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COMPUTATIONAL INVESTIGATION OF PESTICIDE INDUCED OXIDATIVE STRESS AND ITS IMPACT ON THE CHRONIC KIDNEY DISEASE OF UNKNOWN ETIOLOGY (CKDU)

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Abstract: The chronic kidney disease of unknown etiology (CKDu) has been a major health issue in Sri Lanka within the last three decades. Many investigative efforts have been carried out to identify its unknown origin and several risk factors which have been associated. A possible link between oxidative stress and the progression of the disease has been identified. The environmental factors which favor the development of oxidative stress are prevalent in those affected areas. The study of "pesticide induced oxidative stress" has been a topic of research interest. Alterations in the balance between the production of free radicals and the antioxidant defenses were recognized as one of the main causes. Four major pesticides were docked with different enzymes which directly related to mechanisms in generating oxidative stress, using Auto Dock molecular docking program. The strength of the binding of the pesticide in the binding site of the corresponding enzyme was used to emphasize its potential interaction with Cytochrome P450 A34enzyme. According to molecular docking investigations, it was evident that three organophospahates; Profenofos, Diazinon and Chlrofyrifos possessed relatively similar binding energies at the active site compared to the inducer for Cytochrome P450 A34 enzyme. These organophosphates behave as the potent enzyme inducers as well as substrates which involved in bio-activation. The computational findings directed to disclose how reactive oxygen species were generated to cause oxidative stress and it can be utilized to predict mechanistic steps related to the pesticide induced oxidative stress.
Keywords: CKDu, oxidative stress, organophosphates, cytochrome P450.

INTRODUCTION

Chronic Kidney Disease (CKD) is the loss of kidney function over a period of time. It is a global health problem where diabetes, hypertension, and the various forms of glomerulonephritis are well-recognized etiologies [1]. The disease is said to be "chronic" because it takes many years for symptoms to develop. The etiology is uncertain to the date; hence the disease is known as the Chronic Kidney Disease of uncertain etiology (CKDu). This has been reported in Sri Lanka, several Central American countries, Andhra Pradesh in India and the El-Minia Governorate in Egypt [2]. The disease is characterized by substantial morbidity and mortality, resulting in death of adult patients. Progression of CKDu is

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generally symptomless until the advanced stages of disease where the kidneys are damaged irreversibly resulting in mortality unless dialysis or transplantation occur.

Given its unknown origin, CKDu has spurred a variety of investigative efforts in recent years. A number of research studies have been completed to identify the risk factors. But, up to the present date, no specific reason has been proven scientifically to be the exact cause. There are so many risk factors related to environment, agricultural work and farming practices, diet and nutrition, and genetics are being suspected for the contribution for CKDu in the areas which are considered as the hot spots for CKDu [3]. Pesticides, fertilizers, heavy metals (Cd, As, Pb) [4,5], fluoride concentration and hardness of water and cyanobacteria toxins are considered as some of reported factors clinically investigated at those CKDu reported areas. These factors include, the negative effects from overuse of agrochemicals, the effect of heavy metals and other xenobiotic agents present in the environment. The potential interactions and synergism between probable agents have not been studied thoroughly.

Within the area of research interest on the multi factorial origin of CKDu, the condition "oxidative stress" can be given a special importance. The experimental evidence from research done globally as well as in Sri Lanka highlights the relationship between Oxidative stress and CKDu [6,7]. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. It occurs when excess reactive oxygen species produced in cells overwhelm the normal antioxidant capacity. Then, the internal defense mechanisms such as antioxidants fail to prevent the oxidative damage to proteins, lipids, and DNA, which could lead to failure of many biological systems [8].

In relation with CKDu in Sri Lanka, the up-regulation of oxidative stress related genes in CKDu prevalent areas has been revealed [9]. The study suggests that the expression of the GCLC gene which in relation to the glutathione (GSH) protein, was seen to be up-regulated more than 3.16-fold (compared to healthy individuals from non- CKDu areas) in all CKDu patients. GSH functions as a major cellular anti-oxidant which helps to maintain the balance between ROS and antioxidant defenses [10,11]. GCLC catalyzes the rate-limiting step in the production of GSH and synthesis of GSH is not possible in the absence of GCLC. Its activity is highest in the liver, followed by the lungs and kidneys. The GSH effect depends on the balance of depletion, regeneration, and synthesis of the molecule and when the GSH is depleted, the GSH synthesizing systems start making more GSH [12]. The increased production of this enzyme indicates increased production of GSH to counter oxidative stress.

The healthy group from the area had the highest expression of this gene at 7.27-fold upregulation and this is can be a reason for them to be healthy as they have more GSH to counter oxidative stress.

As the majority of the CKDu patients are from rural farming communities, pesticide exposure is suspected as a cause, although it is not a proven etiological factor for chronic renal failure. Many commonly used pesticides in the world have reported to contribute for oxidative stress [13].The increase of production of ROS or decrease in the antioxidant defenses need be responsible for these outcomes. Pesticide-induced oxidative stress as a possible mechanism of chronic toxicity of pesticides has been a focus in toxicological research. But, the mechanisms have not been completely understood yet as research suggests [12]. Cytochrome P450 (CYP) 3A4 is the most abundant hepatic and intestinal phase-I enzyme that metabolizes approximately 50% marketed drugs. A number of important drugs have been identified as substrates, inducers and/or inhibitors of CYP3A4 [14].

Various mechanism for reactive oxygen species generation during cytochrome P450 mediated metabolism of various drugs and endogenous molecules are found in literature. ROS are generated in the intermediate steps of the catalytic cycle. The cytochrome P450 system can give rise to ROS by futile cycling, if it is unable to metabolize a substrate, via an uncoupling of the normal catalytic cycle. Therefore, if the pesticides have the ability to alter the activity of the enzyme, continuous exposure to these classes of pesticides would affect to cause oxidative stress [15].

Organophosphorus pesticides are the most frequently used pesticides in the world, with a wide range of spectrum. This is a group of pesticides with the highest acute mammalian toxicity that is used in Sri Lanka. Profenofos, Diazinon, and Chlorpyrifos (shown in Figure 1) are commonly used organophosphorus pesticides globally. Profenofos and Diazinon are still used in large scale in CKDu hotspot areas at present. Chloropyrifos was also used in large scale until it was banned in 2014 in Sri Lanka. Chloropyrifos was also included in the study to compare its toxic effects. Although it was banned, it might still remain as residues in the environment due to the poor regulation of pesticides among the community. Studies done in 2011 confirmed that these three pesticides were the ones detected most frequently as pesticide residues in the environment [16].The other pesticide; Imidacloprid has also been in frequent use in CKDu prevalent areas for many years. Studies have shown that these pesticides significantly contributed towards “pesticide induced oxidative stress” in animal studies [15].

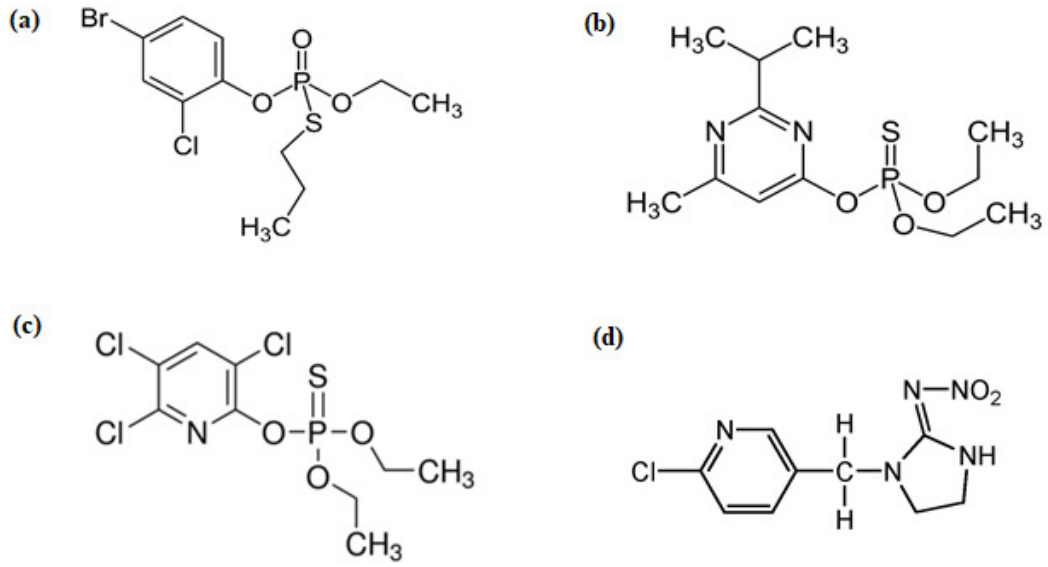

Figure 1. Chemical structures of (a) Profenofos;(b) Diazinon;(c) Chloropyrifos and (d) Imidacloprid.

Ketoconazole is a drug which is known to be a strong inhibitor of CYPA34 while Phenobarbital is a drug which has the capability to induce CYPA34. So they have been used in the docking studies as reference compounds to compare it with the binding site and the binding affinities of the pesticides [17].

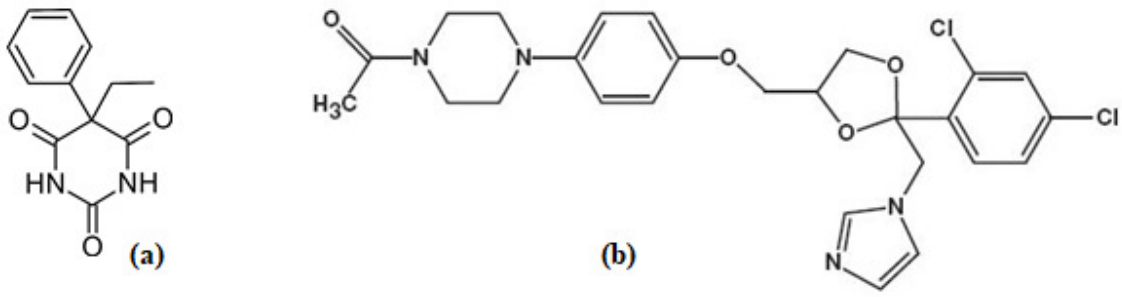

Figure 2. Chemical structures of (a) Phenobarbital (CYPA34 inducer) and (b) Ketoconazole (CYPA34 inhibitor)

Molecular docking is used to investigate how molecules of organophosphate pesticides interact with the Cytochrome P450 A34 which is one of the major human metabolic enzymes. The types of possible interactions calculated can be used to predict their toxic effects on human metabolism. The free energy of binding and the inhibition constant (K_I) were used as major thermodynamic parameters to investigate how pesticides interact with the Cytochrome P450 A34 enzyme. The inhibition constant (K_I) is the concentration required to produce half

maximum inhibition. The smaller the K_I, the greater the binding affinity and the smaller amount of substrate needed in order to inhibit the activity of that enzyme.

MATERIALS AND METHODS

Linux version of Autodock4 computer software package was employed using the desktop computer having Linux Mint 16 Cinnamon 64 bit operating system at a processing speed of 3.3-3.6 GHz with an Intel© Core i7 5820K CPU, 32 GB RAM. To prepare the input files and to view the docking results, Linux versions of Pymol molecule viewer, UCSF Chimera molecule viewer and Avogadro software packages were used. To investigate the binding of active compounds constituted in pesticides with the target enzymes, the free energies of binding and inhibition constant values were computed.

Preparation of input files

The protein data bank (.PDB) files for the corresponding enzymes were obtained from RSCB protein data bank. When selecting the files, the structure with the lowest resolution was chosen to get the highest accuracy. If the structures already contained other bound ligands and water molecules, they were removed using UCSF chimera. Then, the finalized structure was used as the macromolecule in the docking process. The 3D structure of the active compound present in each of the pesticides was obtained from the Pubchem database in the SDF file format. The downloaded file was converted into the PDB file format by using the Avogadro software package. The energy of the structure was minimized using steepest descent algorithm. The finalized structure was used as the ligand in the docking process. The computed macromolecule and ligand input files were opened in Autodock tools, the GUI supported interface for Autodock. The files were saved as PDBQT file format, which is the required file extension to carry out the docking process.

Analysis of results

Analysis of the docking results was performed using AutoDock tools using the final output file. The docking results for the top 10 conformations with highest binding affinities (lowest binding energies) werecomputed. The conformation with the highest binding affinitywas chosen to compare the interactions between the pesticides and the enzyme.

RESULTS AND DISCUSSION

Ketoconazole as the inhibitor and phenobarbital as the inducer were docked with the enzyme, CYT450 as references to compare computed results of these selected pesticides (as shown in Figure 3).

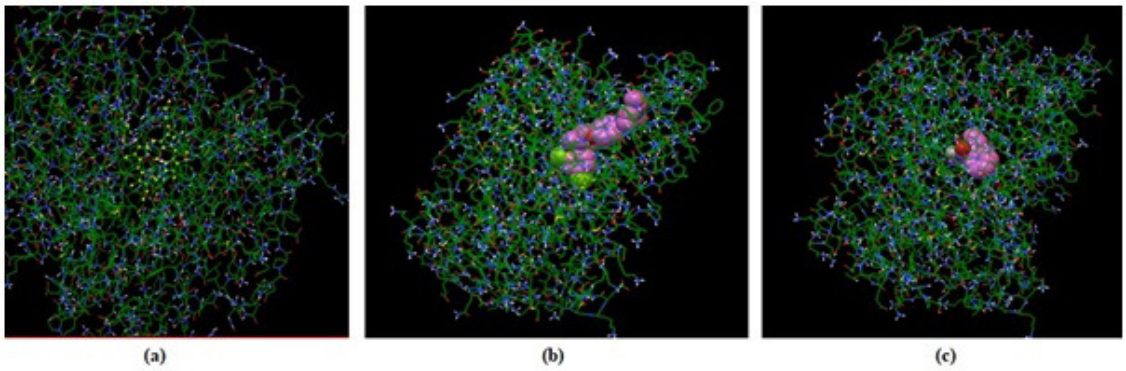

Figure 3. Molecular docked diagrams of (a) active site of CYP450 (marked with yellow crosses); (b) interactions of ketoconazole (inhibitor) with the binding pocket of CYT450; (c) interactions of phenobarbital (inducer) with the binding pocket of CYT450

The most stable ten conformations for substrate binding with the enzyme (CYT450) were generated using Gibbs free energies of binding as illustrated in Table 1. The location of the binding pocket and the strength binding can be used to interpret whether the substrate behaves as a substrate or inducer or inhibitor. If the substrate (pesticide molecule) binds with a higher binding energy and a smaller inhibition constant, there is a high possibility for it to act as an inhibitor.

Table 1. The most stable ten conformations for substrate (inhibitor/ inducer/pesticide) binding with the enzyme (CYT450)

Cluster Rank	Binding Energies (kCal/mol)					
	Ketoconazole (inhibitor) with CYT450	Phenobarbital (inducer) with CYT450	Profenofos (pesticide) with CYT450	Diazinon (pesticide) with CYT450	Chloropyrifos (pesticide) with CYT450	Imidacloprid (pesticide) with CYT450
1	-9.36	-5.44	-5.96	-5.22	-5.73	-5.78
2	-8.00	-5.08	-5.52	-4.95	-5.25	-5.20
3	-7.44	-5.07	-5.36	-4.82	-5.05	-5.18
4	-6.92	-5.03	-5.33	-4.67	-4.98	-5.09
5	-6.32	-4.75	-5.12	-4.54	-4.67	-4.99
6	-6.19	-4.71	-5.08	-4.38	-4.65	-4.98

7	-5.42	-4.66	-5.07	-4.34	-4.53	-4.88
8	-5.38	-4.11	-4.98	-4.32	-4.53	-4.69
9	-5.11	-4.38	-4.96	-4.14	-3.79	-4.62
10	-4.66	-4.76	-4.53	-3.44	-3.69	-4.18

It was evident that, the known inhibitor, Ketoconazole showed the most negative energy (-9.36 kCal/mol) of binding compared to the other substrates docked to enzyme CYT450. This demonstrated that its binding to the enzyme strongly as an irreversible inhibition. The known inducer, Phenobarbital and three pesticides (Profenofos, Diazinon and Chloropyrifos) showed similar binding energies (as shown in Table 1) at the same active site which contains Fe as a co-factor. Those values were much less negative than the values of the inhibitor possessed. This suggests that these three pesticides might bind in a reversible manner, acting as substrates or inducers to the enzyme. The free energies of binding and inhibition constants (K_I) were computationally investigated (Table 2), corresponding to the lowest energy conformation (among top ten confirmations analyzed) of these substrates (the standard inhibitor, the standard inducer and the pesticides used).

Table 2. The free energies of binding and inhibition constants (K_I) for substrate (inhibitor/ inducer/pesticide) binding with the enzyme (CYT450)

	Ketoconazole (inhibitor)	Phenobarbital (inducer)	Profenofos (pesticide)	Diazinon (pesticide)	Chloropyrifos (pesticide)	Imidacloprid (pesticide)
Free Energy of Binding with CYT450 (kCal/mol)	-9.36	-5.44	-5.96	-5.22	-5.73	-5.78
Inhibition Constant, K_I (μM)	0.138	103.05	42.89	148.88	62.94	58.07

From the experimental data, it was found that the Ketoconazole (inhibitor) has a inhibition constant of 0.072 μM for the inhibition of nifedipine (substrate) oxidation [18]. The theoretical docking studies agreed with these experimental evidences. The calculated inhibition constant of 0.138 μM falls in the Nano molar range (138 nM) which suggests that a very small concentration is sufficient to effect 50% of the inhibition.

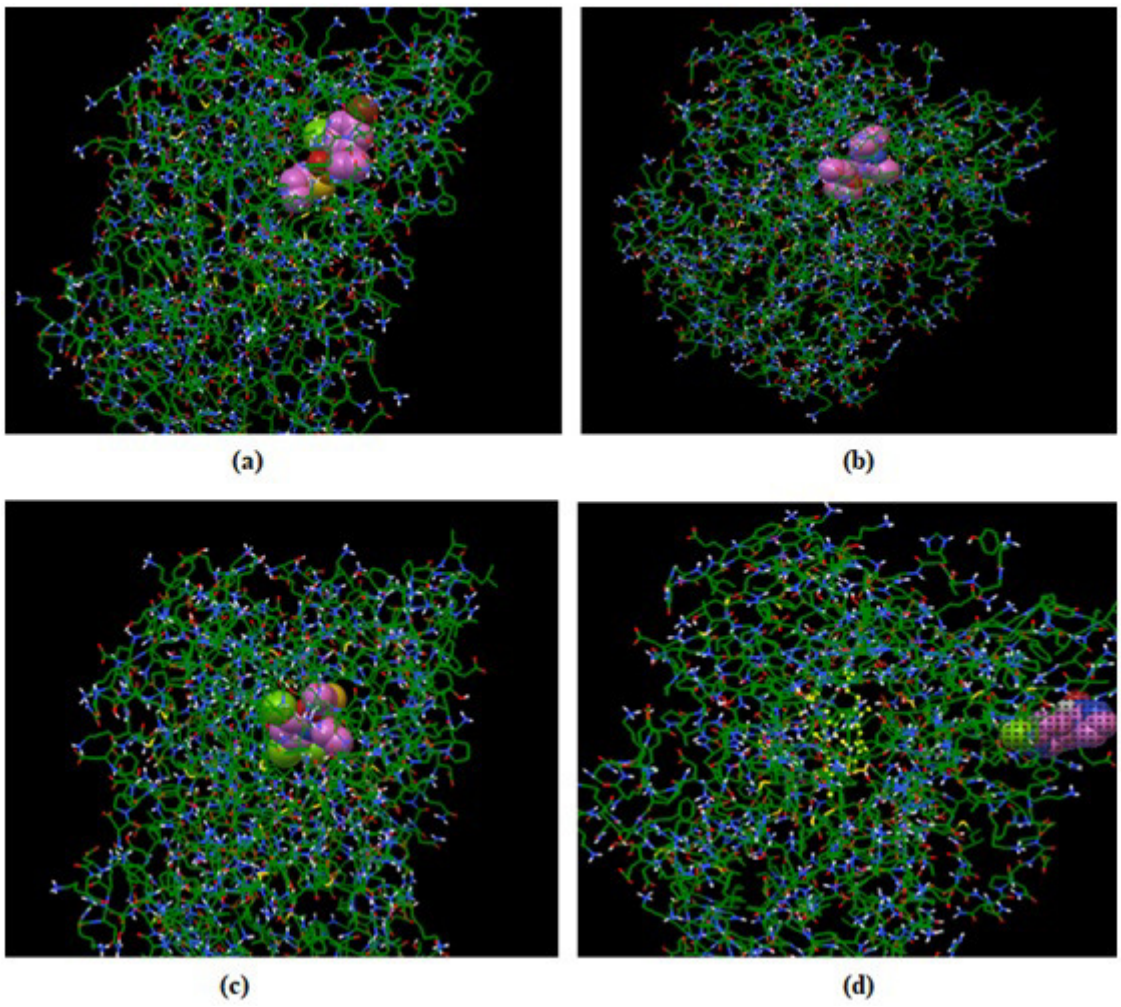

Figure 4. Molecular docked diagrams of (a) interactions of Profenofos with CYT450; (b) interactions of Diazinon with CYT450; (c) interactions of Chlorofyrifos with CYT450 and (d) interactions of Imidacloprid with CYT450

Three organophosphate pesticides; Profenofos, Diazinon and Chlorofyrifos showed significant interactions with the enzyme in the similar region of the binding pocket as shown in Figure 4. The structural similarity of the compounds might be a potential cause for this. The binding energies of these three pesticides were in values close to the binding energy of the inducer too. This suggested that the pesticides might have the tendency to act as inducers to increase the activity of the enzyme or act as substrates and undergo bio-activation. This occurs at the detoxification phase of the organophosphate pesticides and thus itcan be identified as a possible mechanism for the pesticide induced oxidative stress. However, Imidacloprid didn't show any stable conformations in the close proximity of the active site of the molecule. The structural difference of the Imidacloprid pesticide might have resulted it to

interact with a binding pocket away from the active site as shown in Figure 4. It is possible to predict that it is not metabolized by this enzyme.

CONCLUSIONS

According to the molecular docking investigations, it is evident that three organophospahates; Profenofos, Diazinonand and Chlrofyrifos possess relatively similar binding energies at the active site compared to the inducer for Cytochrome P450 A34 enzyme. Among those pesticides, Profenofos showed the most negative value of Gibbs binding energy. The computational studies predicted that these pesticides might serve as potent inducers or substrates for the enzyme. Since Imidacloprid was not metabolized by the enzyme, it didn't exhibit any interactions at the active site. None of the pesticides would act as potent inhibitors for this Cytochrome P450 A34 enzyme. Either these organophosphates behave as potent enzyme inducers or substrates, which involve in bio activation can be directed to a feasible mechanism for generation of reactive oxygen species and hence it may cause oxidative stress. These computational investigations link to the chronic toxicity of pesticides and hence a possible causative factor in the progression of the chronic kidney disease.

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