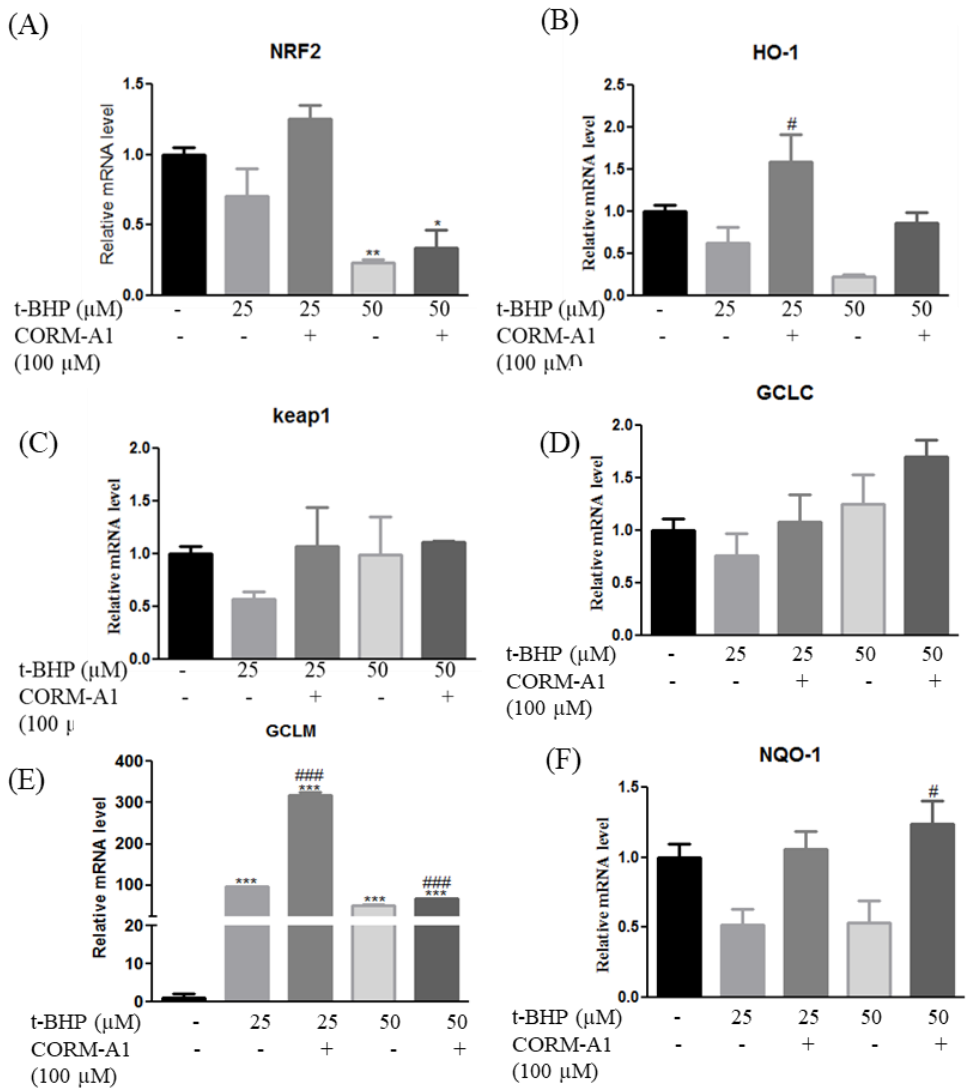


Fig.1.3. HepG2 cells supplemented with CORM-A1 were exposed to t- BHP for 2 h. mRNA levels of Nrf2 and ARE related genes (A) Nrf2 (B) HO-1 (C) Keap1 (D) GCLC (E) GCLM and (F) NQO-1. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective t-BHP treated group (n=3).

(A)

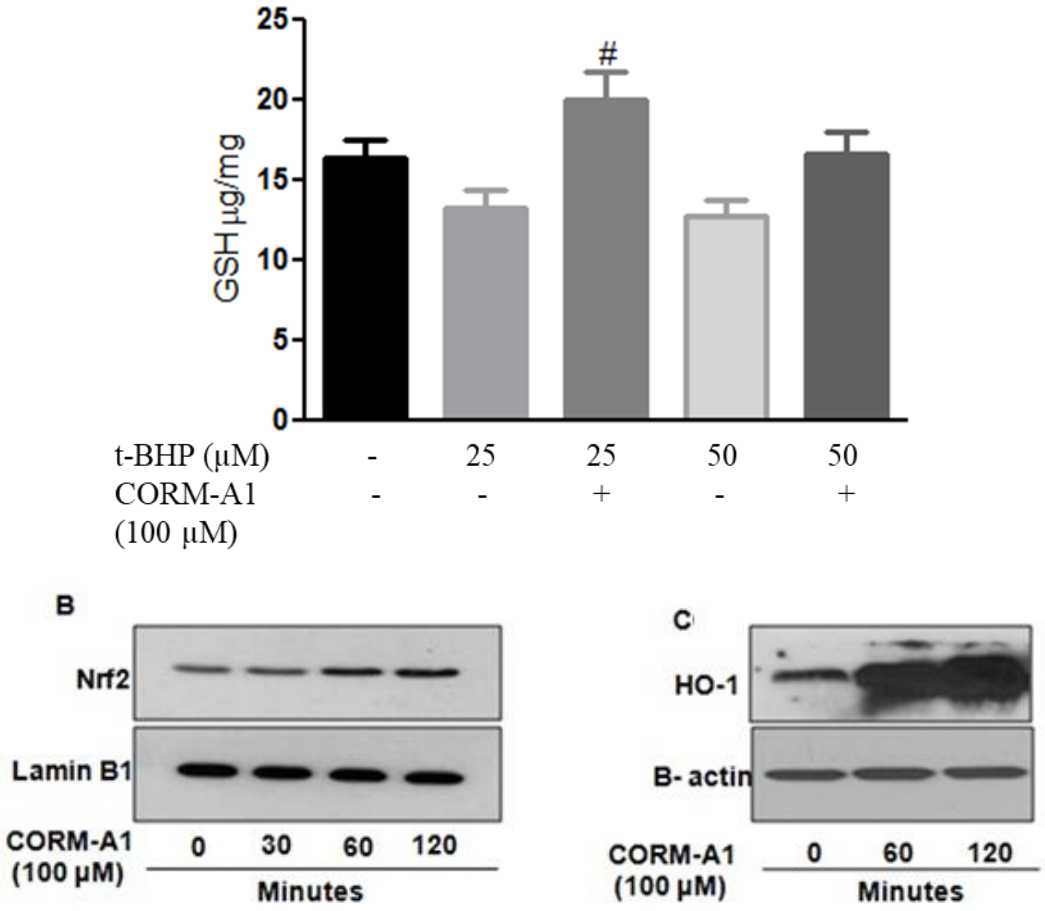


Fig.1.4. (A) Quantification of intracellular GSH. Immunoblot analysis of (B) Nrf2 (in nuclear fraction) and (C) HO-1(in cellular fraction) showing CORM-A1 inducing Nrf2 protein at 60 and 120 min correspondingly. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective t-BHP treated group (n=3).

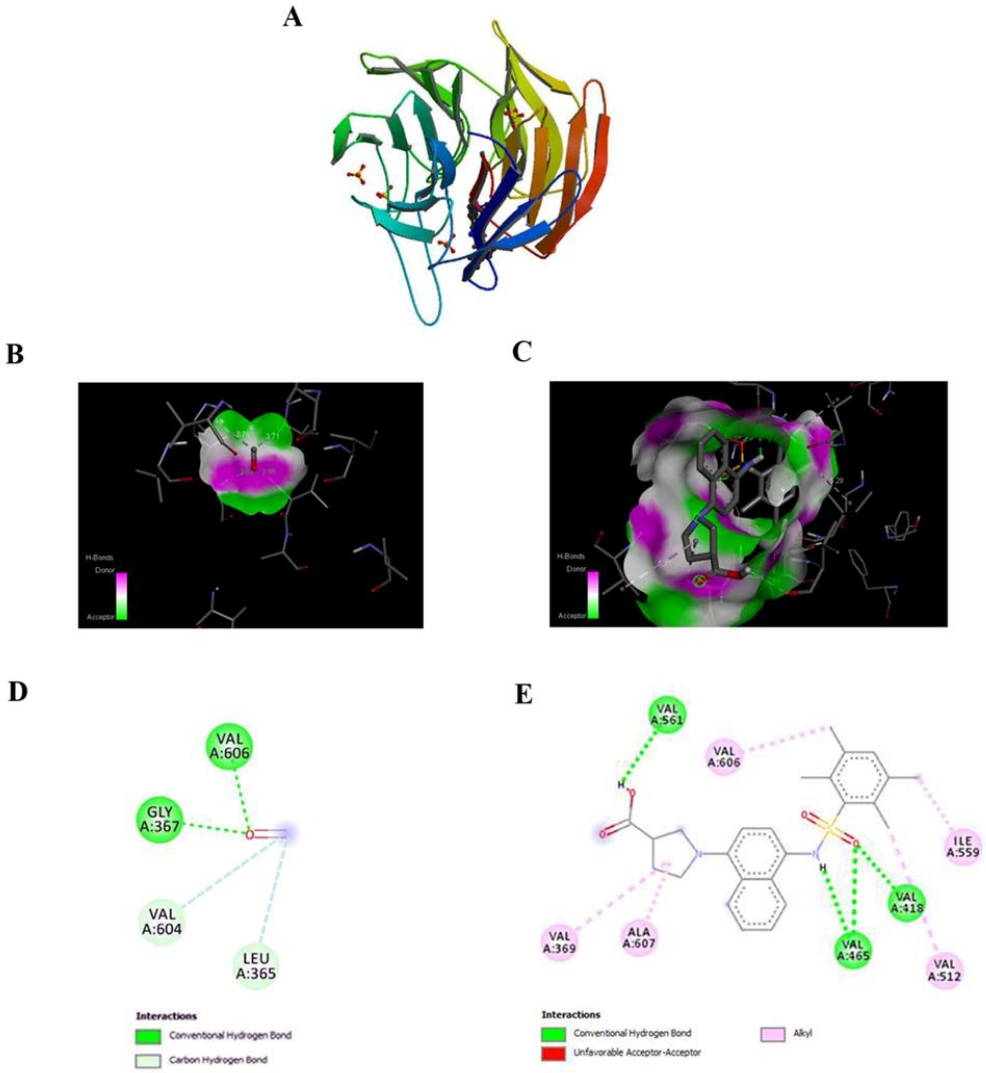


Fig.1.5. Molecular docking analysis of CO with Keap1 Protein (A) Keap1 Protein PDB ID:5CGJ Crystal structure of murine Keap1 in complex with RA839, a non-covalent small-molecule binder to Keap1 and selective activator of Nrf2 signaling. (B) Top view of Keap1 in complex with CO (C) Top view of Keap1 in complex with RA839 (D) and (E) 2D interaction map of Keap1 with CO or RA839. The color codes indicate potential interactions between amino acid residues and CO or RA839.

Discussion

t-BHP is mainly used as a model for assessing underlying mechanisms of cellular oxidative stress. Metabolism of t-BHP involves two pathways, and both induce oxidative stress. The first uses cytochrome p-450 leading to production of peroxyl and alkoxyl radicals. These radicals induce lipoperoxidation of membrane phospholipids which further alters membrane fluidity and permeability (Davies, 1989). Second pathway employs glutathione peroxidase in which t-BHP is detoxified to *tert*-butanol and GSH is depleted by oxidation to its disulphide form (GSSG) (Kučera *et al.*, 2011). Cellular redox imbalance, accumulation of ROS and depletion of antioxidants are crucial in pathology of oxidative stress induced liver injuries. Oxidative injury following t-BHP exposure in HepG2 cells resultant in free radicals initiate lipid peroxidation and DNA damage that often culminate into cell death (Kučera *et al.*, 2014). Therefore, t-BHP induced in vitro liver injury model is used herein to garner prima facie evidence on CORM-A1 mediated Nrf2 activation with possible implications on intracellular GSH levels.

In preliminary studies, cell viability was measured using MTT assay. Herein, t-BHP treated HepG2 cells showed significant decrement in cell viability. Results of this study are consistent with other studies indicating dose dependent decrement in cell viability in t-BHP treated HepG2 cells (Sohn *et al.*, 2005). CORM-A1 co-treatment had higher number of viable cells. These results are in agreement with reported improved cell viability and upregulation of Nrf2 cascade in t-BHP induced cytotoxicity (Qi *et al.*, 2017).

Heightened ROS levels in HepG2 cells accounts for severe damages to the cell organelles, nucleus and cytoplasmic enzyme machinery (Bergamini *et al.*, 2004). Further, it leads to impairment of mitochondrial membrane and subsequent leakage of mitochondrial enzymes in the cytoplasm (Kowaltowski *et al.*, 1999). In this study, t-BHP induced oxidative stress is compensated by CORM-A1 co-treatment to HepG2 cells. CORM-A1 mediates its effects by reducing oxidative stress in hepatocytes. Previous reports had suggested that exogenous CO and tricarbonyl dichlororuthenium (II) dimer (RuCO; a CO releasing ligand), induces Nrf2 activation and increases transcription of HO-1 in HepG2 cells (Lee *et al.*, 2006). Immunoblots of nuc-Nrf2 implies towards CORM-A1 mediated nuclear translocation in HepG2 cells.

Our findings are also comparable to the reports on RuCO mediated Nrf2 activation but RuCO is a faster releaser of CO (Lee *et al.*, 2006). Conversely, CORM-A1 has a slow and sustained pattern of CO release (Motterlini *et al.*, 2005). Hence, the CO mediated improved oxidative stress observed herein is also attributable to the said differences in chemical characteristics. Additionally, we utilized a computational approach to determine the mechanism underlying CORM-A1 mediated Nrf2 activation. Our docking analysis indicated that CORM-A1 induced Nrf2 activation is via CO-mediated release of Nrf2 from kelch domain of Keap1. The molecular docking studies provide evidence that, the competitive binding of CO in kelch domain of Keap1 inhibits interaction between Nrf2 and Keap1. CO-Keap1 interaction is assumed to stabilize the Keap1 protein that facilitates release and activation of Nrf2 to induce ARE genes.

Keap1 is a substrate adaptor component in the Cullin3 (Cul3)-based ubiquitin E3 ligase complex, that recognizes Nrf2 by protein-protein interaction and negatively regulates Nrf2 by polyubiquitination (Jadeja, *et al.,* 2016). Nuclear translocation of Nrf2 and regulatory role of keap1 has been emphasized in transcriptional activation of Nrf2-ARE genes (Jadeja *et al.*, 2016, Matsumoto *et al.*, 2006). CORM-A1 induced upregulation of Nrf2 in hepatotoxicity is established but its effect on Keap1 lacks clarity. In our study, significantly lower mRNA levels of Nrf2 in t-BHP treated group is in agreement with down regulation of Nrf2-ARE genes studied herein. Also, reciprocally higher levels of Nrf2 and HO-1 in CORM-A1 treated groups corroborate with high levels of GSH at the same time points.

The highlights of our study are

i) in HepG2 cells, CORM-A1 facilitates nuclear translocation of Nrf2, reduces oxidative stress, upregulates ARE genes, prevents GSH depletion and promotes cell viability.

ii) Docking analyses suggest that CORM-A1-mediated results are due to inhibition of interaction between Nrf2 and Keap1 protein.

Overall, the data indicates that CORM-A1 reduce oxidative stress-mediated hepatocytes damage via Nrf2-ARE activation.