CHAPTER 5
DISCUSSION

The increasing number of patients suffering from urolithiasis represents one of the major challenges which nephrologists face worldwide today. For enhancing therapeutic outcomes of this disease, the pathogenic basis for the formation of renal stones is the need of the hour. Kidney stones invariably are comprised of a combination of inorganic crystals and organic matrix. Proteins are found as major component in human renal stone matrix. Protein modulators of CaOx crystal nucleation and growth from different sources have been proposed to play an important role in kidney stone disease for several decades [Aggarwal et al. 2000; Aggarwal et al. 2005; Kaur et al. 2009; Bijarnia et al. 2009; Aggarwal et al., 2013c]. Identification of additional stone modulators (proteins) was hampered in the past by limitations in protein identification methods thereby making the identification of novel proteins quite difficult without prior knowledge of their involvement in this process. Recent advances in the technologies are therefore mandatory to study Calcium Oxalate (CaOx) crystallization modulators for better understanding of the pathophysiology and pathogenesis of kidney stone disease. Many proteins present in the organic matrix play an important role in kidney stone formation [Aggarwal et al., 2000; Aggarwal et al., 2013c; Maalouf, 2012] but till date only few of them are identified and fully characterized. Therefore, the purpose of the present study was to use bioactivity guided purification methods and recent advances in mass spectrometric protein identification to characterize and identify novel calcium oxalate crystal nucleation and growth modulators from the organic matrix of calcium oxalate human renal stones.

Proteins were extracted from the human renal calculi having calcium and oxalate as their major components to investigate the matrix proteins involved in the formation of calcium oxalate stone
process. Protein estimation of the EGTA extract, separated into >3 kDa and <3 kDa fractions was done by Lowry's method. It was found that the protein concentration of the whole extract was 296.65 μg/ml, while that of >3 kDa and <3 kDa was 192.31 μg/ml and 101.52 μg/ml respectively. The bioactivity of whole extract, >3 kDa and <3 kDa fractions was investigated through CaOx crystal nucleation and growth assay systems. The whole extract contained both stimulatory and inhibitory activity against CaOx crystal nucleation where as stimulatory activity was observed against CaOx crystal growth. The >3 kDa fraction showed stimulatory activity towards CaOx crystal nucleation and inhibitory activity towards CaOx crystal growth assay system. The <3 kDa fraction showed stimulatory activity towards CaOx crystal nucleation whereas both stimulatory and inhibitory activity was observed towards CaOx crystal growth assay system. The above observations support the fact that the role of matrix proteins is different in the formation of the stone centre and in the subsequent build up of the stone [Khan and Hackett, 1986]. The same protein which inhibits crystal nucleation might promote the growth of the crystal. Thus, the same protein acts differently at different stages of stone formation.

Based on the above observations, the >3 kDa fraction exhibiting highest activity against CaOx crystal nucleation and growth assay and was thus chosen for further investigating its bioactivity against oxalate injured MDCK renal epithelial cells. The >3 kDa fraction majorly reflected promoter activity thereby leading to an increased cell death in a dose dependent manner in the presence of oxalate. These results suggest that the proteins which are promoters of crystallization in nature mask the activity of the proteins which are inhibitory in nature, thereby leading to an enhanced cell injury, and consequently cell death. The observations are in conformity with the observations that that renal epithelial damage can lead to increased crystal attachment. The studies indicate that the mixture of >3 kDa proteins in the matrix of human renal stones augment the renal epithelial cell injury induced by oxalate and thereby may act as promoters of calcium oxalate crystal nucleation and growth. The effect of promoters masks the inhibitors in the protein mixture thereby leading to enhanced renal cell injury. Therefore, any crystallization that occurs most certainly is facilitated by promoters and our results are in conformity with the above facts.
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1D SDS PAGE and 2D SDS PAGE of >3 kDa proteins suggests that plentiful proteins are present in the matrix proteins. A total of 66 spots were detected in the 2D SDS MAP which are of both high and low molecular weight. Also both anionic and cationic proteins are present. Seven most prominent spots spread across the isoelectric points (pI) were further analysed using MALDI-TOF MS and MASCOT server. The identified proteins are Disheveled-associated activator of morphogenesis, Glutamate receptor delta-1 subunit, Caspase recruitment domain-containing protein, Albu Bovin, Chymotrypsinogen A, Plasminogen, VP7 glycoprotein precursor.

More than 3 kDa which exhibiting highest activity on CaOx crystal nucleation and growth assay was subjected to chromatography for purifying proteins using a strong cation and anion exchanger individually. The purification was performed systematically using cation/anionic and molecular-sieve chromatography followed by bioactivity testing against CaOx crystal nucleation and growth assay and SDS-PAGE analysis after each purification step. After conducting anion exchange chromatography it was found that the eluted fractions P3, P4 and P5 exhibited inhibitory activity against CaOx crystal nucleation system, while P3 exhibited inhibitory activity and P4, P5 exhibited promoter activity towards CaOx crystal growth assay system. SDS-PAGE analysis showed presence of few bands in each fraction. After these fractions P3, P4 & P5 were purified individually by molecular-sieve chromatography; the purified proteins obtained from each fraction were tested for their bioactivity against CaOx crystal nucleation and growth. The most potent fractions MP1 and MP2 obtained from purification of P3 possessed promoter activity against both CaOx crystal nucleation and growth assay system. NP1 and NP2 were the most potent fractions obtained from purification of P4. NP1 possessed inhibitory activity against both CaOx crystal nucleation and growth and NP2 contained inhibitory activity against both CaOx crystal nucleation and promotery activity against CaOx crystal growth assay system. The most potent fractions OP1 obtained from purification of P5 possessed inhibitory activity against both CaOx crystal nucleation and growth. When the effect of these purified proteins was tested on oxalate injured Madin Darby Canine Kidney (MDCK) renal epithelial cells for their activity,
it was found that MP1, MP2, NP2 enhanced cell injury whereas NP1 and OP1 diminishes the injury caused by oxalate. The purified potent protein fractions MP1, NP2 showed the presence of single band of ~44 kDa & ~38 kDa respectively when analyzed by 10% SDS PAGE. Whereas the fractions MP2, NP1 & OP1 showed the presence of multiple bands when analysed by 10% SDS PAGE, but showed the presence of single bands when further analyzed by NATIVE PAGE of MW ~181kDa, ~175kDa, ~183kDa respectively. The homogeneity of the purified fractions was confirmed on RP-HPLC which showed a single peak. Analysis of fractions MP1, MP2, NP1, NP2 & OP1 by MALDI-TOF-MS resulted in peptide mass fingerprint which when followed by database search on a MASCOT server matched significantly with Ethanolamine-phosphate cytidylyltransferase, Ras GTPase-activating-like protein, UDP-glucose:glycoprotein glucosyltransferase 2, Macrophage-capping protein, RIMS-binding protein 3A. Our finding suggest presence of the above mentioned, as novel anionic proteins present in the matrix of CaOx stones with capability to modulate CaOx crystallization.

The identified Ethanolamine-phosphate cytidylyltransferase as a promoter protein which promotes calcium oxalate crystal nucleation and growth. Also, it was observed that this protein enhances the injury caused by oxalate to renal epithilum cells (MDCK) thereby leading to cell death. Ethanolamine-phosphate cytidylyltransferase (EC 2.7.7.14) catalyzes the rate-controlling step in a major pathway for the synthesis of phosphatidylethanolamine (PtdEtn) [Gibellini and Smith, 2010; Bladergroen and Golde, 1997]. S R Khan et al. have reported that calcium oxalate stones contain cardiolipids, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol in a lipid extract [Khan et al., 1996; Khan et al., 2002]. Endogenous oxalate synthesis makes an important contribution to the amount of oxalate excreted in urine and hence leading to the development of calcium oxalate kidney stones. Lange et al. have proposed that glyoxal metabolism may be an important contributor to urinary oxalate excretion. In addition, it has been suggested both glyoxal production and oxalate synthesis can be associated with oxidative stress. The glyoxal can be generated endogenously by various sources including, the catabolism of carbohydrates, proteins,
fats and metabolism of glycine, ethanolamine, glycolaldehyde and ascorbic acid. The ethanolamine in turn can be either derived from diet or by the hydrolysis of PE. Therefore, taking cue from the results of cell viability studies, thus it can be suggested that this protein; Ethanolamine-phosphate cytidylyltransferase probably plays a crucial role in promoting stone formation, which is also reflected by the presence of PE in the stone lipid content.

The second protein which is Ras GTPase-activating-like protein IQGAP2 is a protein that in humans is encoded by the IQGAP2 gene [Weissbach et al., 1994; Li et al., 2005]. The protein contains three IQ domains, one calponin homology domain, one Ras-GAP domain and one WW domain. It interacts with components of the cytoskeleton, with cell adhesion molecules and with several signaling molecules to regulate cell morphology and motility [Brandt and Grosse, 2007]. This protein also associates with calmodulin, a calcium-binding messenger protein which is expressed in all eukaryotic cells. It contains four EF-hand motifs, each of which binds a Ca^{2+} ion [Brill et al., 1996; Stevens, 1983]. Also Ras GTPase-activating-like protein IQGAP2 could serve as an assembly scaffold for the organization of a multimolecular complex that would interface incoming signals to the reorganization of the actin cytoskeleton at the plasma membrane [Fukata et al., 1997]. This protein is expressed in the placenta, lung, and kidney. It was found that Ras GTPase-activating-like protein IQGAP2 promotes calcium oxalate crystal nucleation and growth. Also, it was observed that this protein enhances the injury caused by oxalate to renal epithilium cells (MDCK) thereby leading to cell death. This is in accordance with the fact that this protein contains an IQ domain which interacts with calmodulin which is known to bind with Ca^{2+} ions thereby increasing the probability of stone formation. In addition, by down regulating the ras pathway this protein would lead to decreased cell survival and enhanced apoptosis, which has also been demonstrated in the study.

The third protein was identified as UDP-glucose:glycoprotein glucosyltransferase 2 (UGGT) is an enzyme that resides in the endoplasmic reticulum (ER). UGGT recognizes incompletely
folded glycoproteins with N-linked Man9-GlcNAc2 glycans, and transfers a glucose residue from UDP-glucose to produce the Glc1-Man9-GlcNAc2 glycan [Parodi, 2000]. This monoglucosylated glycan is recognized specifically by the lectin-like chaperones calnexin and calreticulin, which retain incompletely folded glycoproteins in the ER and recruit ERP57 to accelerate disulphide bond interchange for further folding [Tessier et al., 2000; Zapun et al., 1997; Zapun et al., 1998; Schrag et al., 2001]. Higher levels of this enzyme have been found in kidney, pancreas, heart, and skeletal muscle. It has been observed that urolithiasis can also be a result of misfolding of proteins like, THP [Weichhart et al., 2005]. UDP-glucose: glycoprotein glucosyltransferase 2 adds glucose residues to these misfolded proteins allowing calnexin and calreticulin to bind these proteins thereby, preventing their transport from ER to Golgi and ultimately sends these proteins for degradation [Arnold et al., 2000; Bazan, 1990]. Therefore, it is probable that this enzyme present in the renal stone matrix could be an adaptive response and thereby prevent further injury. Indeed it was seen that cells treated with this protein were rescued from oxalate induced injury.

The fourth protein was identified as RIMS-binding protein 3A which belongs to the RIMBP family. RIM-binding proteins (RIM-BPs) were identified as binding partners of the presynaptic active zone proteins RIMs as well as for voltage-gated Ca\(^{2+}\) channels. They were suggested to form a functional link between the synaptic-vesicle fusion apparatus and Ca\(^{2+}\) channels. RIM-BP gene family diversified in different stages during evolution, but retained their unique domain structure. RIM-BP3 is exclusively expressed in mammals. All RIM-BP genes encode proteins with three SH3-domains and two to three fibronectin type-III domains. The Fibronectin type III domain is an evolutionary conserved protein domain that is widely found in animal proteins [Tsujihata, 2000]. Fibronectin type III domain has been found to be involved in regulation of wound healing in tumor fibrosis which is considered as a dysregulated wound healing response. Fibronectin which has been earlier reported as an inhibitor of stone formation in urolithiasis contains 16 copies of this domain [Tsujihata, 2000]. Thus, the inhibitory activity of RIMS-binding protein 3A can be related with fibronectin by virtue of having the same domain i.e. Fibronectin type III domain.
The fifth protein viz. Macrophage-capping protein in humans is encoded by the CAPG gene found in macrophages and macrophage-like cells. This protein is a member of the gelsolin/villin family of actin-regulatory proteins [Dabiri et al., 1992]. It reversibly blocks the barbed ends of F-actin filaments [DiNubile et al., 1995] in a Ca\(^{2+}\) and phosphoinositide-regulated manner, thus contributes to the control of actin-based motility in non-muscle cells [Young et al., 1994; Pellieux et al., 2003]. Macrophages play a role in phagocytosis, or engulf and then digest, cellular debris and pathogens. These cells play a central role in protecting the host and also contribute to the pathogenesis of inflammatory and degenerative diseases. It has been reported that crystals of calcium oxalate monohydrate (COM) that adhere to the cell surface can be internalized by endocytosis [Lieske et al., 1992; Lieske and Deganello, 1999]. Macrophages and other inflammatory cells can take care of these crystals and destroy them [Khan et al., 1996]. Large crystal masses with or without insufficient protection by macromolecules will adhere to the tubular cell. The crystals might alter the plasma membrane so that endocytosis occurs; whereas crystals of reasonable size can be taken care of and destroyed by the cell, larger crystal agglomerates might cause cell death [Koul et al., 1996]. When the crystals that are bound to the basolateral membrane or deposited interstitially are too large, the capacity of macrophages and inflammatory cells will probably be insufficient to cope with the crystals. This protein Macrophage-capping protein was found to have a dual activity wherein on one hand this protein inhibits CaOx nucleation, on the other hand this protein promotes CaOx growth. Such proteins which can play different roles at different stages of stone formation like THP are already reported [Jaggi et al., 2007; Sumitra et al., 2005]. In this study one such identified protein with dual activity which was found to be present in macrophages; which play a vital role in stone formation.

On the other hand, after conducting cation exchange chromatography it was found that the eluted fractions F3 & F4 fraction exhibited inhibitory activity against CaOx crystal nucleation and growth both. Further its SDS-PAGE analysis showed presence of few bands. The fractions F3
and F4 were further purified by molecular sieve chromatography. Fractions were collected and pooled as F1', F2' and F3' and examined for inhibitory activity against CaOx crystal nucleation and growth. All the three fractions showed the presence of single bands when analyzed by 10% SDS-PAGE having molecular weight ~53kDa, ~44kDa, ~42kDa. When the effect of these purified proteins was tested on oxalate injured Madin–Darby Canine Kidney (MDCK) renal epithelial cells for their activity, it was found that F1', F2' & F3' diminished the injury caused by oxalate. The homogeneity of the purified fractions F1', F2' and F3' was confirmed on RP-HPLC which showed a single peak respectively. Analysis of fractions F1', F2' and F3' by MALDI-TOF-MS resulted in peptide mass fingerprint which when followed by database search on a MASCOT server matched significantly with Histone-Lysine N-Methyltransferase, Inward Rectifier K Channel and Protein Wnt-2.

Histone-lysine N-methyltransferase is an enzyme which is encoded by the EZH2 gene in humans and belongs to family of histone modifying enzymes [Khan et al., 1984; Mushtaq et al., 2007]. This gene encodes a member of the Polycomb-group (PcG) family. PcG family members form multimeric protein complexes, which are involved in gene silencing over successive cell generations. Histone-lysine N-methyltransferase contains 1 SET Domain. Protein lysine methylation by SET Domain enzymes regulates chromatin structure [Thamilselvan and Khan, 1998]. There are reports that renal ischemia–reperfusion injury upregulates histone-modifying enzyme systems and alters histone expression at proinflammatory/profibrotic genes [Coe and Parks, 1990]. EZH2 acts as a key regulator of hematopoiesis involved in terminal myeloid differentiation and in the regulation of hematopoietic stem cell (HSC) self-renewal by a mechanism that involves DNA methylation. The highest levels of expression were observed in fetal thymus and kidney. The product of this enzyme acts on the substrate S-adenosyl-L-methionine (SAM) and histone L-lysine to produce S-adenosyl-L-homocysteine (SAH) and histone N (6)-methyl-L-lysine. (SAH) is a sensitive indicator of risk for cardiovascular disease and for renal disease. Overproduction of SAM leads to oxidative stress in kidney. Renal stone patients have been diagnosed with ischemic renal necrosis and fibrosis which are influenced by renal stone size.
The second protein Inward rectifying K channels (Kir) are now recognized as forming a superfamilly of seven members (Kir1 to Kir7) [Chauvet and Ryall, 2005]. Members of this channel family play critical roles in cellular signaling processes regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume regulation. Naturally occurring mutations in various K1 channels have been identified in Bartter's syndrome (severe salt-wasting, hypokalemic alkalosis and hypercalciuria.) [Lieske et al., 1996]. Of the 5 major genetic variants associated with Bartter syndrome [Chen et al., 1996], cortical and medullary renal calcifications [Fiskus et al., 2006] are found in patients with Bartter syndrome types I and II, which arise from hypercalciuria. Loop diuretic treatment of premature babies, a pharmacological analog of the reduced TAL reabsorption seen in Bartter syndromes, can cause renal calcifications, 50% of which regress over time [Cao et al., 2002].

The third protein, Wnt-2 protein belongs to a Wnt family. The Wnt gene family, which encodes secreted growth and differentiation factors, has been implicated in kidney organogenesis. The Wnts control both ureteric bud development and signaling, but they also serve as inductive factors to regulate nephrogenesis in the mesenchymal cells. Several of the Wnt genes are expressed in the developing kidney, and gene knock-out studies have revealed specific developmental functions for these. Consistent with this, changes in Wnt ligands and pathway components are associated with many kidney diseases, including kidney cancers, renal fibrosis, cystic kidney diseases, acute renal failure, diabetic nephropathy and ischaemic injury. Wnt signalling is essential for renal development; however, the specific molecular underpinnings involved are poorly understood. Recent research has revealed an unexpected intersection between Wnt signaling and polycystic kidney disease. Some polycystic kidney disease proteins, such as Inversin and Bardet–Biedl syndrome family members, were found to use components of the Wnt signaling cascade to orient cells along a secondary polarity axis within the plane of the epithelium. These spatial cues may be needed to position nascent tubules with a defined
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geometry [Trievel et al., 2002]. Another study revealed the mRNA levels of Wnt-2 in unilateral ureteral obstruction (UOO) kidneys. Kida et al. were first to report the level of Wnt-2 mRNA in fibrous kidneys. They revealed that the Wnt-2 mRNA level was increased 3 days after UOO expression levels of Wnt-2 mRNA in UOO day 7 and UOO day 10 kidneys were 16.5 times and 32.6 times as high as those in control kidneys, respectively [Zagcr and Johnson, 2009]. WNT2 gene is one of the targets for pharmacogenomics in the field of oncology. Wnt-2 acts as a tumor marker of gastric and colorectal cancers. Because WNT2 up-regulation leads to carcinogenesis through activation of the WNT-β catenin pathway, WNT2 specific antagonist might be applied for chemoprevention or treatment of cancer [Fiskus et al., 2006].

The finding suggest presence of Histone lysine N methyl transeferase, Inward Rectifier K channel and protein Wnt2 as cationic proteins present in the matrix of CaOx stones which possess antilithiatic activity. It is known that damaged cell membrane enhances the binding of calcium oxalate monohydrate crystals to anionic membrane because crystal binding sites could be unmasked when cells are injured while under physiological conditions these sites are minimally exposed [Gill et al., 1982; Khan et al., 1984]. Thus it might be possible that Histone lysine N methyl transeferase, Inward Rectifier K channel and Protein Wnt2 attaches to the anionic side of the membrane which is exposed after injury, possibly in hyperoxaluric condition [Mushtaq et al., 2007]. Presence of Histone lysine N methyl transeferase, Inward Rectifier K channel and Protein Wnt2 in the matrix of CaOx stones, suggest that it may bind to membrane phospholipids, exposed after injury, hence, playing a role in stone genesis.

Of all types of renal stones, calcium oxalate (CaOx) is the most common composition found by chemical analysis [Coe et al., 1992]. Modulators of CaOx crystallization (proteins, lipids, glycosaminoglycans, and inorganic compounds) have been proposed to play an important role in renal stone disease [Zerwekh et al., 1983]. During the last few years, more and more research has been done at the cellular and molecular levels. In spite of these advances however, the clinical
The attention was focused on the interaction of the purified proteins: Ethanolamine-phosphate cytidylyltransferase, Macrophage-capping protein, Inward rectifier K channel and Histone lysine N methyltransferase with calcium oxalate using in silico molecular docking studies. In silico results revealed that both the acidic and basic amino acids in the binding site are essential for molecular interactions of CaOx with these purified proteins. Whether a protein or another macromolecule acts as an inhibitor of growth and aggregation or a promoter of nucleation and aggregation implies that there must be some mechanism to explain the interaction with the mineral oxalate surfaces. Determining the molecular mechanisms by which urinary constituents modulate calcium oxalate crystallization is crucial for understanding and controlling urolithiassi in humans. More negative the docking score, stronger is the binding between ligand and protein’s active site [Bijarnia et al., 2008]. The strong interaction between active sites of purified proteins and calcium oxalate predicts inhibition of the same. High docking scores with the active sites of proteins indicates good binding of modeled protein with the ligand calcium oxalate. LIGPLOTS of all the proteins showed involvement of various (acidic and basic) amino acids calcium oxalate. The interaction between calcium and acidic amino acids is certainly plausible,
but it is equally conceivable that basic residues that are normally protonated at urinary pH and positively charged might experience an attraction toward negatively charged oxalate groups. In either case steric constraints from 3D conformation of the molecule might limit the number of these simple interactions. This was further substantiated when substitution of basic and acidic acidic amino acids with alanine and upon phosphorylation, the interaction of the proteins with calcium oxalate was altered.

Among all types of kidney stones, the frequency of calcium stone is 70–80%, struvite stone 5–10%, uric acid stone 5–10%, and cystine stone 1% [Morton and Wooltorton, 2002]. Calcium oxalate is the primary component of 70–80% of calcium stones with calcium phosphate being the predominant component in the rest of calcium stones. The predominant proteins found in organic matrices of CaOx crystals induced in the urine of healthy controls were prothrombin related proteins followed by albumin and osteopontin. Osteopontin (OPN) and Tamm-Horsfall protein (THP) are two major urinary macromolecules that exhibit various activities that can influence calcium crystallization in vitro [Devuyst et al., 2005; Kumar and Licske, 2006]. OPN is a ubiquitously expressed phosphoglycoprotein that regulates bone biomineralization and ectopic calcification [Giachelli and Steitz, 2000].

In the present study, Histone-lysine N-methyltransferase, Inward rectifier K channel, Protein Wnt-2, Ethanolamine-phosphate cytidylyltransferase, Ras GTPase-activating-like protein, UDP-glucose:glycoprotein glucosyltransferase 2, RIMS-binding protein 3A and Macrophage-capping protein were identified as a novel modulators of CaOx crystallization from human renal stone matrix. The selective mineralization process could be attributed to certain biomolecules present in the body fluids which can control the mineralization process by acting as either inhibitors or
promoters of mineralization [Tandon et al., 1999]. As there are proteins biomolecules reported in kidney stone which are involved in inhibiting kidney stone formation as well as in stimulating the same [Aggarwal et al. 2000; Aggarwal et al. 2005].