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Melamine (tripolycyanamide) is an industrial chemical having a chemical formula of C₃H₆N₆ which is composed mainly of nitrogen, carbon and hydrogen (Chan et al., 2008, Tyan et al., 2009). Due to presence of 66% nitrogen content, it can be considered as non-protein nitrogen source for the ruminants (Lu et al., 2009). However it was observed that melamine could not be a satisfactory nitrogen supplement for animals due to slower hydrolysis in cattles (Newton and Utley, 1978). Melamine crystallizes in the form of colorless monoclinic prisms and it has lesser solubility in water and many organic solvents (Tyan et al., 2009). Under some environmental conditions, it is also an intermediate product of metabolic process of cyromazine, an insecticide of plants (Lori et al., 1990). Melamine compound has also been frequently used in laminates, glues, adhesives plastics and resins production to promote thermal resistance (Chan et al., 2008, Zeng et al., 2011, Guan et al., 2013 and Wang et al., 2013). Melamine is not metabolized in mammals and excreted out through urine (Filigenzi et al., 2007, Dobson et al., 2008, Baynes et al., 2008). Hydrolysis of melamine produces cyanuric acid which is a cyclic trimer of hydrocyanic acid. Like melamine, cyanuric acid is also used as a component of herbicides, disinfectants, and bleaching agents. Melamine, melamine-cyanurate and its other derivatives can be utilised as flame retarding materials due to its intense capacity to emancipate nitrogen after burning or heating and also as drug delivery vehicles (Dalal and Goldfarb, 2011). It is also used in the manufacturing of super plasticizer for making high-resistance concrete. It is also a major component of pigment yellow 150 which is a colorant for inks and plastics and derivatives of arsenical drugs such as Melarsoprol for the treatment of African sleeping sickness (Yang et al., 2009, Hau et al., 2009). Melamine is a chemical intermediate that is used to manufacture amino resins. The pKb (dissociation constant of a weak base) value of melamine is nine due to the presence of several amino groups that show basic properties. Melamine is also used in the manufacturing of plastics and other industrial processes. Cyanuric acid which is related triazine compound of melamine sometimes detected with melamine and is used in pool chlorination. Cyanuric acid as well as other chemically related triazines can also be obtained during plastic manufacturing (Baynes and Riviere, 2010). It is an organic base and is synthesized in industries from urea and cyanuric acid as an intermediate product. Other byproducts such as ammeline and ammelide are also formed during the reaction.
Melamine had been apparently added to raw materials especially of milk products sold by food distributors to boost their artificial protein content (Zhang et al., 2011, Vanachayangkul and Tolleson, 2012). Beverages, eggs, and candies were also reported to be melamine contaminated (Ingelfinger, 2008, Xia et al., 2009). This adulteration cannot be easily identified by routine methods such as Kjedahl and Dumas methods (Saint-Denis and Goupy, 2004, Sun et al., 2010, Nascimento et al., 2015). And additional exposure to leached melamine can occur if chlorine

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**Table 2.1** Chemical properties of melamine (Hau et al., 2009)

<table>
<thead>
<tr>
<th>Chemical Properties of melamine</th>
<th>Illustration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance (room temp.)</td>
<td>Whitish crystalline solid</td>
</tr>
<tr>
<td>Solubility</td>
<td>Partially soluble in water</td>
</tr>
<tr>
<td>Melting point</td>
<td>357°C</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.573 g/cm³</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>126.12 g/mol</td>
</tr>
</tbody>
</table>
containing disinfecting agents used for the cleaning of melamine-made dinnerware (WHO, 2008). Leaching of melamine from plastics into food may occur in small amounts and therefore do not considered harmful. It can also be absorbed in foods through crop fertilizers as melamine are also added in crop fertilizers. The extent of melamine contamination is not clearly known. It has been determined that these sources of melamine conferred inconsiderable quantities of melamine to the meals (Ingelfinger, 2008, Buka et al., 2009). Melamine is not considered as a normal ingredient of animal feed, still it was found in poultries, pigs and fish feeds (Nestle and Nesheim, 2007, Anderson et al., 2008).

\[
6\text{C}_2\text{O(NH}_2\text{)}_2 \rightarrow \text{C}_3\text{H}_6\text{N}_6 + 3\text{CO}_2 + 6\text{NH}_3
\]

\text{Urea} \quad \text{Melamine}

\text{Fig. 2.2 Melamine formation (Tyan et al., 2009)}

### 2.1 Melamine adulteration

In 2004 and 2007, melamine contamination in pet foods was reported to be the reason behind two major international incidents where domestic cats and dogs were dead due to acute renal failure in the United States and Europe. Moreover, those pet food scraps were exported from China. This was confirmed after testing levels of melamine in these pet food scraps which were similar to that of melamine contaminated infant formulas. Autopsy reports of animals died during the years under report were also comparable. In 2008, ingestion of melamine contaminated animal feed caused deaths of many raccon dogs in China because of kidney failure (Brown et al. 2007, Puschner 2007, Burns 2007, Bhalla et al., 2009). Spoke-like crystals were formed in the kidneys of melamine affected animals was suspected due to dysfunction of kidneys which lead to acute renal failure and mortality of cats and dogs (He et al., 2008). In 2008, melamine nephrotoxicity found in Chinese infants who were exposed to melamine contaminated milk products with concentrations ranging from 0.1 ppm to 2563 ppm (Xin and Stone, 2008, Ingelfinger, 2008). 22 commercial brands of milk powder were found to have detectable levels of melamine and melamine content of some products were far above the daily tolerance levels of the human body (Bhalla et al., 2009). All these incidents raised the global concerns towards melamine adulteration in food and feed (Gossner et al., 2009, Puschner and Reimschuessel, 2011). Kidneys of infants and children were severely injured (Ho et al., 2009, Wang et al.,
Out of approximately 3170 infants exposed to melamine, one child had confirmed renal stones, seven were suspected renal deposits and results of 208 children were found positive for hematuria (Baynes and Riviere, 2010). In 2008, World Health Organization reported that almost 294,000 Chinese infants were symptomatic which resulted in 51,900 hospitalizations and six deaths due to renal failure (Guan et al., 2009, WHO 2008). Statistical reports said approximately 99% of infants were less than 3 years, 0.8% were above 3 years and no one was older than 4 (Yang et al., 2009). Melamine levels that is not declared on product label were also detected in the nutritional supplements products (Gabriels et al., 2015). As melamine is not only exposed through food and protein sources but also through daily-use melamine–made table ware, so it has become very difficult task to estimate the duration of melamine exposure and quantity of daily consumption of melamine by individuals through various sources (Chien et al., 2011).

Various standard protein assessing tests measure nitrogen content in order to assess protein concentration of the melamine contaminated milk as it falsely elevated the protein content of the milk (Hau et al. 2009, Vanachayangkul et al. 2012, Suchy et al. 2009). Adding 1g of melamine to 1 litre of milk can allegedly boost the protein content up to 0.4%. and while 3.1 g of melamine dissolved in water can falsely increase protein level by 1.2%. So this suggests that 30% can overestimate protein content in liquid milk and risk of increased melamine concentration increased in case of powdered milk (Hau et al., 2009). In toxicology studies, it has been proved that melamine’s analogues primarily cyanuric acid if present in the kidney along with melamine can together cause renal pathology (Afoakwa, 2008, Brown et al., 2007). The most oral lethal dose of melamine that kills almost one-half of the tested rats was 3161mg/kg bw/d. A study done on pigs and rats showed that 400mg/kg/day upto three days of melamine was a safer dose (Dalal and Goldfarb, 2011).

![Melamine-cyanurate complex](image)

**Fig2.4** Melamine-cyanurate complex
2.2 Toxicological studies

Lu et al. (2009) conducted the experiment on chickens to check melamine levels in their tissues after feeding with melamine contaminated diets. Diets were given to the 4 similar groups (12 birds in each cage) in ten doses starting from 0 to 1000mg of melamine for 42 days, followed by no melamine diet for 7 days. On day 28, 42 and 49, it was observed that one bird was killed from each cage and melamine levels were checked in tissue samples of different organs. The work illustrates that melamine levels found in broiler tissues were increased with high doses i.e. at 500 and 1000mg of melamine doses, and melamine distribution was different in different organ tissues with kidneys having higher concentration. Moreover, 7 days of withdrawal period cleared the melamine from tissues.

In Thailand, melamine and cyanuric acid were detected by gas chromatography mass spectrometry in swine herds suffered from an unknown syndrome. New syndrome was determined when five pigs of 4-8 weeks of age were sent for the necropsy examination and it was observed that all the pigs were underweight, having rough and pale coats and kidneys were in abnormal phase. Round and yellowish-brown crystals were found to be distributed in proximal and distal tubules and elevated levels of urea nitrogen and creatinine in blood were detected. All the findings reportedly suggested that melamine and cyanuric acid adulterated feed was responsible for all the complications in these pigs (Nilubol et al., 2009).

Melamine-cyanurate toxicity test was also conducted on Pekin ducks by feeding them with contaminated diets with different ages (3-21 days). 223 male ducks of three years old were assigned to one of ten different dose groups. Doses made of basal diet (BD) with no melamine and cyanuric acid, BD with increasing melamine concentrations, BD with increasing cyanuric acid concentrations and BD with increasing concentrations of melamine and cyanuric acid. Results suggested that birds fed with melamine (>1.0%MEL) contaminated feed affected more as compared to cyanuric acid and melamine-cyanurate complex and pathology reports showed that they suffered from acute renal failure. Interestingly the results showed that the cyanuric acid alone wasn’t toxic upto 1.5% while cyanuric acid lessens the toxicity of melamine when the two were given in combination (Landers et al., 2013).

Strakova and members studied the toxic effects of melamine and cyanuric acid on hematological and biochemical blood indicators among male broiler chickens. Six different treatment groups were made and fed diets with different doses of melamine, cyanuric acid and in combination and
a control group without melamine or cyanuric acid. No significant changes were observed in hematological examination, but highly significant changes were found in total protein (TP), Glu, Ca, P, Na, K and Mg present in blood plasma. Moreover, it was inferred that diets contaminated with melamine, cyanuric acid and combination of both results in renal impairment and partial liver damage (Strakova et al., 2014).

Rat models were also used to investigate the crystal formation in kidneys after ingesting melamine and cyanuric acid (M+CA) combination diet to understand deeply the renal toxicity caused by them. M+CA did not cause any direct nephrotoxicity but after the administration of three days, nephrotoxicity occurred which was confirmed by increased blood urea nitrogen and serum creatinine levels, diminished creatinine clearance and expanded kidneys. It was observed that at 50mg/kg concentration, crystals were observed in medulla while at 100mg/kg, crystals were distributed from cortex to medulla which was further investigated by MALDI-Q-TOF. All these results concluded that M+CA ingestion could cause crystal formation in renal tubules and these crystal formation lead to M+CA induced nephrotoxicity (Kim et al., 2010).

Renal toxicity caused by melamine when given with cyanuric acid was observed by exposing male rats with the combination for three days. Melamine alone showed negligible effect while melamine plus cyanuric acid elevated the blood urea nitrogen and creatinine levels and kidney weight. Combined dose of melamine and cyanuric acid cause more severe renal toxicity with golden brown crystals and damage in renal tubules as compared to melamine alone. And all these results showed that melamine cyanurate complex induce more damage and it was suggested that tolerable daily intake of melamine should be revised again when exposed with cyanuric acid (Park et al., 2011).

In vivo investigations were done to get the information about the possible target cells during melamine plus cyanuric acid per se crystal formation. Lethal doses were provided to rats and analyzed after different intervals and it was found that Melamine and Cyanuric acid induced degeneration in the proximal tubules which started at 12 h and increase by the time. After post treatment, some needle shaped crystals were seen in the cytoplasm and large crystals in the renal tubules observed by ultrastructure study which indicated the physical damage of the renal cells (Chen et al., 2014).

Brown et al. (2007) observed sixteen animals (2 dogs of 2004, 10 cats and 4 dogs of 2007) affected by pet food associated nephropathy during two incidences were observed for
histopathologic, toxicologic and clinicopathologic changes. All these animals were died or euthanized because of serious uremia. Polarizable crystals were found in distal tubules of all the animals and interstitial fibrosis and inflammation was found in some animals. All the clinical, histological, and toxicological studies of animals from two different renal failure outbreaks showed the identical evidences which proved that melamine and cyanuric acid were the cause behind the outbreaks.

A study on cats showed that both the adulterants, melamine and cyanuric acid were responsible for serious kidney failure in pets during pet food outbreak. The aim of investigation was to analyze the toxicity potential of melamine and cyanuric acid in cats. In this study, 0.5% and 1% of melamine was added to the food of two cats respectively, and on the other hand cyanuric acid was added to the diet of one cat in increasing concentration during 10 days course. Melamine and cyanuric acid in combination was given to one cat per dose group. There was no effect on kidney function in cats with diet of melamine and cyanuric acid alone while the cats administered with the combination were euthanized after 48 hours of dosage. Study revealed the presence of fan shaped and birefringent crystals within distal tubules of nephron and severe renal interstitial edema in cats (Puschner et al., 2007).

Melamine-cyanurate crystals were also formed in the renal tubules of rainbow trout (Oncorhynchus mykiss) after feeding on diets containing melamine and cyanuric acid for 10 weeks. Prooxidant effects of both the compounds were assessed by oxidative stress markers like catalase, GST and malondialdehyde. Crystals were remained in the kidneys of trout which was treated with the highest dose after six withdrawal weeks. It could be inferred that crystal formation depends on time and dosage. Also it was quite clear from the activities of oxidative stress markers that melamine (alone or in combination) showed higher pro-oxidant effect than cyanuric acid (Pacini et al., 2014).

Effects of intake of feed contaminated with melamine and cyanuric acid were also examined in red tilapia. Diet 1 was without melamine and cyanuric acid, diets 2 to 4 contained both melamine and cyanuric acid at 2.5, 5 and 7.5 g/kg diet, respectively and diets 5 and 6 contained either melamine or cyanuric acid at 10 g/kg diet. Diet containing only melamine affects both growth and FCR while the diet with only cyanuric acid tends to reduce only FCR of fish. The renal tubules of fish ingested melamine plus cyanuric acid diet, had melamine-cyanurate crystals. Diets containing only single substance did not induce any crystal formation. Both in combination
(i.e. melamine and cyanuric acid) and single chemical (melamine or cyanuric acid) diet increased the levels of Hsp70 in the liver of red tilapia. Combination of melamine and cyanuric acid at inclusion levels greater than 5 g/kg diet affect the activities of catalase present in liver and glutathione peroxidase present in liver and kidneys. So, this study also suggested that these adulterants (melamine and cyanuric acid) have adverse effects on fish and should not be added in fish feeds (Phromkunthong et al., 2015).

Fujio and co-members found that KLF5 (Kruppellike factor5) have a prominent role in modulating the responses to a renal injury and it is expressed in epithelial cells of collecting ducts (Fujiu et al., 2011). Reports of all these experiments conducted on different animals showed the acute renal toxicity and crystals formation in renal tubules which suggest the common mode of toxicity that is due to exposure of melamine and its analogue cyanuric acid.

### 2.3 Melamine detection methods

Since the reporting of food and feed adulteration incidents, a number of methods have been developed for detecting melamine and its related toxins. Cation-exchange chromatography, liquid chromatography, chemiluminescence, gas chromatography and HPLC (high performance liquid chromatography) have been used to analyze melamine and its metabolites (Muniz-Valencia et al., 2008, Sun et al., 2010, Wen et al., 2010, Pan et al., 2013 and Zhang et al., 2011). Real-time MS has also been used to track the presence of melamine in pet food (Tyan et al., 2009). LC-MS/MS has been used to determine melamine in porcine muscle tissue. Diode-array detectors, UV and MS detectors are used for detection (Ehling et al., 2007, Filigenzi et al., 2008). The sample pretreatment of melamine is mostly done in a complex matrix in which polar solvent is used for liquid extraction and further clean-up by using solid phase extraction. Analysis of melamine and its related compounds is carried out by liquid or gas chromatography in combination with mass spectrometry. Other newly developed methods for screening melamine are the use of antibodies, molecularly imprinted polymers, gold nanoparticles and capillary electrophoresis to develop assays and biosensors to melamine (Liu et al., 2012).

Fluorimetric methods for detection of melamine in milk were also proposed in various studies (Attia et al., 2011, Liu et al., 2012, Wang et al., 2011 and Zhang et al., 2012). Other approaches used for determining melamine are surface-enhanced Raman spectroscopy, enzyme immunoassay, ELISA (enzyme-linked immunosorbent assay) and isotope dilution. There is a technique of stable isotope dilution i.e. LC-MS/MS which is used in quantitative analysis of
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melamine and cyanuric acid in pet food formulas (Dobson et al., 2008, Varelis and Jeskelis, 2008, Fodey et al., 2011, Ma et al., 2013). ELISA test kits have also been developed for the quantitative analysis to meet the requirements of high-throughput screening of samples (Garber, 2008, Kim et al., 2008). An Electromembrane micro-extraction technique combined with HPLC-UV detector was used in the quick extraction and determination of melamine present in dairy products (Fashi et al., 2015). A novel method, MISPE-UPLC (molecularly imprinted solid phase extraction-ultra-performance liquid chromatography) was also developed for determining the levels of cyromazine, melamine, ammelide and ammeline in milk samples (Ge et al. (2015).

2.4 Health impacts of Melamine

Melamine is a pure substance and has low oral acute toxicity (FDA, 2007). If direct contact, then melamine could led to irritation in skin and eyes whereas if inhaled could cause irritation in breathing tract. When ingested orally, melamine poses harm to digestive tract in the form of nausea, vomiting or diarrhea (Jeong et al., 2006). Animal studies showed that exposure of melamine can be responsible for toxicities like nephrolithiasis, chronic kidney inflammation, bladder stones and reproductive toxicity (Ogaswara et al., 1995). Kidney is the organ which is the common target of melamine induced toxicity (Brand et al., 2012, Early et al., 2013). Kidney stone formation is mostly observed in males as compared to females (3:1). Several investigations were done earlier to find out the association between melamine exposure and consumption of melamine contaminated milk formulas and nephrolithiasis among infants and children (Ho et al., 2009, Hocking, 2009, Ji et al., 2009, Li et al., 2010). However, these observations reveal the relationship between dose of melamine and occurrence of kidney stones. Melamine induced toxicity can be further elevated by the presence of other analogues especially cyanuric acid (Afoakwa, 2008). Urinary pH may also responsible in the formation of insoluble crystals of melamine and its by product, cyanuric acid. Low urinary pH is associated with higher risk of melamine induced nephrolithiasis, so melamine-cyanurate crystals can be easily formed in acid urine. There is 1.78 times increased risk of melamine related kidney stone in acidic urine as compared to normal urine (Lu et al., 2011). Melamine and cyanuric acid together combine to form a lattice structure by hydrogen bond formation at pH 5.8 (Canelli, 1974). Recently crystal structure of melamine and cyanuric acid complex was also re-determined which showed that true unit cell was approximately two times the volume of previously reported cell. Crystal data for the
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Melamine cyanurate complex revealed that ordered array of melamine and cyanuric acid and hydrogen bonded sheets stacked perpendicularly between two components were reported (Prior et al., 2013). Children are more prone to melamine toxicity as compared to adults due to their greater body size and consumption of different foods (Wu et al., 2014). Liver abnormalities were also observed among the children who consumed melamine-tainted milk (Zhang et al., 2009, Hu et al., 2012). Acute uric acid nephropathy is a mechanical obstruction that led to renal damage due to the uric acid crystal “spherulites” or “spherically symmetrical and radiating crystal aggregates” that can occur in humans with gout (Fiechtner and Simkin, 1980, Fiechtner and Simkin, 1981). Kidney failure may result from both intra renal crystal-associated obstruction and an elevation in renal pressure that reduces renal blood flow (Herz et al., 1972, Ejaz et al., 2007). Melamine has adverse effects on the reproductive system also. Clear rectangular crystals of melamine and cyanuric acid were found after their combined exposure which in turn caused reproductive sequel in melamine treated animals and even fetal deaths (Yin et al., 2013, Stine et al., 2014). Melamine can damage the ovaries by inducing oxidative stress (Dai et al., 2015).

Yin and co-members studied about the toxicity of melamine and melamine-cyanurate complex in male mice after treating mice with increasing doses of melamine and melamine-cyanuric acid combination. It was observed that melamine induced renal failure at high doses with abnormal sperm morphology as compared to melamine-cyanuric acid combination which was toxic even at low doses. More severe apoptosis was examined in the rats administered with both melamine and cyanuric acid. All the mice died before day 6 who were exposed with melamine and cyanuric acid in combination with no sperm abnormality confirmed. The study demonstrated that melamine caused toxicity on the male testis especially at high concentration and can be useful in stating about the toxicity of melamine on male reproductive system (Yin et al., 2013).

Studies on pregnant rats proved that the reproductive toxicity of adulterants, melamine and cyanuric acid when given in high doses on the developing fetus. As amniotic fluid contained melamine and cyanuric acid after being exposed to high doses transfers these toxic substances from mother to the developing fetus. It was found clear rectangular crystals of melamine and cyanuric acid after combined exposure which in turn caused fertility related issues and melamine exposed animals were more prone to fetal deaths as compared to cyanuric acid treatment (Stine et al., 2014).
Yasui et al. (2014) evaluated renal function on a rat model by combined exposure of melamine and cyanuric acid considering their age and sex. 12 male rats with ages 6, 10 and 26 weeks respectively were administered for 12 mg/kg/day melamine and cyanuric acid for 28 days and six weeks male and female rats were also given melamine and cyanuric acid for 28 days. After the examination of urine and kidney samples, it was stated that younger rats were severely affected by renal failure with more crystal formation and male rats were affected more as compared to females.

Duan et al. (2015) studied in vivo toxicity of melamine on oocyte development and fertility. Three groups of mice were assigned and fed with a melamine contaminated diet at different concentrations for 8 weeks and then in vivo effect was studied. Techniques like Immunofluorescent staining, western blotting and qRT–PCR were used to observe the effect of melamine on oocyte development. The study showed melamine can induce toxicity in oocyte which affects its quality and fertility due to abnormal oocyte cytoskeleton, apoptosis and autophagy induction (Stine et al., 2014).

Dai et al. (2015) observed that melamine exposure may affect the development of follicles and ovaries. Data obtained revealed that melamine induced increased reactive oxygen species levels and led to apoptotic death of granulosa cells and follicle atresia. Further analysis of mechanism behind melamine toxicity was done by examining the expression and activities of SOD and GPx enzymes in controlled and melamine treated ovaries. Concentration of malondialdehyde was also compared between controlled and melamine-treated ovaries. The results showed the expression and activities of both SOD and GPx was changed in melamine-treated mice. Therefore, results stated that melamine may damage the mice ovaries via oxidative stress mechanism.

Oxidative stress is an important pathogenic mechanism in the renal diseases developed by increased ROS levels via inflammation (Kuo et al., 2013). Guo et al. (2012) demonstrated in a study that melamine can cause oxidative damage in NRK (normal rat kidney)-52e cells. By using fluorescence microscopy, it was found that melamine increased the intracellular ROS levels of the NRK-52e cells and acridine orange or ethidium bromide staining showed dose-dependent increase in percentages of apoptotic and necrotic NRK-52e cells. MTT assays and flow cytometry studies revealed that SB203580 is an inhibitor of p38MAPK pathway which enhanced the proliferation NRK-52e cells and lowered the levels of apoptotic and necrotic NRK-52e cells. Western blotting further stated that melamine activated p38 phosphorylation in the NRK-52e
cells and inhibitor i.e. SB203580 inhibited the increase of p38 phosphorylation caused by melamine (Lali et al., 2000). All these studies together suggested that melamine causes apoptosis of NRK-52e cells through the increased ROS levels and activated p38 MAPK pathway and also revealed the molecular process behind the melamine induced cytotoxicity inside renal tubular cells (Guo et al., 2012a). Low dose of melamine for long term leads to a high risk of urolithiasis which is further associated to chronic kidney disease (CKD) (Chen et al., 2009, Saucier et al., 2010). CKD is linked to interaction of inflammation, oxidative stress and TGF-β which further cause increase in extracellular matrix genes and cell apoptosis and finally adverse renal disease with nephron loss (Evan et al., 2003). In another study, human renal proximal tubular HK-2 cells were stimulated with melamine at different concentrations for different time intervals and effects were examined on molecular process behind occurrence of Chronic Kidney Disease. Results concluded that melamine activated MAPKs, NF-κB and ROS which can be further responsible for elevation in TGF-β1 in HK2 cells and increase in TGF-β1 promote cell apoptosis (Kim et al., 2002). The whole study demonstrated that melamine may be a dangerous sign for the tubular cell loss and finally tubulointerstitial damage (Hsieh et al., 2012).

Kuo et al. (2013) investigated oxidative stress and inflammation induced by melamine in macrophage-like cell line (RAW 264.7) and human embryonic kidney cell line (HEK293). Results showed melamine induced NF-κB activation through increasing IκB-α degradation and NF-κB p65/p50 DNA binding activity and also by increased COX-2 expression and prostaglandin E2 production. Melamine also activated NOX with an elevated ROS production and ROS production can be attenuated by NOX inhibitor, apocynin (Petronio et al., 2013). The whole work suggested that melamine activate NF-κB/COX-2 and NOX/ROS pathway which increase inflammation and oxidative stress and also revealed the pivotal role of NOX in melamine-induced ROS production along with prospective of NOX inhibitor against melamine toxicity.
Fig2.5 Mechanism behind melamine causing renal proximal tubular cell injury (Hsieh et al., 2012)

2.5 Melamine and urinary biomarkers

Various traditional methods were proposed in the past to detect early changes in kidneys occurred due to melamine adulterated infant formula and other milk foods. These early changes may help in evaluating kidney damage and impairments (Schnellmann, 2008). In the recent years, various urinary associated proteins have been utilised as important biomarkers to detect injury in kidneys.

Bandele and coworkers proposed urinary renal biomarkers to demonstrate initial stages of kidney problems after the establishment of link between melamine intake and nephropathy and these biomarkers were sensitive enough to evaluate the sequel of melamine and its analogues. Kim-1, clusterin and osteopontin were proved useful in distinguishing the severity of consequences of melamine or other analogues in pregnant female rats and non-pregnant female rats when given in high doses of 1000mg/kg/day for 10 days. All the rats were adversely affected by the exposure
but the pregnant rats were affected the most, indicated by the increased kidney weight, decreased body weight and presence of urinary stones. And biomarker levels were started elevating after day2 which further supported the observations. This work proved the efficiency of biomarkers in detecting early effects of melamine induced nephropathy (Bandele et al., 2014). Zhang et al. (2012) also studied about effectiveness of urinary biomarkers in detecting renal damage caused due to melamine-cyanurate exposure. Male and female rats were orally exposed with melamine and cyanuric acid supplemented diet of specific doses and urine was obtained from day 0 to 28 at specific intervals. Urinary biomarkers like osteopontin, albumin, RPA-1, kidney injury molecule-1, alpha-GST, GST-Yb1, cluster in and neutrophil gelatinase-associated lipocalin were used and the result indicated that RPA-1 was the most sensitive one as it showed elevation at 120 ppm dose in males and 180 ppm in females on day 28. Observations revealed severe renal damage and increased levels of blood urea nitrogen and serum creatinine after 28 days of exposure. Results concluded the fact that RPA-1 may work as the most sensitive biomarker for detecting and monitoring melamine-cyanurate induced nephropathy. Initial stage diagnosis in renal injuries could prevent the extremity of the kidney diseases. Therefore, there is an urgent need to find out novel and highly sensitive biomarkers to assess the renal injuries at the starting stages.

2.6 Human Serum Albumin (HSA)

Human serum albumin is a very important protein present in blood plasma and its concentration in blood is 5mg/ml. HSA is composed of 585 amino acids with three homologous domains and have molecular mass of 66,500 Da (Sugio et al., 1999, Fujiwara and Amisaki, 2008). HSA is produced in the liver and a crucial protein constituent in blood plasma which transports various compounds like fatty acids, bilirubin and a wide variety of therapeutic agents. Drug binding to HSA is one of the important factors which determine its pharmacokinetics behavior due to its prolonged in vivo half-life after binding (Fujiwara and Amisaki, 2008, Fasano et al., 2005). Not only these compounds but other products like lipopolysaccharides, many bacterial products such as lipoteichoic acid and peptidoglycan, nitric oxide, ROS can bind to it. Reversible binding capacity of HSA regulates its inflammatory reactions as molecules can be transferred to different sites within the cell (Arroyo et al., 2014). A study showed a decrease in the levels of serum albumin (>0.3 g/dL) which persisted for more than six weeks and this response is related to the
activation of inflammation. Inflammation is the main cause of decrease in levels of serum albumin (Kaysen et al., 2004).

Sugio and co-members obtained triclinic shaped crystal of HSA from pool plasma (pHSA) and Pichiapastoris expression system (rHSA), extracted from polyethylene glycol solution. 3D structures of both pHSA and rHSA were deduced at resolution of 2.5 Å using molecular placement technique and atomic coordinates got from already known tetragonal crystal structure. Both HSA molecules can superimpose each other with an RMSD of 0.28 Å. It was found that Cys34 was the only cysteine having free sulfhydryl group and doesn’t make disulfide bridges with any ligand bind to it. It was found that maximum number of hydrophobic and positively charged residues were present in the pockets of Domains II and III so large number of ligands can be fitted inside the pockets and long chain fatty acids bind specifically at the surface of the domains (Sugio et al., 1999).

Petitpas and co-workers presented its high resolution crystal structure in complex with two fatty acids i.e. oleic acid and arachidonic acid and it was observed that both the ligands bind to all the seven active sites of HSA with the similar conformation. But the multiple cis bonds present in arachidonic acid lead to different binding configuration at some binding sites which can be further responsible for different binding affinities of both the fatty acids for the major binding sites (Petitpus et al., 2001).

PIP i.e. Prolactin inducible protein is a glycoprotein and it has a vital role in many biological processes like fertility, antimicrobial activity, apoptosis etc. HSA in complex with PIP was studied using chromatographic techniques, gel electrophoresis, MALDI-TOF mass spectrometry and co-immunoprecipitation and western blotting techniques. In silico protein-protein docking was used to see the interacting residues and behavior was analysed by dynamic light scattering. The complex showed many intermolecular hydrogen bonds which maintain its stability. HSA preserve the motility of sperm so its strong binding with PIP showed a direct correlation with male fertility/infertility (Kumar et al., 2012).

Bhattacharya et al. (2000) described about the high resolution crystal structure of HSA with two most popular anesthetic drugs, propofol and halothane alongwith a fatty acid, myristate. After proper analysis, it was seen that propofol bound to the two different sites and halothane bound to three distinct sites but those were the same binding sites where fatty acid used to bind. Increase in halothane concentration induced it to occupy some other sites. Results inferred that all the
high affinity binding sites of anesthetics are of amphiphilic nature, have polar and apolar residues at the binding sites and induce only slight reshaping of the local structure of the albumin after binding.

Various computational studies had also been done to study the interactions of HSA with many drugs. Interactions of betulinic acid with human serum albumin (HSA) were studied using molecular docking and molecular dynamics simulations. Betulinic acid is a natural product having anti-cancer, anti-inflammatory and anti-retroviral properties. Molecular docking studies of HSA and betulinic acid showed that betulinic acid bound at sub-domain IIA and IIB of drug binding site I of HSA by hydrogen bond and hydrophobic interactions and it was found to interact with the residues of the binding pocket. Betulinic acid interacted with Phe206, Arg209, Ala210, Ala213, Leu327, Gly328, Leu331, Ala350 and Lys351of HSA by hydrophobic interactions and Phe206 and Glu354 of HSA showed hydrogen bond interactions. Molecular dynamics simulation helped in understanding the conformational changes came in HSA after binding to betulinic acid and the stability of the protein-ligand complex in water. This work may help in understanding the binding of betulinic acid with HSA and in designing new drugs with structure similar to betulinic acid (Malleda et al., 2012).

Keshavarz and co-members studied the interaction of a group of anti-cancer drugs with HSA by molecular docking and molecular dynamics simulation. Activities of the drugs were analysed in terms of their docking scores, binding sites and structural descriptors. The results showed that all the drugs bind to the cavity 1 which is present in the cleft between domains I and III and is the major binding site for the drugs. This is a huge spaced cavity with minimum or no steric hindrance which helped in the stability of the HSA-drug complexes by inducing different type of interactions with the binding site residues. Still, some drugs strongly bind to other cavities also instead of cavity 1 because of specific structural features. So, steric factor plays a pivotal role in transporting of drugs by HSA (Keshawarz et al., 2012).

Garg et al.(2013) studied the interactions of synthesized coumarin derivatives with HSA at pH 7.2 by using various methods. Coumarin is an anti-oxidant, anti-cancer, anti-coagulant drug and also used to cure many diseases like arthritis, asthma, inflammation etc. Fluorescence spectroscopy showed that maximum fluorescence intensity was decreased after the binding of coumarin derivatives to HSA. And CD spectroscopy results revealed that secondary structure of HSA was partially unfolded upon addition of coumarin derivatives. Molecular docking studies
contributed in knowing that coumarin derivatives bind at the sub-domain IB with hydrogen bonds and hydrophobic interactions. Molecular dynamics helped in understanding the stability of the HSA-drug complex in the water and conformational changes came in HSA after binding to drug. The whole work focused on understanding of binding of coumarin to HSA and to design coumarin inspired drugs to fight against various dangerous diseases.

2.7 Extracellular Receptor Kinases
Ras/Raf/MEK/ERK signal cascade is an important signal pathway activated by number of extracellular stimuli which transmit signals to other substrates (Hao et al., 2011). MAPKs are the important enzymes for signaling system and driving number of cellular functions like growth, differentiation, stress and apoptosis (Almog et al., 2008). MAP kinases are class of serine or threonine kinases which work as a critical intermediary of signal transduction and cause serious diseases like asthma and rheumatoid arthritis, chronic inflammatory autoimmune disease etc. (Choy et al., 2001, Dinarello, 1991, Palladino et al., 2003). ERKs are principal signaling proteins whose function is to phosphorylate different proteins after binding with them. CML-modified albumin does quick temporary activation of extracellular signal-regulated kinase1 and 2, p38 mitogen-activated protein kinase and tyrosine phosphorylation, except c-Jun NH2-terminal kinase (Yeh et al., 2001).

Fantz et al.(2001) studied that ERK binds with the substrate proteins with the help of docking sites having FXFP motif and the D-domain. A considerable binding affinity was noticed at position 1 and 3 after substituting phenylalanines there in contrast to position 2 and 4. These FXFP motif and the D-domains present in the proteins like ELK-1 and KSR-1 were also analysed in different positions and arrangements. The results of this work showed that FXFP motif and the D-domain docking sites perform two major functions: first is to regulate the affinity of substrate for ERK by changing the number, type, position and arrangement of residues of these docking sites and second is to direct the phosphorylation of specific serine or threonine residues. Like in ELK-1, FQFP motif is sufficient to direct phosphorylation of serine 383 while D-domain leads to the phosphorylation of other(serine/threonine) residues.

As male and female need various patterns of gonadotrophin secretion for the fertility. The process behind these gender specific profiles of gonadotrophin is unknown but these are important for the understanding of sexually dimorphic control of reproductive system. Various
studies showed that ERK 1 and 2 are the pivotal modulators behind the production of pituitary gonadotrophin and the reproduction. In a study, a variety of physiological parameters including fertility were observed in a mice generated with deficiency of ERK 1 and 2. It was find out after a study that ERK signaling is needed in ovulation and fertility in females while male reproductive system doesn’t need this signaling. The results represent that ERK is the basis of gender specific regulation of reproductive system and sexual dimorphic control of fertility (Bliss et al., 2009).

Mature spermatozoa need progressive motility only after ejaculation and when entered inside female reproductive tract progressive motility is suppressed. Almog et al.(2008) reported that ERK1/2 and p38MAPK, both are located in the tail of matured human spermatozoa while c-Jun N-terminal kinase 1/2 was not found to be expressed. ERK1 and 2 stimulation is downstream to protein kinase C(PKC) activation which is also present in the tail of male sperm. Both the enzymes ERK1/2 and p38 helps in inhibition of motility of sperm and involved in acrosome reaction. Inverse correlation was found to be obtained between the ERK1 expression or relative p38 activation and sperm morphology, progressive and forward sperm motility, so poor sperm quality can be predicted due to high expression of ERK1 and activation of p38. Seo et al.(2010) stated that ERK is involved in promoting osteolysis by identifying the ERK pathway as a chief osteolysis pathway with elucidation of pro-inflammatory capacity of osteoblasts. PD98059, an ERK inhibitor helps in inhibiting inflammatory reactions induced by different cell types which elucidated the importance of ERK signaling in cell mediated inflammatory osteolysis and osteoblastic innate immunity. All these findings increase the understanding about inflammatory osteolysis and support discoveries of new targeted therapies against ERK to treat osteolytic diseases.

Tubular necrosis in acute kidney failure is related to excessive production of reactive oxygen species but the process behind ROS induced tubular necrosis has not been completely understood (Zhuang et al., 2008).

2.8 Nuclear Factor kappa B

Not only ERK pathway, but also nuclear factor-κB(NF-κB) has a pivotal role in the understanding of inflammatory reactions (Maeng et al., 2006). NF-kappa B is a heterodimeric
transcription factor and plays a vital role in inflammatory and immune responses (Laird et al., 2000, Sakamoto et al., 2003, Ponce et al., 2009). Catalytic subunits i.e. IKK alpha and IKK beta and a regulatory subunit, IKK gamma (an essential modulator of NF-κB) combines to form IKK complex (Perkins 2000, Solt and May, 2008, Hayden and Ghosh, 2008). Out of both catalytic subunits, IKK beta is crucial for NF-κB activation particularly in response to proinflammatory activities. NF-kappa B dimers are located in cytoplasm by interacting with inhibitory proteins i.e. I kappa Bs. After the cell stimulation by proinflammatory cytokines, a multi-subunit protein kinase and I kappa B kinase (IKK), NF-κB is activated and phosphorylates two critical serines located at the N-terminal regulatory domain of I kappa Bs.

Schreiber et al. (1998) investigated whether the increased activation of NF-κB is essential in inflammatory bowel disease (IBD) and anti-inflammatory treatment could down-regulate this. The study showed increased nuclear levels of NF-κB p65 in lamina propria biopsy samples taken from patients with Crohn’s disease and increased activation of NF-κB in lamina propria mononuclear cells obtained from patients with active IBD. Corticosteroids stabilize the cytosolic inhibitor I kappa B alpha against induced degradation which confirms its inhibition of NF-κB activation both in-vivo and in-vitro experiments. The whole work suggests that increased activation of NF-κB may play an important part in the regulation of inflammatory responses in both IBDs. And inhibition of NF-κB activation may represent the process by which steroids can induce an anti-inflammatory effect in IBD.

In a study, the neuronal cultures were exposed to chemical ischemia i.e. iodoacetic acid, followed by reperfusion (I/R insult) due to which neurons were injured and it manifested the increased LDH release and decreased cellular content of IκBα which further induced NF-κB activation. The antioxidants and and inhibitors of NF-kappaB activation protected the neurons against the insult and prevented the decrease in cellular IκBα alpha content. In contrast, inhibiton of NF-κB translocation by SN50 in both uninsulted and insulted neuronal cultures resulted in a 2.9 fold and 2.4 fold increase in LDH release, respectively. The results indicated that the insult-induced oxidative stress activates transcription factor NF-κB associated with the induction of protection and suggested that constitutive activation of NF-κB under physiological conditions act to protect the neurons against physiological injury (Kratsovnik et al., 2005).
2.9 Antioxidant enzymes

Reactive oxygen species i.e. ROS like O$_{2-}$, H$_2$O$_2$ and .OH formed due to partial reduction of oxygen cause toxicity inside the cells (Tan et al., 1998, Sharma et al., 2012). MAPK pathways like ERKs or p38 MAPKs or JNKs are not only affected by receptor-ligand interactions but also by different factors present in the cell and one of the factor which can induce activation of MAPK pathways is oxidative stress occurred due to reactive oxygen species (ROS). When ROS production is increased, ERKs and other MAPK pathways become activated (Son et al., 2011). Cellular ROS can be generated either by the mitochondrial oxidative phosphorylation process or interactions with exogenous sources. ROS affects the cellular defense system either by its elevated levels or decrease in antioxidants, oxidative stress occurs (Vasdev et al., 2006). Oxidative stress is the direct or indirect damage of biomolecules like proteins, lipids and nucleic acids and can lead to pathological conditions (Bandyopadhyay, 1999, Ray et al., 2012).

Catalase helps in regulating intracellular levels of hydrogen peroxide and hydroxyl radical, so role of catalase deficiency in progressive renal fibrosis was investigated (Ismail et al., 2012, Sindhu et al., 2005). Homozygous acatalasemic mutant mice and control wild-type mice were nephrectomized and EMT peroxidation, antioxidant enzyme activity and gene expression of EMT-related molecules were compared between the two groups after different time intervals. Nephrectomized mice showed albuminuria, decreased renal function and tubulointerstitial fibrosis and these changes were significant in acatalasemic mice as compared to wild-type mice. EMT peroxidation, tubulointerstitial deposition of lipid peroxide products and glomerular sclerosis were found to be developed in acatalasemic mice. Low catalase activity was observed in the remnant kidneys of acatalasemic mice without compensation of glutathione peroxidase and superoxide dismutase activity. The work suggested that catalase deficiency lead to renal oxidant tissue injury and progressive renal fibrosis which concluded the importance of catalase in the defense against oxidant-mediated renal fibrosis (Kobayashi et al., 2005).

GPx (Glutathione peroxidase) is an essential antioxidant enzyme which catalyze the reduction of both organic and hydrogen peroxides. The main source of GPx activity is kidney proximal tubular cells and oxidative stress participate in increasing renal disease complications (Cachofeiro et al., 2008, Locatelli et al., 2003). Randox commercial kits were used to observe the erythrocyte and plasma GPx in 12 patients suffered from nephroticsyndrome(NS), 48 patients with renal impairment(RI) and 50 patients with chronic kidney failure on hemodialysis(HD) and
50 healthy individuals served as controls. It was observed that plasma GPx activity was reduced in the HD group and RI group while there was no significant difference in comparison to the controls. After hemodialysis, erythrocyte GPx activity was almost similar as it was before hemodialysis while plasma GPx activity was increased after hemodialysis. It was concluded from the results that plasma GPx activity is very important to assess the oxidative damage among patients with renal complications. So, plasma GPx activity mainly relies on physiological renal function but not on erythrocyte GPx activity (El-Far et al., 2005).

In a study, high GPx activity was found in dialysis patients while healthy individuals had low plasma GPx activities. The plasma activity could be precipitated by anti-extracellular GPx activity in normal individuals and plasma GPx activity rises after kidney transplantation but reached to normal within 10 days. Since extracellular GPx in the kidney is primarily synthesized in the proximal tubules, so nephrotoxins were used to disrupt proximal tubule function to assess the affected GPx activity (Perazella, 2009). Beta-lactam antibiotic cephaloglycin rapidly decreased plasma GPx activity in rabbits whereas ifosfamide decreased plasma GPx activity in pediatric osteosarcoma patients (Whitin et al., 1996, Saito et al., 1994). All these interpretations showed that plasma GPx activity is associated with renal function and nephrotoxic drugs can decrease its activity. So plasma GPx activity can be monitored not only to predict the function of transplanted kidneys but also to assess the patients with a high risk of nephrotoxic injury (Whitin et al., 1998).

ROS has been found to be involved in causing various diseases and one of them is male infertility. As mammalian spermatozoa contain large amount of polyunsaturated fatty acids and are easily attacked by ROS and membrane lipid peroxide ion. A balance is maintained between ROS produced and scavenged but cellular damage occurs due to oxidative stress when the equilibrium gets disturbed (Vasdev et al., 2006). Dandekar and Parker (1999) took ejaculates from 83 infertile and fertile healthy individuals and studied the correlation between lipid peroxidation and antioxidant enzymes like catalase, GPx and SOD using water test in male fertility. Results showed that catalase showed no significant change in the pathological samples while SOD and GPx show significant changes and the degree of lipid peroxidation also correlated positively with the poorly swollen sperm tails. Increase in the levels of SOD and GPx in pathological cases indicated the attempt made by them to overcome ROS levels. So, water test
could be an effective test to assess sperm tail damage by ROS as it correlates nicely with lipid peroxidation and antioxidant enzymes (Dandekar et al., 2002).

2.10 Molecular docking and Molecular Dynamics simulations

The integration of computational strategies like molecular docking has been of great value not only in the study of ligand-receptor interactions but also in the identification and development of novel therapeutic compounds. Molecular docking is one of the most frequently used methods for predicting the conformation of small ligands within the appropriate target binding site with a degree of accuracy (Meng et al., 2011, Ferreira et al., 2015). Essential molecular events such as ligand binding modes and the corresponding inter-molecular interactions that stabilize the ligand-receptor complex can be easily done through molecular docking (Huang and Zou, 2010). Moreover, docking programs perform quantitative prediction of binding energies to rank the docked compounds on the basis of binding affinity of ligand-receptor complexes (Huang and Zou, 2010, Lopez-Vallejo, 2011). Most of the docking programs have the ability to successfully predict the appropriate conformation of the ligand inside the target binding site that can be confirmed by comparing predicted complexes with their corresponding crystallographic data. The aim of protein-ligand docking is to predict and rank the structure(s) arising from the association of a given ligand and a target protein of known 3D structure (Sousa et al., 2006). Efficient searching of algorithms involves in the solving of all docking problems which cover the relevant conformational space and selective scoring functions. However, protein-protein docking is also related to recognition, cellular pathways and macromolecular assemblies (Halperin et al., 2002).

Chong et al. (2011) determined interactions between HIV-1 gp120 and mutated CD4 proteins so as to identify a lead structure for therapy based on competitive blocking of the HIV binding receptor in human T-cells. The initial structures were energy minimized in Gromacs and then subjected to docking experiments using different docking tools like AutoDock4, FireDock, ClusPro and ZDock. The Gibbs free binding energy was calculated by molecular dynamics for the gp120-CD4 complexes. The results concluded that ligand should have an extended and conformational flexible aromatic group i.e. biphenyl for efficient blocking of HIV gp120. Docking of Triazine analogues that are MAP kinase inhibitors were performed by using AutoDock along with the calculation of binding energies and regression analysis. It was found
that presence of morpholino or anilino ring is important for the binding of inhibitors and newly
designed compounds to fit inside the binding pockets of MAP kinases (Das et al., 2012).
Molecular dynamics (MD) simulations have been used to interpret experimental data in terms of
detailed atomistic model and map them into the protein structure (Yesylevskyy and Hushcha, 2012). It mimics the physical motions of atoms of a macromolecule present in the natural
environment of a cell. The atoms are allowed to interact for a specific period of time that would
help in computing the trajectories of the molecule. MD simulation helps in getting detailed
information of individual motion of each atom of molecule as a function of time (Patodia et al.,
2014). MD simulations provide the deep understanding of natural dynamics on different time-
scales of protein-ligand complex as well as single protein in solution and calculate thermal
averages of various molecular properties (Malleda et al., 2012). MD simulations use molecular
mechanics force fields to compute the potential energy of the system. Molecular force fields that
are widely used for biomolecular simulations are AMBER03 (Duan et al., 2003), AMBER94
(Cornell et al., 1995), AMBER96 (Kollman, 1996), CHARMM27 (Foloppe and MacKerell,
2000), OPLS-AA (Kaminski and Friesner, 2001), GROMOS87 (Oostenbrink et al., 2004) and
GROMOS96 (Schuler et al., 2001). MD simulation provides a platform for the study of protein–
protein, protein–ligand and protein–nucleic acid interactions (Patodia et al., 2014).

In a study, the interactions between a polyphenolic compound, trans-resveratrol and Bovine β-
lactoglobulin (BLG) were observed by molecular docking and molecular dynamics simulation
methods. Molecular dynamics study has been proved essential in contributing towards knowing
the effect of binding of resveratrol on different conformations of BLG and stability of the
complex in aqueous solutions. Molecular docking predicts that ligand bound to the surface of
BLG by forming two hydrogen bonds. Molecular dynamics studies explained the root mean
square deviation values and radius of gyration of BLG and BLG-resveratrol complex which
reached at equilibrium after a specific simulation time. Another property of rms fluctuations
explained the rigidity of the ligand binding site during the simulation (Sahihi et al., 2013).
Active site of ERK2 was also investigated through molecular docking and molecular dynamics
analysis (Lindin et al., 2014). A 3D QSAR pharmacophore (Hypo 1) model with high correlation
was developed for ERK2 ATP site according to the structure of known inhibitors bound at the
site. Fisher randomization, cost analysis, leave one out method and decoy test were used to
confirm that model can reliably predict ERK2 inhibitors. Various databases were interrogated for
the compounds meeting the pharmacophore features by using Hypo1 as a query. The resulting compounds were then analysed through docking and molecular dynamics methods. It was observed that higher potency compounds should interact with catalytic site, glycine rich loop, hinge region and ATP site entrance residues (Larif et al., 2014).

Benyamini et al.(2009) used protein docking and molecular dynamics methods to obtain the structure model of the complex. Results showed that ASPP2 bind to the NF-κB at the same two sites where its natural inhibitor, IkappaB binds and also the complex was energetically similar to the NF-κB-IkappaB complex which further suggested the novel role of ASPP2 as an NF-κB inhibitor.

Important antioxidant enzyme, catalase was also studied for its affinity for potent natural compounds to keep it active. Two natural products i.e. hesperidin and 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside (THSG) were found which showed high binding affinity with catalase. The results showed that THSG is the most stable in trajectory analysis and it can make the structure more compact during the MD simulation run. So, it was stated that natural TCM compound, THSG is the most potent compound and can be used to make drugs having similar effect to keep catalase in active form (Huang et al., 2015).
2.11 Databases used to access ligands and receptors information

2.11.1 NCBI

National Center for Biotechnology Information i.e. NCBI is a division of the National Library of Medicine (NLM) at the National Institutes of Health (U.S.). NCBI contains a list of databases related to biotechnology and provides distribution of biomedical information and bioinformatics tools like BLAST (Basic Local alignment Search Tool) for sequence alignment. Important Molecular biology databases are GenBank for DNA sequences, PubMed for biomedical literature. They also provide sequence similarity searching tools and entrez search and retrieval tools. The databases and resources have been organized into seven major areas of literature, genomic data, variation, health, genes and their expression, nucleic acids, proteins, and small molecules and biological assays (http://www.ncbi.nlm.nih.gov/books/NBK143764/).

![Fig2.6](image_url) Homepage of NCBI

2.11.2 Protein Data Bank

PDB (Protein Data Bank) was established in 1971 by Brookhaven National Laboratory under the
leadership of Walter Hamilton. It is a worldwide repository that provides information about three dimensional structures of macromolecules including proteins and nucleic acids. Initially, it contained only 7 structures. Structure of a molecule helps in deducing its role in human diseases and drug development. As it is a member of the wwPDB, therefore RCSB PDB also curates and annotates PDB data. The structures present in PDB range from tiny peptides to complex protein structures and bits of DNA to complex molecular machines like ribosomes. Presently it contains 36987 distinct protein sequences, 30047 structures of human sequences and 8085 nucleic acid containing structures (http://www.rcsb.org/pdb/, Berman et al., 2000). In addition to this, RCSB PDB also supports a website where users can ask queries about their data and visualize the results. RCSB PDB can be accessed at http://www.pdb.org.

Fig 2.7 Home page of RCSB Protein Data Bank
2.11.3 Drug Bank

Drug Bank is a highly annotated resource that combines detailed data of drug along with comprehensive information about drug targets and drug actions. It was first released in 2006 and has been widely used in facilitating in silico drug target discovery, drug designing, drug docking and screening, prediction of drug metabolism and drug interaction and general pharmaceutical education since that time. DrugBank is a clinically oriented drug encyclopedia with ability to
provide detailed, updated, quantitative, analytic and molecular information about drugs, drug targets and also the biological and physiological outcomes of drug actions. Also it is chemically oriented drug database that provides many built-in tools for viewing, sorting and searching and also extracting text, image, sequence or structure data (Wishart et al., 2008). Drugbank can be accessed at http://www.drugbank.ca/.

![DrugBank](image)

**Fig 2.9** Home page of Drug Bank

### 2.11.4 PubChem

PubChem is an open database providing chemical substance description, biological activities and biomedical annotations. PubChem is composed of three distinct and interrelated primary databases: Substance, BioAssay and Compound. The Substance database (SID) includes depositor-provided description information of sample that includes chemical depictions, chemical names or synonyms, external registration IDs, comments and cross-links. The BioAssay database (AID) includes depositor-provided experimental result information that combines experiment description, experimental protocol, and results for the chemicals of SIDs tested in a biological assay (Fu et al., 2015). Compound database has been used to aggregate
information from number of PubChem contributors using the chemical structure as the key. The As a part of this, each structure which is recorded with chemical information is conducted with a validation and normalization procedure to ensure that the chemical structure is well-defined, makes chemical sense and provide a standard chemical representations (Bolton et al., 2008, Bolton et al., 2011). NCBI PubChem can be accessed at http://www.ncbi.nlm.nih.gov/pccompound.

![Home page of NCBI PubChem](image)

**Fig2.10** Home page of NCBI PubChem

### 2.12 Computational tools
#### 2.12.1 AutoDock
AutoDock is a set of automated docking tools that is designed to predict the way of binding of small ligand molecules like substrates or drug molecules to a receptor with known 3D structure (http://autodock.scripps.edu/). AutoDock uses a grid-based method to allow rapid assessment of the binding energy of trial conformations of ligands and searching the large conformational
space for the ligand around the protein (Morris *et al*., 2009). Current package of AutoDock consists of two softwares, AutoDock 4 and AutoDockVina. AutoDock 4 further comprised of two main programs: autodock, which performs the docking of the ligand to a group of grids describing the target protein and autogrid that pre-calculates these grids. The atomic affinity grids can be visualised which helps in guiding organic synthetic chemists to design better binders for the receptors. In AutoDockVina, there is no need to choose atom types and pre-calculating grid maps because it calculates the grids internally for the required atom types. AutoDock is available free of cost under the GNU General Public License. And, AutoDockVina is available under the Apache license with commercial and non-commercial use and redistribution (http://autodock.scripps.edu/).

### 2.12.2 RASMOL

RasMol (shadowed RASTERMOLECules) is a graphical visualization software for visualizing molecules having PDB (Protein Data Bank) format. It was originally developed by Roger Sayle and is available for Windows, Macintosh and UNIX operating systems. It displays the molecule in different representations wireframe, spacefill, alpha-carbon backbone, strands, ribbons and Richardson style cartoons and allows to rotate the molecule interactively (Sayle and Milner-White, 1995, www.openrasmol.org/). RasMol-ucb allows viewing of multiple molecules simultaneously. Rasmol can be accessed at www.RasMol.org

### 2.12.3 PyMOL

PyMOL is a molecular modeling program that is used for the construction and 3D visualization of macromolecules either proteins or protein–ligand complexes. PyMOL is also used to visualize .pdb (Protein data bank) files having refinement of a crystal structure. A refinement is a three dimensional structure of a protein that is based on electron density maps obtained directly through X-ray crystallography. PyMOL helps in gaining better understanding of the relationship between protein structure and binding and also better understanding of biochemical literature (https://www.pymol.org/).

### 2.12.4 PEARLS

PEARLS (Program for Energetic Analysis of Ligand-Receptor Systems) is a Web-based online tool developed by BIDD (Bioinformatics and Drug Design group) and used to calculate interaction energies of ligand-protein, protein-nucleic acid, ligand-nucleic acid and ligand-protein-nucleic acid complexes from their 3D structures. These interaction energies provide the
quantitative perception of interactions between molecules that has major role in regulating the function and conformation of proteins. It has also been widely used in the ranking of potential new ligands for virtual drug screening (Han et al., 2006).

2.12.5 SWISS-MODEL

Swiss-Model is an online server used for automated comparative modeling of three dimensional protein structures. It was started in 1993 and is the most widely used free web-based automated modeling tool today. Template selection, alignment and model building are the three main steps done completely automated by the server. In the alignment mode, the modeling process is based on alignment of a target template defined by the user. Complex modeling tasks are handled with the ‘project mode’ using DeepView (Swiss-PdbViewer). All models are sent back to the user via email with a detailed modeling report. All the homology-modeling methods constitute the following four steps:- template selection, target template alignment, model building and evaluation (Schwede et al., 2003).

![Fig 2.11 Home page of Swiss-model](image-url)
2.12.6 SwissParam
SwissParam is a fast force field generation tool capable of generating topologies and parameters for arbitrary small organic molecules based on the Merck molecular force field that is compatible with the CHARMM force field. Computational methods of structure-based drug discovery mainly involve ligand-protein docking and rapid estimation of binding free energy and both require force field parameterization for drug candidates. Output files can be used with CHARMM or GROMACS simulation programs. It uses a rapid binding free energy calculation approach by using SwissParam for ligands and CHARMM22/27 for proteins. SwissParam produces topologies and parameters to describe smaller organic molecules in computer-aided drug design applications along with CHARMM22/27 description of the target protein (Zoete et al., 2011). SwissParam is freely available for academic users at www.swissparam.ch.

![SwissParam Home page](image)

**Fig 2.12** Home page of SwissParam

2.12.7 PRODRG Server
PRODRG is small-molecule topology generator which takes input from existing coordinates or two-dimensional formats and automatically generates the coordinates and molecular topologies. Tests done in crystallographic refinement showed that topologies generated by PRODRG are
generally of equal quality and better than topologies generated by other means. It is also noted that the variety of topologies generated by PRODRG permits the use of consistent descriptions of any molecule in all the steps of inhibitor designing from crystallographic refinement and visualization through structure analysis and also in molecular dynamics or docking studies (Schuettelkopf and van Aalten, 2004).

![PRODRG Home](image)

**The GlycoBioChem PRODRG2 Server**

PRODRG will take a description of a small molecule (as PDB coordinates, MOL file, SYBYL Mol2 file, etc.) and from it generate a variety of topologies for use with GROMACS, WHAT IF, Autodock, CNS, REFMAC5, SHELX, O, and other programs, as well as energy-minimized coordinates in a variety of formats.

Please note that this server is strictly for academic use (max 5 submissions/day) only. For more extensive or commercial use you can obtain your own copy of PRODRG.

A list of some frequently asked questions is available. Please have a look at it if you are having problems with PRODRG’s output. If that does not help, or you have other comments/suggestions, feel free to email [Dean van Aalten](mailto:dean.van_aalten@ub.edu).

When using PRODRG-generated files in a publication, please cite:


Get started...

**Fig 2.13** Home page of PRODRG Server

### 2.12.8 GROMACS

GROMACS stands for Groningen machine for chemical simulations and has a vast collection of programs and libraries for the molecular dynamics simulation studies and in-depth analysis of trajectory data. Apart from normal potential functions like Lennard-Jones, Buckingham and Coulomb, spline-interpolated tables are also used for arbitrary forms of interactions. Simulations can be performed with or without periodic boundary conditions in a general triclinic cell which may be rectangular/rhombic/dodecahedron or truncated octahedron. The interface is simple and easy to use that works by standard command line arguments with self-explaining functionality.
and integrated documentation (Lindahl et al., 2001, Pronk et al., 2013). A wide collection of flexible tools for trajectory analysis are available in GROMACS and gives output in the form of Xmgr/Grace graphs (http://www.gromacs.org).

**2.12.9 XmGrace**

XmGrace is a two dimensional graph plotting tool used in Unix-like operating systems. Grace stands for Graphing, Advanced Computation and Exploration of data which uses the X Window System and Motif for its GUI. It is a descendant of the ACE/gr (Xvgr), a plotting tool based on Xview libraries of Open Windows. Xmgrace is a useful plotting package available with the linux operating system and makes certain choices automatically such as numerical ranges of the horizontal and vertical axis, colors of the line segments connecting the data points etc. Features of Xmgrace include a point-and-click GUI (graphical user interface), precise control in graph features, publication quality and instant plot refresh. It also support a number of vector and raster file formats and can be called from C and Fortran programs. Other features of the tool are unlimited number of graphs, upto 256 customizable colors, 9 dashed line styles, 32 fill patterns and text annotations with subscripts, superscripts, mixed fonts and styles (http://plasmagate.weizmann.ac.il/Grace, Motorola, 1996).