Words are things; and a small drop of ink, falling like dew upon a thought, produces that which makes thousands, perhaps millions, think.

—Byron

Dept. of Pharmacology, L.M. College of Pharmacy, Ahmedabad.
While the incidence of death from coronary artery disease (CAD) and myocardial infarction (MI) has been declining, the overall mortality after surviving from an acute MI is still around 30%, and CAD remains the number one cause of death in the US. Living organisms have developed highly conserved enzymatic system that generates small gaseous molecules, such as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H$_2$S). Regardless of their notoriety, recent findings revealed that trace amount of these gaseous molecules are essential for maintaining the biological system and defending the host from hostile environments. CO is produced endogenously by heme oxygenase (HO) enzyme as a product in the catabolism of heme to CO, biliverdin and iron. A connection between NO and cardiovascular diseases has been demonstrated since decades whereas CO has been emerged as cardioprotective agent in recent years. But this has been criticized because of the low potency of CO as compared to NO. Tricarbonyldichlororuthenium (II) dimmer known as CO-releasing molecule-2 (CORM-2), a lipid soluble molecule that was able to deliver CO in a controlled manner and simulate the cytoprotective action of HO-1 derived CO in biological systems. Subsequently tricarbonyldichloro (glycinato) ruthenium (II) (CORM-3), a watersoluble form has been developed and demonstrated protection against cardiac ischemia-reperfusion (I/R) injury.

The overall objective of this dissertation work was to investigate the role of CO in cardioprotection and to elucidate the molecular mechanisms involved. To achieve our goal we utilized (i) Langendorff’s isolated rat heart model, (ii) doxorubicin (DXR)-induced cardiotoxicity model in H9c2 cells, (iii) DXR-induced cardiotoxicity model in mice and (iv) in vitro and ex vivo platelet aggregation studies as well as in vivo studies using animal models of thrombosis.

In order to find out the cardioprotective potential of CO, we employed CORM-2 as CO releaser and Langendorff’s isolated rat heart model of I/R injury. In the beginning we wanted to find out the concentration of CORM-2, which is necessary to produce cardioprotection. We found that pretreatment of isolated heart with 50 nM CORM-2 produced significant reduction in myocardial injury markers such as creatine kinase (CK) and lactate dehydrogenase (LDH). There was significant improvement in cardiodynamic parameters like left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), dp/dt max (indices of left ventricular contraction) and dp/dt min (indices of left ventricular relaxation). When hearts were preconditioned with CORM-2, we also observed that treated hearts produced significant reduction in myocardial infarct size. It has
been reported that CO and NO has structural and functional similarities. Therefore we also aimed to find out the role of NO in CORM-2-mediated cardioprotection using L-NAME as pharmacological inhibitor of NO synthase (NOS). Addition of L-NAME produced similar improvement in cardiac parameters as CORM-2 alone which suggests that NO has nonsignificant role in CORM-2-mediated cardioprotection. Further, we investigated the role of coronary endothelium in CORM-2-induced cardioprotection using isolated heart. We observed that cardioprotection by CORM-2 was independent of coronary endothelium. Furthermore, we focused on the possible involvement of $K_{ATP}$ channel and found that inhibition of $K_{ATP}$ channel by glibenclamide significantly abolished the CORM-2 induced cardioprotection suggesting that activation of $K_{ATP}$ channel present on vascular smooth muscle cell (VSMC) may be one of the mechanisms for the protective effect. There are many other signaling pathways involved during I/R injury and therefore we also studied the involvement of p38 mitogen-activated protein kinase (p38MAPK), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) in CORM-2-induced cardioprotection using pharmacological inhibitors of these kinases. We demonstrated that activation of p38MAPK beta and PKC are important signaling mediators responsible for cardioprotection by CORM-2 during I/R injury before ischemia. Further, our study results also suggested that CORM-2 may lead to activation of PI3K during reperfusion injury and confers cardioprotection.

Next we assessed the cardioprotective effect of CORM-2 using H9c2 cells. Here we aimed to find out the anti-apoptotic potential of CO using CORM-2 as CO releaser and DXR to induce apoptosis. We found that CORM-2 treated cells showed better cell viability and reduction in LDH in presence of DXR as compared to DXR alone. CORM-2 treated cells also showed significant reduction in caspase-3 activity in presence of DXR as compared to DXR alone in H9c2 cells. Anti-apoptotic activity has also been confirmed using DNA ladder experiment in gel electrophoresis. DXR-treated cells produced characteristic ladder pattern whereas pretreatment with CORM-2 (50 μM, 1 hr) prevented DNA fragmentation and ladder. We also observed the nuclear morphology of H9c2 cells by staining the nucleus of cells with fluorescent dye and found that DXR-treated cells produced nuclear fragmentation whereas CORM-2+DXR-treated cells showed intact nuclei.

Then we investigated the effects of CORM-2 in DXR-induced acute cardiotoxicity in mice and mechanism(s) involved. The major focus of this study was to find out the antioxidant property of CO and to assess the change in mRNA expression of heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), hypoxia inducible factor-1.
Abstract

The results of this study demonstrated that CORM-2 (30 mg/kg, i.p., 10 days) treatment produced significant decrease in myocardial injury markers such as CK and LDH levels in the mouse serum in presence of DXR as compared to DXR alone group. Similarly there was significant decrease in malondialdehyde (MDA) and significant increase in total antioxidant status (TAS) using heart tissues as compared to DXR alone. There was also improvement in hematological profile like red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and % reticulocyte count with CORM-2 (30 mg/kg, i.p.) -treated group as compared to DXR alone. Histopathological observations of heart sections suggest that treatment with CORM-2 showed protective effect whereas DXR-treated hearts showed abnormal morphology. DNA isolated from CORM-2 treated heart showed no laddering pattern and there was prevention of DXR-induced increase in caspase-3 activity in the heart tissues. These results are correlated with our in vitro study observations in H9c2 cells. HO-1 has been considered as one of the endogenous antioxidative defence mechanism against injury. Therefore we measured the HO-1 mRNA expression in mouse heart using real time RT-PCR. Similarly, we have also measured the mRNA expression of iNOS, HIF-1 alpha and VEGF in heart tissues. We found that mRNA expression of HO-1 was elevated whereas mRNA expression of iNOS was decreased in CORM-2 treated hearts which suggests that decrease in oxidative damage may be responsible for cardioprotective effect by CORM-2. It has also been found that there was increase in HIF-1 alpha and VEGF expression, which may cause cardioprotection.

There was no much information regarding the antiplatelet role of CORM and its mechanism(s). Therefore, we demonstrated that CORM-3 (water soluble CO-releaser)-showed antiplatelet effect and this effect might be mediated by inhibition of protease-activated receptor-1 (PAR-1) in human platelets. We also determined platelet inhibitory role of CORM-3 using rat platelets and investigated that antiplatelet property may be attributed to activation of soluble guanylate cyclase (sGC), release of NO as well as inhibition of plasminogen activator inhibitor-1 (PAI-1). Results of the experiments on platelet aggregation assay had also been supported by performing experiments using in vivo thrombosis models in rat. CORM-3 (3 mg/kg/min i.v., 10 min) produced significant increase in time to occlusion (TTO) in FeCl3-induced arterial thrombosis model. This antithrombotic activity may be the combined result of activation of sGC, activation of NOS and increase in fibrinolytic activity due to inhibition of PAI-1 by CORM-3. CORM-3 was not able to reduce the venous thrombus weight because thrombus in vein is RBC rich with few platelets whereas arterial
thrombus is rich in platelets. We also observed that CORM-3 produced less bleeding complications as compared to clopidogrel.

Based on above experimental data we demonstrated that CORM-2-mediated cardioprotective effect which is concentration dependent, independent of NO and coronary endothelium and activation of $K_{ATP}$ channel, p38MAPK beta and PKC before ischemic insult as well as activation of PI3K during reperfusion may protect the heart during I/R injury. Further, we also concluded that CORM-2 produced cardioprotection by reducing apoptosis of cardiomyocyte and reducing oxidative injury in heart. Water-soluble CO-releaser, CORM-3 has antiplatelet activity, which may lead to further cardiovascular benefits. We observed the antiplatelet and antithrombotic activity of CORM-3 and found that inhibition of PAR-1 and PAI-1 as well as activation of sGC and activation of NOS mediated NO might be responsible for CORM-3-mediated antiplatelet and antithrombotic activity.