Clinica Chimica Acta 415 (2013) 181–190

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Novel antilithiatic cationic proteins from human calcium oxalate renal stone matrix identified by MALDI-TOF-MS endowed with cytoprotective potential: An insight into the molecular mechanism of urolithiasis

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ARTICLE INFO

Article history:
Received 17 August 2012
Received in revised form 10 October 2012
Accepted 10 October 2012
Available online 2 November 2012

Keywords:
Calcium oxalate (CaOx)
Madin–Darby Canine Kidney (MDCK)
Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-MS)
Antilithiatic
Calcium oxalate monohydrate (COM)
Urolithiasis

ABSTRACT

Background: No substantial work has been conducted to date in context to cationic proteins with antilithiatic activity. We explored the antilithiatic cationic proteins present in human calcium oxalate (CaOx) stones and also examined their molecular interactions with calcium oxalate crystals in silico.
Methods: Proteins were isolated from the matrix of human CaOx containing kidney stones. Proteins having MW > 3 kDa were subjected to cation exchange chromatography followed by molecular-sieve chromatography. The effect of these purified cationic proteins was tested against CaOx nucleation and growth and on oxalate injured MDCK cells for their activity. Proteins were identified by MALDI-TOF MS. Molecular interaction studies with COM crystals in silico were also investigated.
Results: Three antilithiatic cationic proteins were identified as histone-lysine N-methyltransferase, inward rectifier K channel and protein Wnt-2 (MW ~ 53, ~44, and ~42 kDa respectively) by MALDI-TOF MS based on database search with MASCOT server. Further molecular modeling calculations revealed the mode of interaction of these proteins with CaOx at the molecular level.
Conclusion: We identified histone-lysine N-methyltransferase, inward rectifier K channel and protein Wnt-2 as novel antilithiatic proteins which play a vital role in the kidney function and have been associated with various kidney diseases.

1. Introduction

Urolithiasis, the deposition of stones in urinary tract has plagued man since long and continues to pose a universal health problem even today. Urolithiasis is a relevant clinical problem in everyday practice with a subsequent burden for the health system. It is classically explained as the derangement in the process of biomineralization involving the equilibrium between promoters and inhibitors of crystallization: a deficit of one or several inhibitors or an excess of one or several promoters plays a pivotal role in the stone formation. The revolutionary beginning of the molecular biology in medicine has given a new insight in urolithiasis [1].

Histological and archeological studies have clearly revealed that ancient man suffered from urinary tract stone disease [2]. The earliest evidence dates back to around 4800 B. C. when a bladder stone was found among the pelvic bones of a young predynastic Egyptian. The incidence of urolithiasis appears to have been generally increasing over the last 100 y, particularly in countries which have either hot climate or which have moved from an agriculture economy to one based on industrial and technological development [3]. It is estimated that at least 10–12% of the population in the industrialized part of the world is afflicted by urinary tract stone disease [4]. The so called stone belts of the world are located in the countries of the Middle East, North Africa, Mediterranean regions, North-western state of India and Southern states of US [5]. As far as India is concerned two distinct stone belts having very high incidence of urinary calculi have been identified. The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U.P. The other belt which starts from Jamnagar in west coast extends inwards towards Jabalpur in central India. Very low incidence areas have been in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh [6].

Urolithiasis remains a chronic disease and our fundamental understanding of the pathogenesis of stones as well as their prevention and cure, remains rudimentary. With its multifactor etiology and high rate of recurrences, urinary tract stone disease provides a medical challenge. It is necessary to identify risk factors that might be of

Abbreviations: CaOx, Calcium Oxalate; MDCK, Madin–Darby Canine Kidney; COM, Calcium Oxalate Monohydrate.

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http://dx.doi.org/10.1016/j.cca.2012.10.040

etiological importance and thus get some clues for predicting the further course of the disease. The predominance of proteins in the organic matrix of calculi suggests their significance in stone formation [7]. However, although the presence of these proteins may imply that they fulfill some function in stone formation, it is equally possible that their inclusion in the stone structure may simply have been fortuitous or may have resulted from the injurious effects of the stone itself. Although some proteins have been reported from the renal stone matrix, but the study of function of only few has been done. Organic matrix proteins which have been reported to play a significant role in urolithiasis are mostly anionic in nature. Only a few proteins are reported to be cationic in nature. Stone recurrence in human beings cannot be predicted and is beyond the control of urologists, mainly because the mechanism of stone formation at molecular level is not yet fully understood [8].

2. Materials and methods

2.1. Human renal stone collection

Surgically removed human renal calculi, from the kidney stone patients were obtained from the Department of Urology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Stones were of noninfectious nature and were collected from those patients who were >25 y and were suffering from no other abnormality. After FTIR analysis, the stones with calcium and oxalate as their major components were selected for present study.

2.2. Protein extraction from human renal stones

Proteins were isolated from the matrix of kidney stones containing CaOx as the major constituent using EGTA as a demineralizing agent. Stones were washed in 0.15 mol/l NaCl with gentle stirring for 48 h to remove the adhered blood, tissue etc. They were then dried and pulverized with a mortar and pestle. For extraction of the organic matrix of powdered stone; each gram of stone was suspended in 10 ml of 0.05 mol/l EGTA, 1 mmol/l PMSF and 1% β-mercaptoethanol. The extraction was carried out for 4 days at 4 °C with constant stirring. The suspension was centrifuged for 30 min at 10,000 ×g and at 4 °C. The supernatant of EGTA extract was filtered through Amicon ultra centrifugal filter device with a molecular weight cut-off of 3 kDa at 4 °C and concentrated to a known volume. Whole EGTA extract and greater than 3 kDa fractions were stored at −20 °C for further studies [7].

2.3. Protein determination

Total protein concentration was determined by Lowry's method using BSA as a standard [9].

2.4. Assay to measure inhibitory activity of protein w.r.t CaOx crystal nucleation

The method used was similar to that described by Hennequin et al. with some minor modifications [10]. Solutions of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) were prepared at the final concentration of 3 and 0.5 mmol/l, respectively, in a buffer containing Tris 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5. Both solutions were filtered through a 0.22 μm filter; 1.5 ml of $CaCl_2$ solution was mixed with different concentrations of extracted proteins. Crystallization was started by adding 1.5 ml of $Na_2C_2O_4$ solution. The final solution was stirred at 37 °C repeatedly after an interval of 60 s for 8 min. The absorbance of the solution was monitored at 620 nm after every 60 s. The percentage inhibition produced by the protein extract was calculated as $[1-(Tsi/Tsc)]\times 100$, where Tsc was the turbidity slope of the control and Tsi the turbidity slope in the presence of the inhibitor.

2.5. Assay to measure activity of protein w.r.t. CaOx crystal growth

Activity against CaOx crystal growth was measured using the seeded, solution-depletion assay [11]. Briefly, CaOx crystal seed (from FTIR identified clinical kidney stones) slurry, 1.5 mg/ml was added to a solution containing 1 mmol/l $CaCl_2$ and 1 mmol/l $Na_2C_2O_4$. The reaction between $CaCl_2$ and $Na_2C_2O_4$ would lead to deposition of CaOx on the crystal surface leading to the depletion in free oxalate that is detectable spectrophotometrically at 214 nm. When a protein is added into this solution, the rate of depletion of free oxalate will decrease if the protein inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after incubation with or without protein. The relative inhibitory activity was calculated as follows: % Relative inhibitory activity $=[(C-S)/C]\times 100$, where C is the rate of reduction of free oxalate without any test protein and S is the rate of reduction of free oxalate with a test protein.

2.6. Cell culture

Madin–Darby Canine Kidney (MDCK) cells were obtained from the National Centre of Cell Sciences (NCCS, Pune). The cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) with 2.0 mmol/l L-glutamine adjusted to contain 3.7 g/l sodium bicarbonate and 4.5 g/l glucose. Media was supplemented with 1% penicillin (100 units/ml)–streptomycin (10,000 μg/ml) and 10% fetal bovine serum. Cells were cultured in 25 cm^2 tissue-culture treated flasks at 37 °C and 5% CO_2 in humidified chambers [12].

2.7. Oxalate-induced cell injury

MDCK cells were incubated in DMEM containing 1 mmol/l sodium oxalate in the presence of different concentrations of protein samples for 72 h [13,14]. Cell injury was assessed by measuring the cell viability through monitoring the lactate dehydrogenase (LDH) leakage into the medium.

2.8. Preparation of the protein samples

For cell culture studies, the proteins was dialyzed through Millipore Amicon Ultra Centrifugal Filters, 3 kDa (Catalog UFC 800324) and desalted by ReadyPrep 2-D Cleanup Kit (catalog 163-2130) and was reconstituted in 0.22 μ filtered distilled water using Millipore Millex GV Filter Unit 0.22 μm (Catalog SLGU033RS). This was treated as a stock solution of the proteins.

2.9. LDH leakage assay

MDCK cells were suspended in DMEM with serum and plated into the microwells of 96-well tissue culture plates. Plates were incubated for 24 h at 37 °C in a humidified incubator containing 5% CO_2. Then the medium was removed from the wells. Two hundred microliters of DMEM (without serum) containing different concentrations of proteins with and without sodium oxalate were added into the wells. Lactate dehydrogenase (LDH) leakage assay was performed by the LDH Cytotoxicity Assay Kit (Cayman Chem. Co., Ann Arbor, MI) according to the manufacturer's instructions [15].

2.10. Statistical analysis

Data were expressed as mean values of 3 independent experiments and analyzed by the analysis of variance ($p<0.05$) to estimate the differences between values.

2.11. SDS-PAGE

Mini-Protean III apparatus (Bio-Rad Laboratories) was used for SDS-PAGE analysis. Lyophilized samples were reconstituted in reducing sample buffer and analyzed by one-dimensional discontinuous SDS-PAGE using 1 mm thick, 10% separating and 4.4% stacking gels. Protein bands were stained with silver using ProteoSilver™ Plus Silver Stain Kit (PROTSIL2, Sigma-Aldrich Co.). Broad range and low range molecular weight markers (catalog # 161-0317, # 161-0304 Bio-Rad) were used as standards [16].

2.12. Purification and characterization of proteins

More than 3 kDa EGTA fraction exhibited significant activity on CaOx crystal nucleation and growth assay system, therefore it was subjected to strong cation exchanger Macro Prep® High 25S (Bio-Rad, Hercules, CA). The column (50×1 cm) was previously washed and equilibrated with 20 mmol/l MOPS buffer with 0.1 mmol/l NaCl (pH 7.4). Bound proteins were eluted by incorporating a linear concentration gradient of NaCl (0.1–1 mol/l) in the column buffer while keeping the pH constant at a flow rate of 0.5 ml/min. All fractions were monitored for protein content (A280) and simultaneously their conductivity was measured. Fractions coming under the peak were pooled and dialyzed and their bioactivity was studied. Fraction showing the highest bioactivity was concentrated and loaded on a Bio gel® P-100 gel molecular sieve column (50×1 cm) equilibrated and eluted with the 20 mmol/l Tris buffer (pH 7.4) at a flow rate of 0.1 ml/min [17]. The fractions which eluted out based on their molecular weights were pooled to study their w.r.t. CaOx crystal nucleation and growth as well as on oxalate induced injury on MDCK cells.

2.13. Tryptic in-gel digestion of purified protein

Single band detected after molecular-sieve chromatography was excised from the gel and was destained with destainer provided in the ProteoSilver™ Plus Silver Stain Kit (Sigma-Aldrich Co., St. Louis, MO). Trypsin profile IGD kit (Sigma-Aldrich Co.) was used for in-gel digestion of purified protein. Destained gel piece was dried for approximately 15 to 30 min. Trypsin solubilized in 1 mmol/l HCl and mixed with 40 mmol/l ammonium bicarbonate and 9% acetonitrile was added to the destained gel piece. Gel piece was fully covered by the addition of 40 mmol/l ammonium bicarbonate and 9% acetonitrile (pH 8.2) solution and was incubated for 5 h at 37 °C. After the incubation, liquid was removed from the gel piece and transferred to a new labeled Eppendorf tubes and was preserved for mass spectroscopic analysis [18].

2.14. Peptide mass fingerprinting by MALDI-TOF-MS

Each proteolytic sample was premixed 1:2 with the matrix solution (α-cyano-4-hydroxycinnamic acid) and spotted on the sample stage. It was dried at room temperature then washed with 0.1% TFA and was analyzed by Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectrometer was calibrated by peptide calibration standard II (Bruker). Acquired mass spectra had a resolution of ~6000 (FWHM), which was sufficient to identify the digested peptide. The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com) using all the 3 databases (MSDB, SwissProt, NCBInr). For search, peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. A *Homo sapiens* taxonomy restriction was used, only one missed cleavage was allowed, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.

2.15. Amino acid analysis

Total amino acid content in the protein was estimated after acid hydrolysis of purified protein sample by the method of Elkin et al. [19]. Derivatization of free amino acid after hydrolysis was performed by phenylisothiocyanate (PITC) and loaded on silica based column Pico Tag (Waters, Milford, MA; 3.9 mm×15 cm). Elution was done under high pressure using a gradient of using a gradient of sodium acetate trihydrate in 6% acetonitrile and 60% acetonitrile. The detection was done at a wavelength of 254 nm.

2.16. Homology modeling and structure validation

Homology model building of the identified histone lysine N methyltransferase, inward rectifier K channel and Wnt-2 protein structure was done using molecular operating environment (MOE) [8,20]. The template used for model building of histone lysine N methyltransferase protein was the crystal structure of variegation 4–20 homolg-2 suppressor protein (PDB ID: 3RQ4A). Similarly the crystal structure of Kir 2.2 R186A, a mutant of inward rectifier potassium channel (PDB ID: 3SPGA) was used as a template for model building of inward rectifier K channel protein. Due to lack of significant similarity with any protein from the PDB database for Wnt-2 protein, homology model could not be built. The modeled structures were further refined using molecular dynamics simulation (MD). Gromacs 7.0.1 was used for MD simulation. The quality of built structures was checked with PROCHEK and ERRAT.

2.17. Docking molecular docking of calcium oxalate

Calcium oxalate structure was built and geometrically optimized with the help of molecular builder of Molecular Operating Environment (MOE) package developed by the Chemical Computing Group Inc. Montreal, Canada. Active site of 3RQ4A and inward rectifier K channel protein was predicted by using active site finder tool of MOE software. Molecular docking of calcium oxalate was done using MOE-Dock. MOE-Dock utilizes a Monte Carlo Simulated Annealing (SA) method in docking calculations to search for favorable binding configurations of a small, flexible ligand and a rigid macromolecule in a pre-set box. The docking energy calculation was carried out within a user-specified three-dimensional docking box (3D docking box) using the simulated annealing method under the OPLS-AA force field. The energy grids for docking were generated as grid-based potential fields by the MOE-Dock program. Docking energy was calculated as the sum of the electrostatic, van der Waals, and flexibility energies. The van der Waals parameters were taken from the force field. The electrostatic field was calculated based on force field in the Coulombic manner using the constant dielectric of 1.0 for solvation. MOE-Dock performed 25 independent docking runs and the lowest docking energy conformation for each active site was chosen for LIGPLOT [21].

2.18. Molecular interaction of calcium oxalate and inhibitor proteins

To investigate the precise interaction between calcium oxalate and inhibitor proteins (histone lysine N methyltransferase and inward rectifier K channel) identified in this study we have mutated the amino acids in the binding site. Precisely all the acidic and basic amino acids were mutated to alanine and the amino acids such as tyrosine, threonine and serine in the active site were phosphorylated. After incorporating these mutations, calcium oxalate was docked into the binding site using MOE-Dock with similar parameter as was used for wild type.

3. Results

3.1. Activity study of renal stone extract on COM assay system

Whole EGTA extract, greater than and less than 3 kDa fractions were assayed to measure activity against CaOx crystal nucleation and growth. Whole EGTA extract (296.65 μg/ml protein) as well as greater than 3 kDa fraction (192.31 μg/ml protein) exhibited significant inhibitory activity as compared to the <3 kDa fraction (101.52 μg/ml protein). Whole EGTA extract exhibited a mix activity pattern i.e. both stimulatory and inhibitory activities against CaOx nucleation and exhibited only stimulatory activity against CaOx growth. Whereas, >3 kDa fraction exhibited stimulatory activity against CaOx nucleation and inhibitory activity against CaOx growth. The <3 kDa fraction exhibited stimulatory activity against CaOx nucleation and a mix activity pattern of both inhibition and stimulation was observed against CaOx growth assay.

3.2. Purification of potential cationic inhibitors of CaOx crystal nucleation and growth

Greater than 3 kDa fraction exhibited significant activity towards calcium oxalate monohydrate nucleation and growth assay system and hence was selected for purification purpose. For this more than 3 kDa fraction was loaded on a strong cation exchanger Macro Prep® 25S column. Consecutive fractions were collected with increasing gradient, pooled and were named F1, F2, F3 and F4 (Fig. 1). It was found that F3 and F4 fractions exhibited inhibitory activity against CaOx crystal nucleation and growth (Fig. 2). Further its SDS-PAGE (Fig. 3) analysis showed the presence of few bands. The fractions F3 and F4 were pooled together and were further purified by molecular sieve chromatography (Fig. 4) on a Bio gel® P-100 gel molecular sieve column (50×1 cm). Proteins were eluted at pH 7.4 with 20 mM Tris buffer. Fractions were collected and pooled as F1′, F2′and F3′and examined for inhibitory activity against CaOx crystal nucleation and growth (Fig. 5). All the three fractions were analyzed further by 10% SDS-PAGE (Fig. 6). Single bands were present in the three lanes having molecular weights of ~53,~44, and ~42 kDa indicating that proteins were purified.

3.3. Mass spectrometric identification of novel proteins

The protein bands detected in fractions F1′, F2′and F3′ were excised, in-gel tryptic digested and identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. Purity of the samples was tested using RP-HPLC (Fig. 7). Using the Mascot search engine (http://www.matrixscience.com), the MALDI-TOF data obtained from fractions F1′, F2′and F3′ were matched significantly

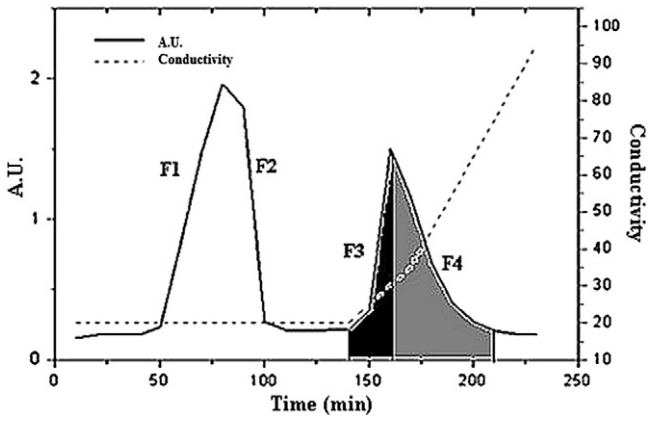

Fig. 1. Elution profile of >3 kDa protein sample loaded on a cation exchanger. F3 and F4 fractions were collected with a linear gradient of NaCl.

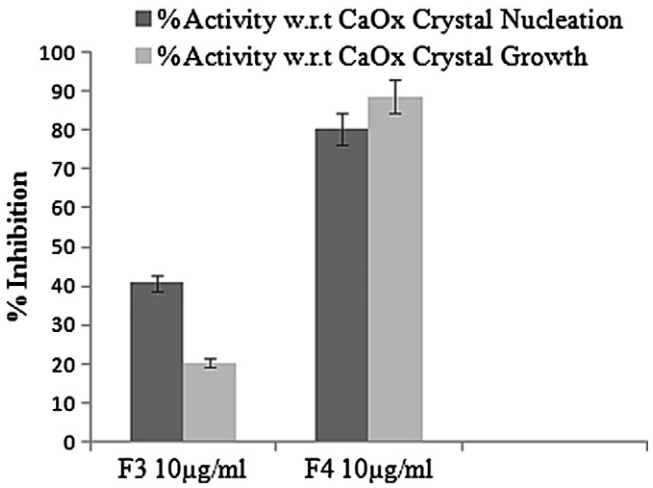

Fig. 2. Percentage activity of pooled fractions of cation exchange chromatography against CaOx crystal nucleation and growth assay.

with histone-lysine N-methyltransferase, inward rectifier K channel and protein Wnt-2. The matching score of mascot search of histone-lysine N-methyltransferase was 33 and sequence coverage was 14%. For inward rectifier K channel matching score of mascot search was 36 and sequence coverage was 26% and for protein Wnt-2 matching score of mascot search was 24 and sequence coverage was 24%.

3.4. Amino acid analysis

Amino acid analysis revealed that the three proteins consisted majorly of tyrosine residues with some portion of cystine in histone-lysine N-methyltransferase and inward rectifier K channel. The amino acid content of each protein is shown in Table 1.

3.5. Reduction of oxalate-induced renal tubular epithelial cell injury by purified protein from human renal stone matrix

The cytoprotective effect of the purified protein was assessed towards the oxalate injured renal tubular epithelial cells, MDCK (Fig. 8). The release of LDH, a stable cytosolic enzyme, was estimated to check the cell injury. In the cells injured with the exposure of oxalate, LDH release was significantly increased from 100% in the untreated cells (control) to 196.72%. The LDH release was significantly reduced in the cells which had the exposure of different concentrations of the purified proteins with oxalate in a concentration dependent manner. The

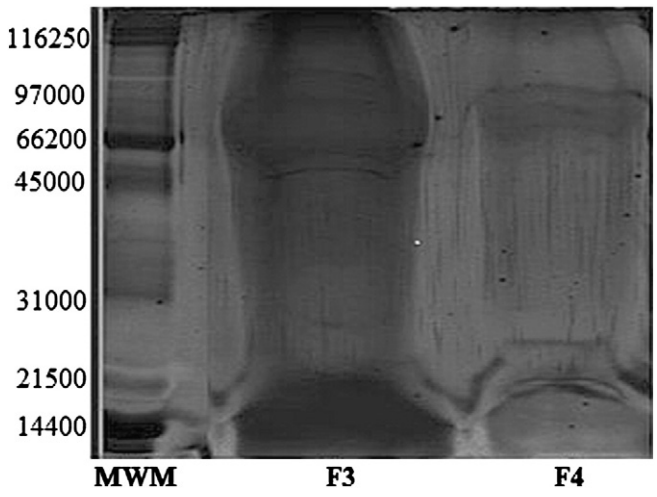

Fig. 3. SDS-PAGE of pooled fractions after cation exchange chromatography.

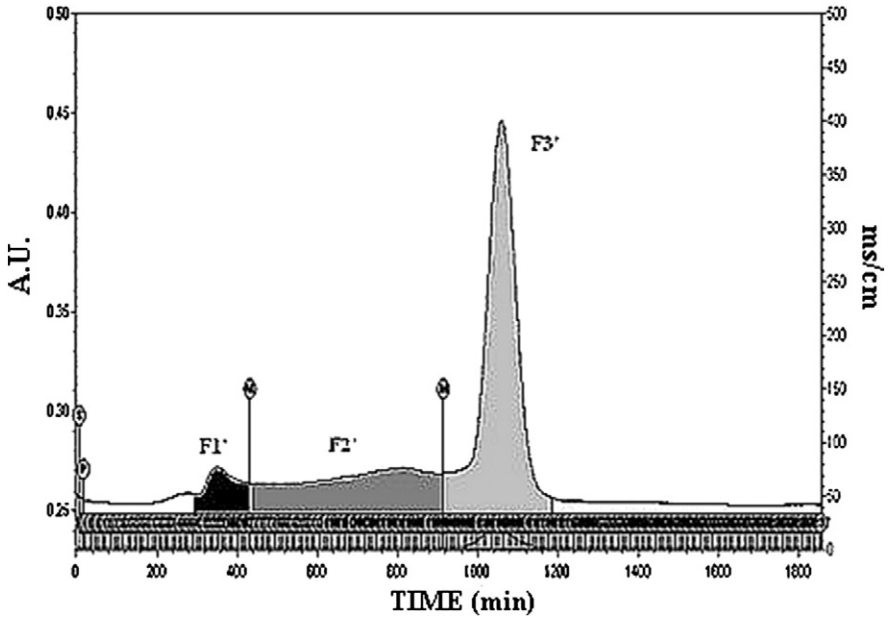

Fig. 4. Elution profile of fractions F2 and F3 loaded on a molecular-sieve chromatography column after cation exchange chromatography.

purified protein proved to have a protective effect towards oxalate induced injury as it decreased the LDH activity significantly in a concentration dependent manner from 5 μg/ml, 10 μg/ml to 20 μg/ml.

3.6. Sequence alignments, secondary structure prediction and protein fold recognition

Out of 462 amino acid sequence of histone lysine N methyltransferase, 246 amino acids were 100% similar to the chain A suppressor of variegation 4–20 homolog 2 (Protein Data Bank ID: 3RQ4A) of *H. sapiens*, which contains a SET Domain. The amino acid sequence of inward rectifier K channel was 51% similar to chain A of inward rectifier potassium channel Kir2.2 R186 a mutant R186 Kir2.2 R186 a mutant in complex with PIP2 (Protein Data Bank ID: 3SPGA) of *Gallus gallus*.

3.7. Homology modeling of inward rectifier potassium channel and docking of histone lysine N methyltransferase and inward rectifier potassium channel and molecular docking with calcium oxalate

The protein inward rectifier K channel was homology modeled based on chain A suppressor of variegation 4–20 homolog 2 (Protein Data Bank 3RQ4A) of *H. sapiens* as template (Fig. 9). The predicted structure was validated using different validation programs (Errat score: 76.358; Verify 3D score: 65.15% of the residues had an averaged 3D–1D score > 0.2; Ramachandran distribution: 69.9% core, 9.4% allow, 0.6% gener, and 0.0% disall). Furthermore, the modeled structure was refined using molecular dynamic simulation (Fig. 9) (Errat score: 88.255; Verify 3D score: 20.81% of the residues had an averaged 3D-1D score > 0.2; Ramachandran distribution: 80.8% core, 16.7% allow, 1.9% gener, and 0.6% disall). For histone lysine N methyltransferase the crystal structure of 3RQ4A was used for the purpose of docking, as this structure contains a SET Domain. Different binding sites of both the proteins were predicted by MOE Site

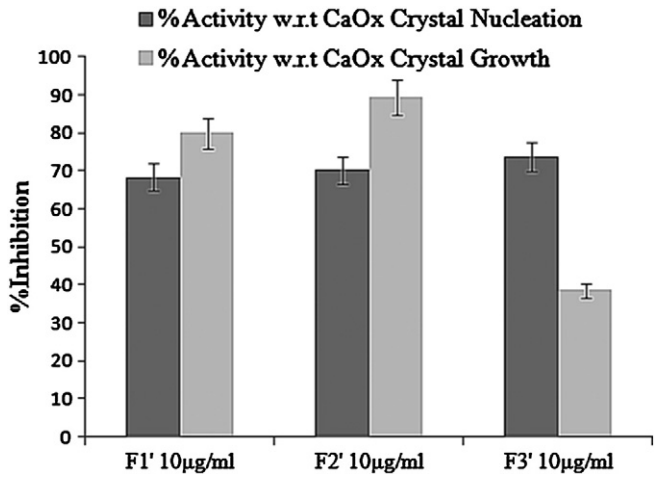

Fig. 5. Percentage activity of pooled fractions of molecular-sieve chromatography against CaOx crystal nucleation and growth assay.

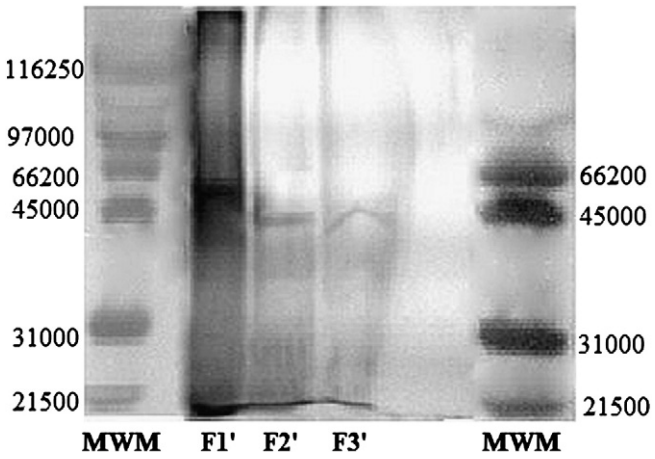

Fig. 6. SDS PAGE of pooled fractions after molecular sieve chromatography.

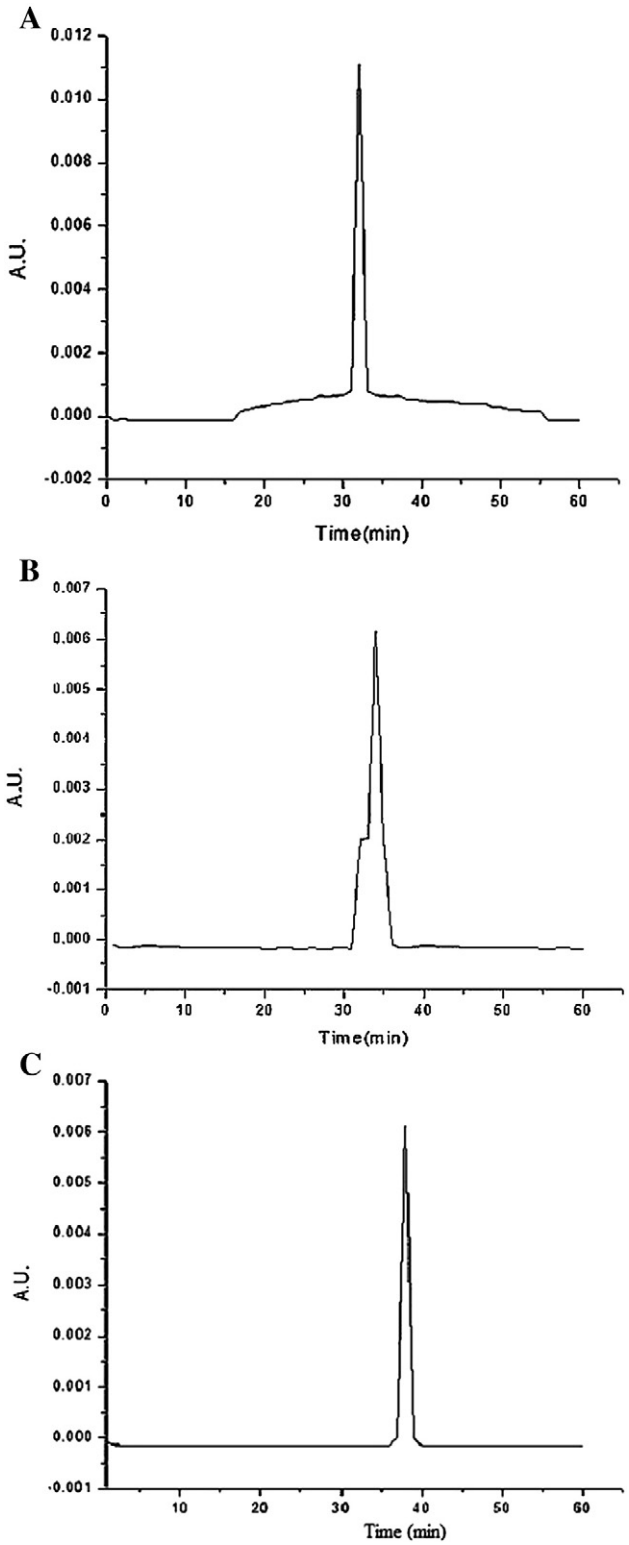

Fig. 7. HPLC analysis of (A) F1′; (B) F2′; and (C) F3′.

Table 1
Amino acid analysis of purified fractions.

F1′	F2′	F3′
Try 94.9% Cys 5.1%	Tyr 95.7% Cys 4.3%	Tyr 100%

finder and used for molecular docking. CaOx was docked onto the binding sites using the Monte Carlo docking procedure of MOE. The best-docked poses of the ligand were identified and energy minimized in the protein, while allowing full side chain flexibility. Docking of calcium oxalate with the binding site of inward rectifier K channel and histone lysine N methyltransferase gave the best docking score of −9.2649 and −8.1249 kcal/mol respectively. In histone lysine N methyltransferase both basic and acidic amino acids seem to be involved in binding with CaOx. We found that, basic amino acids were interacting with the oxalate of calcium oxalate and acidic amino acids were interacting with calcium of calcium oxalate. This was validated when substitution of these basic amino acids with alanine diminished the interaction with calcium oxalate. On mutation the docking score decreased to −7.2466 kcal/mol for mutated basic amino acids from −8.1249 kcal/mol of wild type and increased to −8.8458 kcal/mol for mutated acidic amino acids. Upon phosphorylation the docking score decreased to a value of −7.7729 kcal/mol indicating involvement of these amino acids. LIGPLOTs were generated to show the interaction between identified proteins and CaOx (ligand). LIGPLOT of histone lysine N methyltransferase's wild type showed involvement of aspartate (Asp) at position 195 with calcium and arginine (Arg) 95,98 with oxalate (Fig. 11).

The degree of identity between the template and inward rectifier K channel sequence was 51%, which enabled a preliminary model to be generated. Lysine, phenylalanine, alanine and leucine were found to be interacting with calcium oxalate in the wild type. The more negative the docking score, the stronger is the binding between ligand and protein's active site. The strong interaction between inward rectifier K channel's active site and calcium oxalate predicts inhibition of the same. The highest docking score of −9.2649 kcal/mol indicates good binding of modeled protein with the ligand (calcium oxalate).

We found in inward rectifier K channel that both acidic and basic amino acids were interacting with calcium oxalate. This was further substantiated when substitution of these acidic and basic amino acids with alanine diminished the interaction with calcium oxalate. On mutation the docking score decreased from −9.2649 kcal/mol of wild type to −6.1418 kcal/mol for mutated acidic amino acids and decreased to −8.8168 kcal/mol for mutated basic amino acids (Table 2). Upon phosphorylation the docking score decreased to a value of −7.2632 kcal/mol. LIGPLOT of inward rectifier Potassium channel wild type showed involvement of alanine (Ala) at position 148 with calcium (Fig. 10). Whether a protein or other 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.

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 [22]. This is supported by the presence of basic amino acids too in the inhibitory proteins [23]. In either case steric constraints from 3D conformation of the molecule might limit the number of these simple interactions [22].

3.8. Binding site of wild type protein with calcium oxalate

Binding sites of histone lysine N methyltransferase contained amino acids Asp 4, Arg 5, Val 6, Leu61, Arg84, Ala 87, Ala 88, Lys 90 Thr 91, Tyr 94, Arg 95, Arg 98, Cys 141, Asp 195, Gly 196, Asn 197, and Ala 198 were involved in interaction with calcium oxalate. Binding sites of inward rectifier K channel Lys 9, Gly 10, Asn 11, Met 139, Gly 143, Ala 144, His 145, Ala 148, Glu 149, Ile 266, Thr 267, Thr 268, Gln 269, Ala 270, and Arg 271 were found to be participating in the interaction with calcium oxalate.

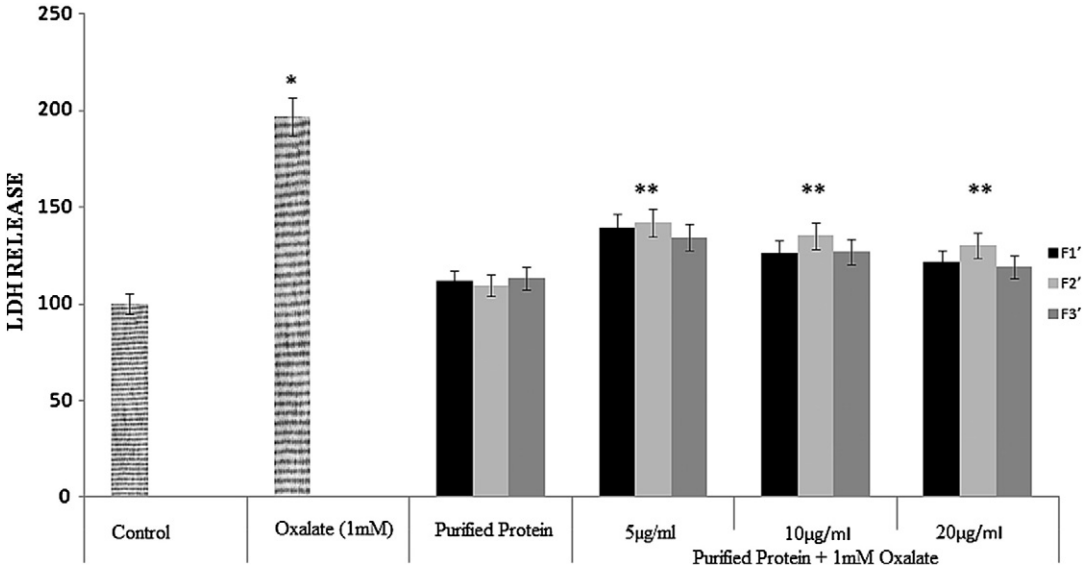

Fig. 8. Effect of purified proteins from human renal stone matrix on the % LDH release. Data are mean ± SEM of three independent observations. *$p<0.05$ versus untreated control, **$p<0.05$ versus oxalate control.

4. Discussion

Proteins are found as a major component in human renal stone matrix and are considered to have a potential role in stone formation, growth and crystal–membrane interaction [24,25]. Recent advances in the technologies are therefore compulsory to study kidney stone formation inhibitors for better understanding of the pathophysiology and pathogenesis of kidney stone disease. Few well-characterized anionic inhibitors of kidney stone formation have been identified earlier. But on the other hand no significant work has been conducted in context to cationic proteins. Therefore, we aim to explore the role of cationic protein from calcium oxalate stones in urolithiasis and to assess their molecular interactions with calcium oxalate monohydrate crystals in silico. Three antilithiatic proteins namely histone lysine N methyltransferase, inward rectifier K channel and protein Wnt-2 have been identified as new components of stones from organic matrix of CaOx stones by MALDI-TOF-MS. The presence of these proteins in the matrix of CaOx stones suggests their putative role in urolithiasis, that they may be related to the process of stone formation. 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 [26,27]. Thus it might be possible that these three proteins attach to the anionic side of the membrane which is exposed after injury, possibly in hyperoxaluric condition [28]. The crystallization assays used in this study provide sufficient evidence to assert that proteins isolated from renal stone with MW > 3 kDa have a tendency to both inhibit and promote crystallization and aggregation of calcium. Previous reports suggest that certain crystal binding proteins are upregulated in damaged kidney during renal disease state. These proteins could coat crystal and may either block or direct to their adhesion to cells. Anionic cell surface sialic acid residues appear to function as COM crystal receptors that can be blocked by specific cations or lectins. Elution profile of cation exchanger suggests that cationic proteins are present in the stone matrix. This mixture of cationic proteins exhibited an inhibitory effect towards both the assay systems, further, followed by purification of three cationic proteins by molecular sieve chromatography. The purified proteins showed a significant inhibition towards calcium oxalate crystal nucleation and growth and showed to have a protective effect towards the cell injury caused by oxalate. Thus, we identified three novel cationic proteins with antilithiatic properties as 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 [29,30]. 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 [31,32]. There are reports that renal ischemia–reperfusion injury upregulates histone-modifying

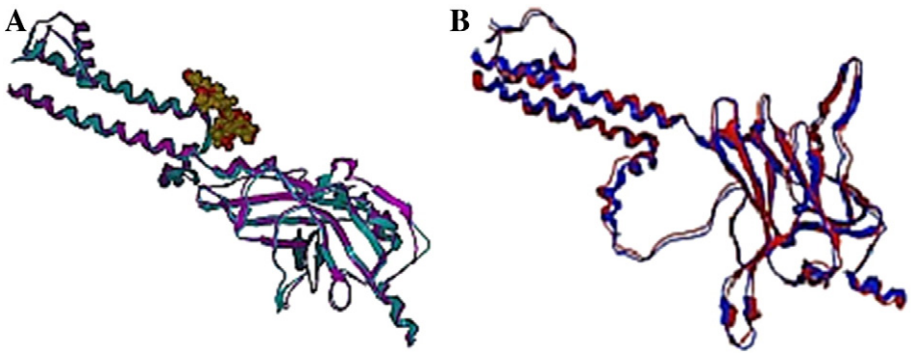

Fig. 9. (A) Superposition of modeled structure and the template of inward rectifier K channel (RMSD value: 0.798) and (B) superposition of refined structure (after molecular dynamics simulation) with the template (RMSD value: 0.877).

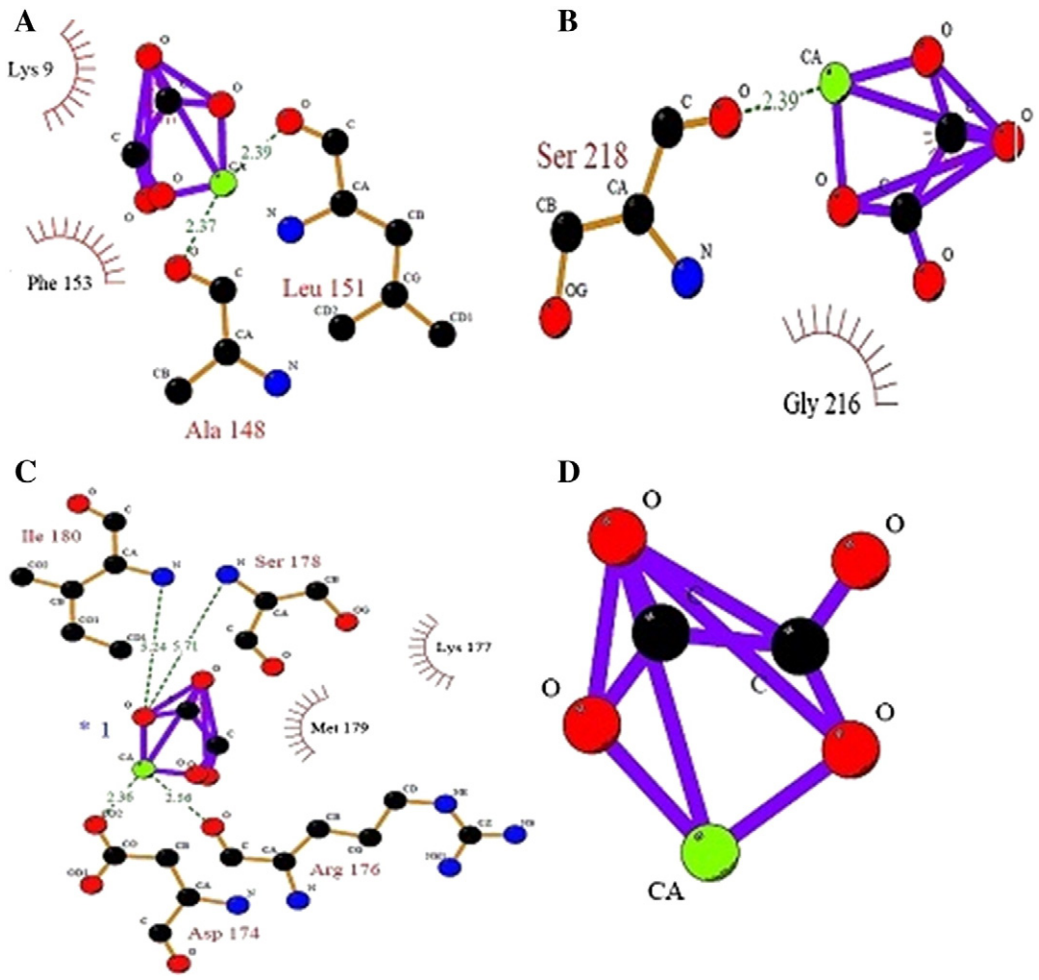

Fig. 10. LIGPLOT of inward rectifier K channel (A) wild type; (B) M1; (C) M2; and (D) M3; where M1: mutation of acidic amino acid with alanine; M2: mutation of basic amino acid with alanine; and M3: phosphorylation of tyrosine and threonine. The hydrogen bonds involved in the interaction between CaOX and the protein are represented in dotted line.

enzyme systems and alters histone expression at proinflammatory/profibrotic genes [33]. 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. Inwardly rectifying K channels (Kir) are now recognized as forming a superfamily of seven members (Kir1 to Kir7) [34]. 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.) [35]. Of the 5 major genetic variants associated with Bartter syndrome [36], cortical and medullary renal calcifications [37] 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 [38]. 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 signaling 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 geometry [39]. Another study revealed the mRNA levels of Wnt-2 in unilateral ureteral obstruction (UUO) 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 UUO expression levels of Wnt-2 mRNA in UUO day 7 and UUO day 10 kidneys were 16.5 times and 32.6 times as high as those in control kidneys, respectively [40]. 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

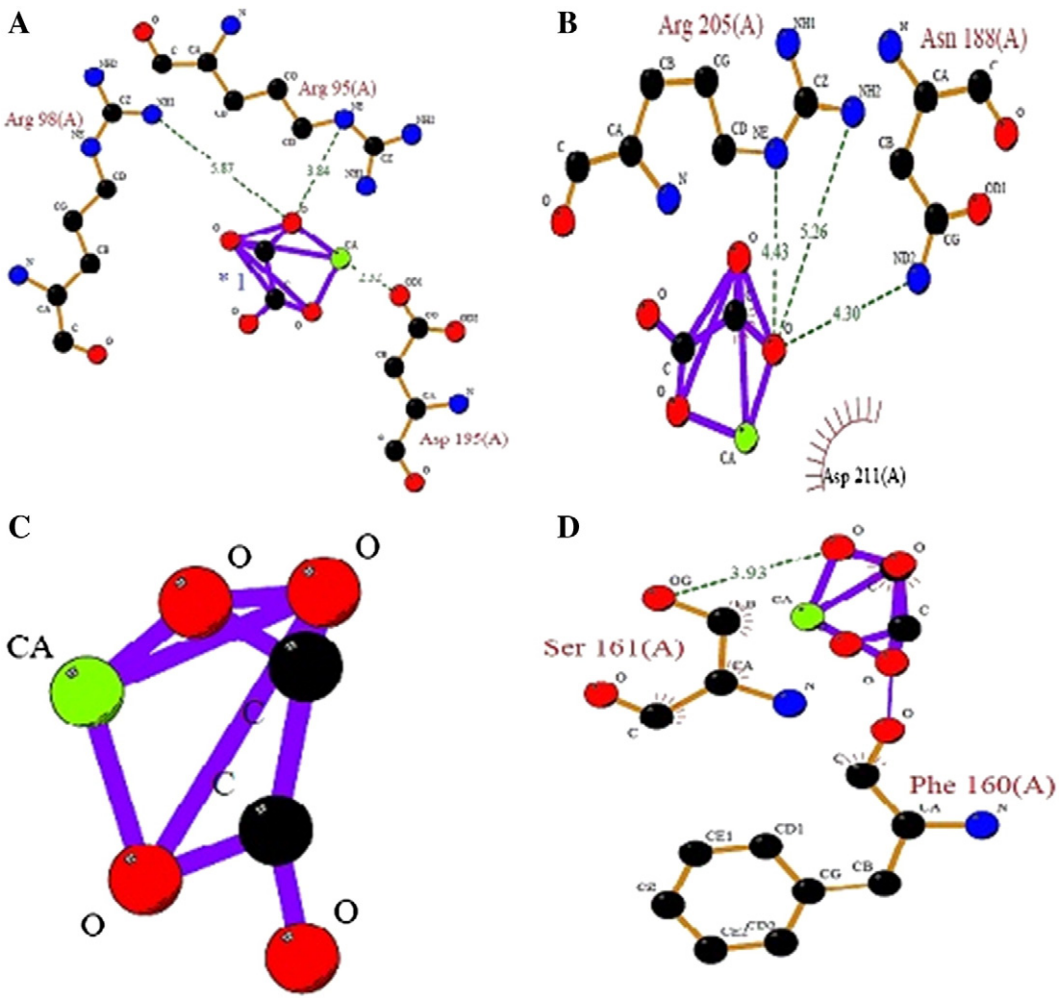

Fig. 11. LIGPLOT of histone lysine N methyltransferase (A) wild type; (B) M1; (C) M2; and (D) M3; where M1: mutation of acidic amino acid with alanine; M2: mutation of basic amino acid with alanine; and M3: phosphorylation of tyrosine and threonine. The hydrogen bonds involved in the interaction between CaOX and the protein are represented in dotted line.

antagonist might be applied for chemoprevention or treatment of cancer [41,42].

We focused our attention on the interaction of the purified proteins with calcium oxalate using molecular docking. In silico results revealed that both the acidic and basic amino acids in the binding site are essential for molecular interactions of CaOX with the antilithiatic cationic proteins. Specifically the amino acids such as lysine, phenylalanine, alanine and leucine were found to be interacting with calcium oxalate. 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. 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. This is supported by the presence of basic amino acids too in the inhibitory proteins. In either case steric constraints from 3D conformation of the molecule might limit the number of these simple interactions.

Table 2
Docking scores of histone lysine N methyltransferase and inward rectifier K channel where M1: mutation of acidic amino acid with alanine; M2: mutation of basic amino acid with alanine; and M3: phosphorylation of tyrosine and threonine.

Inward rectifier K channel	Docking score kcal/mol	Histone lysine N methyltransferase	Docking score kcal/mol
Wild type	−9.2649	Wild type	−8.1249
M1	−6.1418	M1	−8.8458
M2	−8.8168	M2	−7.2466
M3	−7.2632	M3	−7.7729

Acknowledgments

The authors thank the Indian Council of Medical Research (ICMR) and Jaypee University of Information Technology, Solan, HP, India for providing funds to carry out this research work. We also express our gratitude to the Department of Urology, Post-Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India for providing kidney stones. Also, we would like to thank Sneha Santoshi for the technical support in bioinformatics area.

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