Chapter IV

DISCUSSION
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Fibrosis is a progressive, irreversible disease of unknown etiology which is a final manifestation of variety of and possibly diversified chronic inflammatory disorders. The disease most commonly affects middle-aged adults, although infants and children are also affected. It is characterized by injury with loss of epithelial cells lead to abnormal and highly compromised tissue repair, resulting in replacement of normal tissue with ECM proteins causing deterioration of organ architecture and subsequent dysfunction and ultimate failure of organ [Kis et al., 2011].

In the present study, based on the important roles of ET-1 and PDGF signaling pathways in the pathogenesis of fibroses, bosentan and imatinib that block these key signaling pathways respectively were used to investigate the combined effects in lung and kidney mice models of fibroses. Both bleomycin induced PF and UUO induced kidney fibrosis are established animal models in which pathogenic mechanisms are similar to humans.

The main finding of the present study is that combination with both bosentan and imatinib retards the development of PF and kidney fibrosis induced by bleomycin and UUO respectively in a more profound way than either by bosentan or by imatinib alone. Combination treatment prevented mortality and the loss of body weight of PF mice, improves the kidney function in UUO mice, suppressed early inflammation, reduced MMP-9 and MMP-2 activities indicating reduced migration of inflammatory or mesenchymal cells and overall, decreased the fibrosis of lung and kidney tissues in respective animal models.

Combination treatment with bosentan and imatinib ameliorated bleomycin induced reduction in body weights of PF mice more profoundly than individual treatment. This indicates drug combination therapy offers better safety for bleomycin induced pulmonary toxicity than single agent alone. In the case of UUO induced kidney fibrosis, elevated levels of serum BUN, creatinine and proteinuria levels indicate that loss of kidney function. According to previous reports, since, ET-1 and PDGF signalings were responsible for albuminuria and loss of kidney function [Fligny et al., 2011], in present study, the combination therapy with the inhibitors of these signaling pathways bosentan and imatinib improved kidney physiology more effectively than individual therapy, which was reflected by the reduction in serum BUN, creatinine and urinary protein levels in UUO mice. Overall, combination treatment ameliorated the fibrotic
conditions especially, with reference to improved cellular, molecular, biochemical and histological parameters of lungs and kidneys in respective fibrosis models.

Despite the lack of efficacy of anti-inflammatory agents in treatment of fibrotic disorders, inflammation is the early key incident that drives the progression of fibrosis. In this study, tissue infiltration of inflammatory cells in both lungs and kidneys of respective animal models may be due to ET-1 signaling which facilitates infiltration of CD-4 positive cells [Mutsaers et al., 1998] by inducing expression of E-selectin and intracellular adhesion molecule-1 [Zouki et al., 1999]. Moreover, increased synthesis of TNF-α in macrophages by ET-1 augments chemotaxis of monocytes, neutrophils [Bellisai et al., 2011]. In addition to chemo-attractant of leukocytes, ET-1 also stimulates the expression of pro-inflammatory cytokines. However, in addition to ET-1, PDGF/PDGFR signaling pathways are closely associated with inflammation [Huang et al., 2009]. Previous studies reported that leukocyte derived PDGF induces chemokine expression, which may lead to recruitment of additional leukocytes creating amplification loop for renal inflammation [Eitner et al., 2008]. Hence, these two signaling pathways are contributing the infiltration of inflammatory cells in distinct and non-overlapping way in both lung and kidney fibroses. Indeed, in current study, simultaneous inhibition of ET-1 and PDGF signaling with bosentan and imatinib exerted more profound attenuation of inflammation than individual agents alone. This was reflected by decrease in number of total inflammatory cells, macrophages, lymphocytes and neutrophils along with total protein content in BALF, MPO activity, wet/dry lung weight ratio (indicator of edema) in lung tissues, leukocytes infiltration in lung tissue sections on day seven. Similar results were noticed in kidney fibrosis, combination treatment with bosentan and imatinib profoundly attenuated the tissue infiltration of inflammatory cells, confirmed by reduced MPO activity, indicates decreased intrusion of neutrophils into kidney tissues on day seven of post UUO. This aspect clearly highlights the interaction of both these drugs at molecular level. Though bosentan and imatinib act through different pathways involving ET-1 and PDGF respectively, their anti-inflammatory effect through both the pathways may be conspicuous and quantitatively distinct, thereby, the drug combination paved the way for the enhanced (near additive) efficacy.

These observations were consistent with previous findings that both bosentan and imatinib have been individually shown to exhibit strong anti-inflammatory effects.
Bosentan reduced leukocyte adhesion and inflammation in murine model of inflammation [Anthoni et al., 2005]. It also reduced pulmonary hypertension by decreasing the pro-inflammatory cytokines. Another study reported that bosentan attenuates the inflammation in monoarthritic mice [Imhof et al., 2011]. Bosentan attenuated inflammatory response by suppressing the cytokine release in human pulmonary vascular smooth muscle cells induced by bacterial endotoxins [Knobloch et al., 2013]. Bosentan inhibited angiotensin-II induced NF-κB and AP-1 activation and subsequent inflammation [Muller et al., 2000]. Protein tyrosine kinases that include BCR/Abl, PDGF-R, c-KIT, c-fms, TCR/Abl, Lck, FLT-3 and MAPKs are intimately associated with inflammatory disorders. Imatinib, a selective inhibitor of these tyrosine kinases has therapeutic benefit for wide range of autoimmune rheumatic diseases including scleroderma, pulmonary arterial hypertension, spondyloarthropathies, and rheumatoid arthritis [Azizi et al., 2013]. On other hand, imatinib has anti-proliferative and immuno-modulatory effects in lymphocytes, macrophages, mast cells and with abrogating multiple signal transduction pathways involved in pathogenesis of autoimmune diseases e.g., inhibiting IFN-γ, TNF-α, IL-1β and IL-17 pro-inflammatory cytokines [Azizi et al., 2013]. It showed potent anti-inflammatory effect by modulating the TNF-α production in macrophages [Wolf et al., 2005]. Another study reported that imatinib or nilotinib decrease bleomycin induced lung inflammation and fibrosis [Yoon et al., 2010].

A number of previous studies reported that cellular imbalance between the ROS and antioxidant enzymes play a key role in the pathogenesis of fibrosis. Infact, lung injury in bleomycin animal model is due to production of ROS by bleomycin and also by recruited inflammatory cells during PF [Strausz et al., 1990; Hay et al., 1991]. Similarly, ROS are also generated in chronic obstructive kidney diseases which damage the tissues leading to perpetuation of disease pathogenesis [Haugen et al., 1999]. Previous studies reported that ET-1 develops oxidative stress by stimulating the production of ROS (O$_2^-$). A mechanism underlying the generation of ROS by ET-1 is initial vasodilation of blood vessels followed by their subsequent long lasting contraction, resulting in ischemia of internal organs and the dysfunction of the endothelium, consequently lead to abundant generation of ROS [Lopez-Sepulveda et al. 2011]. Another mechanism of generation of ROS is escalation of lipid peroxidation and reduction of intracellular glutathione levels by ET-1. In such a scenario, the imbalance results in the reduced activities of antioxidant
enzymes such as catalase, SOD and glutathione peroxidase in PF and kidney fibrosis [Santos-Silva et al., 2012]. Consistant with these previous findings, in the present study, admnistratration of belomycin resulted in significant reduction of SOD and catalase activities. However, in this scenario, only bosentan contributed to the amelioration of antioxidant enzyme activities, but, imatinib did not show any effect in amelioration of these activities. Even, the combination of both the agents was not more effective than bosentan alone, which emphasizes the involvement of ET-1 but not PDGF signaling in this event. Similarly, in kidney fibrosis, administration of bosentan alone enhanced the UUO induced decrease of SOD and catalase activities in kidney tissues. These observations are in sharp contrast to the observed additive and/or near additive anti-inflammatory effects when these two drugs are combined, also adds strength to the notion that many cellular targets may be the key in the pathogenesis of fibrosis.

These results were in agreement with the previously reported studies. In various animal models bosentan increased the serum antioxidant activity, decreased oxidative stress and restored cellular antioxidant levels [Chen et al., 2010]. Bosentan administration ameliorated the decreased SOD, catalase activities and lipid peroxidation levles in experimental myocardial ischemia and reperfusion [Gupta et al., 2005]

Owing to the production of ECM protiens, myofibroblasts are major perpetrators in the pathogenesis of fibrosis. ET-1 promotes stimulation of myofibroblasts phenotype in different cell types include fibroblasts, epithelial/endothelial cells, bone marrow derived monocytes etc., consequently, augments collagen deposition [Fonseca et al., 2011]. Whereas, PDGF is a potent mitogen and chemoattractant for fibroblasts/myofibroblasts for recuritment and proliferation of these cell types at sites of injury [Bonner, 2004]. Hence, in the present study, both ET-1 and PDGF signaling pathways may be contributing to significant elevation of gene/protien expression of α-SMA in lung tissues of bleomycin instilled mice as well as in kidney tissues of UUO mice, which was determined by immuno-histochemistry α-SMA. In this scienario, as both ET-1 and PDGF signaling pathways are responsible for deposition/proliferation of myofibroblasts (consists of α-SMA as a marker), combination therapy with both the inhibitors of these signaling pathways (bosentan and imatinib respectively) attenuated α-SMA expression more profoundly than individual agents to the extent of near additive efficacy.
These findings suggest that the combination treatment with bosentan and imatinib significantly decreased the proliferation and differentiation of fibroblasts/myofibroblasts and in turn, reduced collagen production in lung and kidney tissues. Decreased collagen-I and -III gene expression, decrease in hydroxyproline content in tissues, reduced collagen deposition reflected by Masson’s trichrome and picrosirus red stained in lung tissue sections in PF model and in kidney tissue sections in UUO kidney fibrosis model further confirmed that combination of both agents showed significant effect in amelioration of collagen deposition than either bosentan or imatinib to the extent of near additive efficacy. These findings were consistent with previous studies demonstration of bosentan inhibiting bleomycin induced PF [Park et al., 1997], renal interstitial fibrosis [Tian et al., 2003] renal vascular fibrosis [Boffa et al., 2001] and skin fibrosis [Kuhn et al., 2010].

Previous studies reported that combination of bosentan with valsartan ameliorates renal interstitial fibrosis by inhibiting the ECM accumulation [Zhang et al., 2005], Imatinib on the other hand, was shown to reduce bleomycin induced PF [Aono et al., 2005] and UUO induced renal fibrosis in vivo by blocking non-Smad TGF-β pathway [Wang et al., 2005]. This reduced ECM deposition in the present study is because of decreased myofibroblasts population. As mentioned before, TGF-β and CTGF are the key cytokines responsible for differentiation and proliferation of myofibroblasts. ET-1 and PGDF exerted their activity of myofibroblasts differentiation through TGF-β and CTGF. ET-1 is a downstream mediator of pro-fibrotic actions of TGF-β that induces CTGF, a stimulant of ECM production in mesenchymal cells such as fibroblasts and mesangial cells, suggesting that TGF-β and ET-1 act together to promote fibrogenesis through CTGF. Similar to ET-1, PDGF induces the chemotaxis and proliferation of myofibroblasts through ERK and PI-3 kinase signaling pathways. PDGF also stimulates the expression of CTGF through TGF-β1, suggesting that PDGF mediates collagen deposition through actions of TGF-β and CTGF. Hence, in the current study, simultaneous inhibition of ET-1 and PDGF signaling using bosentan and imatinib combination abrogates the action of TGF-β on differentiation of myofibroblasts thereby, decreasing the ultimate collagen deposition more effectively than individual treatment.

Fibrosis is characterized by an aberrant remodeling of ECM and disruption of basement membranes in which MMPs play substantial role and lead to disease progression. MMP-2 and MMP-9 are two major proteinases facilitating inflammatory or mesenchymal cell movement by disrupting the basement membrane [Tan et al., 2006].
A previous study reported that ET-1 enhanced MMP-9 and MMP-2 activities in osteosarcoma cells and human osteosarcoma tissue [Felx et al., 2006]. Another study illustrated that ET-1 signaling increased the expression and activity of MMP-2 and MMP-9 [Felx et al., 2006]. PDGF signaling also contribute to expression and secretion of MMP-2 and MMP-9 influencing monocyte migration [Wagsater et al., 2009]. Several studies have implicated elevated MMP-2 and MMP-9 levels in lungs and BALF of IPF patients [Suga et al., 2000] and in experimental animal models of PF [Kim et al., 2009]. MMP-2 and MMP-9 also play important role in kidney fibrosis through induction of tubular cell EMT [Cheng et al., 2003].

In present study, the results of gelatin zymography in PF model reiterated that bosentan or imatinib individually and more profoundly in combination, decreased bleomycin induced increase in MMP-9 and MMP-2 activities on day seven. Similarly, in UUO induced kidney fibrosis model combination treatment with bosentan or imatinib decreased the activities of MMP-2 and MMP-9 to a greater extent than individual treatment. These observations are consistent with previous reports that both bosentan [Chen et al., 2010] or imatinib [Blom et al., 2008] individually reduced both MMP-9 and MMP-2 activities. As both ET-1 and PDGF signalings are responsible for the expression and secretion of MMP-2 and MMP-9 in pathogenesis of fibrosis, this may be the reason why combination therapy with inhibitors of these signaling pathways exhibited additive efficacy in attenuation of MMPs activities.

Since, both ET-1 and PDGF signalings distinctively contributing to inflammatory and fibrotic processes in pathogenesis of lung and kidney fibroses, the drug combination may have paved the way for the enhanced efficacy in attenuation of these parameters. The results of current study explicitly emphasized the molecular interaction of both these drugs. Protease-activated receptor (PAR-1) is the cellular target of ET-1 and PDGF and is expressed in many cell types including fibroblasts which promote proliferation via autocrine production of PDGF [Datta et al., 2011]. Also, ET-1 induces CTGF production in myofibroblasts through PAR-1 [Kambas et al., 2011]. The observed additive efficacy could be due to modifications of downstream cellular targets of ET-1 such as CTGF, PAR-1 and cellular targets of PDGF such as PI3 kinase, Ras, FAK, c-raf, PLC-g and other known targets of PDGFR downstream pathway leading to modulation of STAT-1, JNK and ERK pathways which alter gene expression in the nucleus. These observations suggest that interference with pathways affecting different cellular targets of ET-1 and
PDGFR system is the key and more effective in modulating multiple cell components of different cells that contribute to fibrosis.

The results of this study indicate that bosentan and imatinib combination profoundly suppressed the interstitial inflammation and total collagen accumulation in lung and kidney tissues in respective animal models of fibroses. Quite intriguingly, the combination of both drugs used in the present study led to near additive efficacy in a majority of the parameters studied in inhibiting inflammation and fibrotic lesions in both lung and kidney fibroses models. Hence, this gross observation of the current study mainly highlights the importance of combination rather than monotherapy to encounter fibroses due to its heterogeneous etiology. Owing to the heterogenic nature of fibrosis, if some more biomarkers get identified and validated, polytherapy may be more efficacious than monotherapy for treatment of this dreadful disease. Hence, in future, such attempts of mutitherapy may also be optimised for the individual drug candidates for the maximum combination effect working out the proportions for a potential synergy.