Research Article

Role of *in-silico* COX2 molecular docking bioinformatics tool and *in-vivo* carrageenan induced various inflammatory phase in mouse and rat models, in evaluation of anti-inflammatory activity of molecules

Chetan Nimgulkar[1], Sravani Edula[1], Venu Racha[1], M. Chalamaiah[1], P. Shyam[2], A. Ravindranath[3], *B. Dinesh Kumar[1]

1. Food and Drug Toxicology Research Centre, National Institute of Nutrition, (ICMR) Jamai Osmania, Hyderabad 500 007, India.
2. Bio-Medical Informatics Centre, NIN, (ICMR), India.
3. University College of Technology, Osmania University, Hyderabad, 500 007, India.

ABSTRACT

*In- silico*molecular docking study is used to screen new molecules for anti-inflammatory activity to evaluate their potency by *in-vivo* studies in animal. In the present investigation, aspirin was used as a ligand for docking with COX2 inflammatory protein by Accelrys Discovery Studio v2.1. *In-vivo* anti-inflammatory activity of aspirin was evaluated in C57Bl/6 mice and Wistar rats. Carrageenan was injected in intraplantar region of mice (300µg/paw) and rat (4000µg/paw). Aspirin was administered 91mg/Kg and 60mg/Kg P.O. Paw volume was measured by plethysmometer. Aspirin docked at 3rd and 4th Active Binding Site (ABS) of COX2 protein with 36.346 and 49.9 dock score with one hydrogen bond with Trp^{387} and four hydrogen bonds with Lys^{166}, Lys^{468}, and Glu^{465} amino acid respectively. In C57Bl/6 mice of 7 to 8 weeks old, and 30 to 35g body weight biphasic inflammatory phases (acute and chronic) were observed whereas in rat only acute phase of inflammation was observed. Aspirin could potentially inhibited late inflammatory response of 72nd h (79%) than 7th h (25.3 %) in mice. However, aspirin inhibit inflammatory phase at 7th h (62%) in rat. Results showed the correlation between *in-silico* and *in-vivo* studies. In drug development process, new molecules initially screened for molecular docking with COX2 protein, and only higher dock score molecules forward for *in-vivo* anti-inflammatory mice paw edema using C57Bl/6 of 7 to 8th week and 30 to 35gm weight.

Keywords: Carrageenan paw edema, biphasic edema, anti-inflammatory, in-silico molecular docking, COX-2, aspirin

Received 06 March 2014 Received in revised form 25 March 2014 Accepted 28 March 2014

***Address for correspondence:**
B. Dinesh Kumar
Food and Drug Toxicology Research Centre, National Institute of Nutrition, (ICMR) Jamai Osmania, Hyderabad 500 007, India.
E-mail:nindineshpct@gmail.com

INTRODUCTION

Inflammation is a complex biological protective response by the vascular tissues of organism to remove the injurious stimuli and initiate the healing process (1). The characteristics of acute inflammatory response of a body are increased vascular permeability and cellular infiltration that lead to extravasations of fluid, proteins, and accumulation of leukocytes at the leading inflammatory site to oedema formation (2, 3). Inflammatory stress is a multi-mechanistic process, which induces many diseases. Cyclooxygenases (COX) is an inflammatory key enzyme responsible for prostaglandin synthesis, inflammation progression, pain, and hyperpyrexia. COX is having two main isoforms i.e., cyclooxygenases −1 (COX-1) and cyclooxygenases-2 (COX-2). The COX-1 is responsible for formation of important biological mediators like prostanoids, including prostaglandins, prostacyclin, and thromboxane and is involved in pain causing, blood clotting and protecting the stomach whereas COX-2 is involved in the pain by inflammation and plays a major role in prostaglandin biosynthesis in inflammatory cells and central nervous system. Many NSAIDs inhibit both COX1 and

COX2 enzymes, which reduces inflammation, lining of the stomach leading to gastric problems like stomach upset, ulceration, and bleeding from the stomach and intestines. Antagonizing specific COX2 enzyme is an attractive therapeutic target to develop potent anti-inflammatory drugs.

*In-silico*bio-informatics docking study and its calculations provide stochastic information regarding the binding patterns within a protein complex, along with a rough estimation of the principal interactions involved (4). Thus, in order to evaluate the docking results, aspirin as a ligand was molecularly docked with COX-2 protein. Protein-ligand integration may be gained after the study of dynamic properties such as the flexibility of the systems, dominant hydrogen bonding interactions, conformational changes, and hydrophobic environment inside the binding cavities. With the help of in-silico bio-informatics docking study, many new anti-inflammatory molecules can be screened and lead molecule can be translate in drug candidate by trans-disciplinary and exploratory in-vivo studies.

It is well established that intraplantar injection of carrageenan induced rat paw oedema is a widely used animal model to evaluate the anti-inflammatory activity of the test compounds (5-7). Carrageenan produces a monophasic oedematogenic response in the rat paw (8). However, mouse paw oedema has been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation (3). In 1969, Levy described that injection of carrageenan 1% in the mouse paw causes an oedema similar as time course to the rat, but less powerful in proportion (9). While Sugishita in 1981 further characterized the acute phase of mouse paw oedema using 3 percent carrageenan (10). Whereas Henriques and co-workers reported that carrageenan, injection into the mouse paw induces a biphasic oedema (3; 11). Where in the first phase it is characterized by an oedema of little intensity and unrelated to the dose of carrageenan used, while the second phase is develops after 24^{th} h, displaying a more pronounced oedema with a maximum effect between 48^{th} and 72^{nd} h (3, 8, 10, 12). Recently, it has been demonstrated that carrageenan 1% induces a marked powerful oedema in BALB/c mice, but in this case only the second phase has been shown (3, 8, 10, 12). Thus, at the present stage, much confusion was generated by the fact that there are many reports where only one phase is considered either the first Njamen in 2003(13) or the second for example, Wu in 2002 (14) and different doses of carrageenan are used. Addition of carrageenan 1% was followed by protocol, (3; 8; 9; 12; 15) and 3% (10).

The species was selected not only for interest to understand the mechanism but also facilitate developing the compound, which has potential anti-inflammatory activity. In the present investigation, an attempt was made to develop an *in-silico* and in-vivo correlation for anti-inflammatory drug development. Additionally standardization of age, weight, and species of animal for in-vivo anti-inflammatory study was done to obtain biphasic reproducible response *in-vivo*, which will help to predict detailed anti-inflammatory activity and its correlation with *in-silico* molecular docking study.

MATERIALS AND METHODS

Ligand-protein interaction (*in-silico*)

Protein Structures

Molecular docking was done by Accelrys Discovery Studio v2.1 (ADS) (Discover 2.7) package from (Biosystems Technologies, San Diego, CA, USA), on an O2 (R12000) workstation (Silicon Graphics, Mountain View, CA, USA). The 3D crystal structure of enzyme cyclooxygenase-2 (COX-2 PDB code: 4COX) X-Ray diffraction with resolution of 2.90 (A^{o}) were retrieved from the Protein Data Bank.

Ligand Structures

3D structure of aspirin was retrieved from Pubchem database.

Protein and Ligand Preparation and Minimization Methodology

COX2 protein was cleaned by standardizing atom orders, ordering the bonds, protein naming, alternate conformations, adding incomplete residues and modifying hydrogen with clean protein tool. COX2 protein was prepared by a Prepare Protein Protocol and energy was minimized (Generalized Born Solvent Model) using

Smart Minimizer Algorithm in MinimizationProtocol by applying CHARMm force field on the protein. Ligand (aspirin) was prepared by standardizing charges, using Prepare Ligand protocol and minimized using Ligand Minimization protocol and Smart Minimizer Algorithm.

Protein-Ligand Docking Methodology

3D protein structure of COX2 docked with aspirin by standard protocol of Discovery Studio using Ligandfit module of ADS. In a protein created sphere around the active site about 12°A. After docking experiments, poses viewed by Dock Sore (DS) viewer. Hydrogen bond interactions and docking score of aspirin was sequenced for COX2 protein. Various characteristic of protein ligand interaction was calculated by docking function (PLP1 and PLP04), empirical fitting functional (Jain, LigScore1 and LigScore2) and statistical functions (PMF).

Animals

Institutional Animal Ethical Committee (IAEC) (CPCSEA) approved the protocol. IAEC approval number is P31/2-2010/BDK and P29/8-2010/BDK. Mice: Male C57Bl/6 of age: 6 to 7 weeks old and weight: 30–35 g, Rats: Wistar-National Institute of Nutrition of age: 8 to 10 weeks old, and weight: 200 -220 g, for the study obtained from animal breeding of National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN, ICMR), Andhra Pradesh, India. All the animals were maintained in individual ventilated cages with sterilized bedding materials. Environmental conditions (throughout the year) viz. temperature: 25°C ± 2°C, relative humidity: 45-55%, ventilation (number of air changes): 16-20 fresh air changes/hour, light-dark cycle: 12 hour light / 12 hour dark cycle, light intensity: 350-400 lux one meter above the floor level, light source: Fluorescent tube lights fixed in such a way to facilitate uniform distribution of light. Hygienic conditions maintained throughout the study. Food and water was provided*ad libitum.*

Carrageenan induced Paw oedema (*in-vivo*)

Study design: Completely Randomized Design was used (CRD) in which 18 rats and 18 mice were randomly divided into three respective groups (*n=6* each group). Group I and II were taken as negative control, and positive control respectively. Group III was considered as standard group.

Mice paw oedema

Experimental procedure was followed as per Henriques(3, 8, 12). Briefly, Group I and II mice received vehicle (distilled water 10mL/Kg P.O) and Group III mice were given standard drug Aspirin 91 mg/Kg P.O. After 30 minutes mice paw oedema was induced in mice of group II and III with 50 µL sub plantar (intraplantar) injection, into right hind paw containing carrageenan (300 µg/paw s.c) in normal saline. Group I mice paw received 50µL sub plantar (intraplantar) of normal saline, and was used as the negative control.

Rat paw oedema

While in case of rat paw oedema, Group I and II rats received vehicle (distilled water 10mL/Kg P.O) and Group III rats were administered standard drug Aspirin 60 mg/Kg P.O. After 30 minutes rat paw oedema was induced in rats of group II and III with 200 µL sub plantar (intraplantar) injection into right hind paw, containing carrageenan (4000 µg/paw) in normal saline. Group I rats paw received 200 µL of normal saline and was considered as the negative control.

Paw oedema measurement

Paw oedema was measured by means of a plethysmometer, (LE7500Panlab S.I.) at several time points (1/2, 1, 2, 4, 6, 24, 48, 72 and 96 h) after the injection of carrageenan. It was expressed in microliters (µL) as the difference between the right and left paws.Percentage of inhibition of oedema,

$= \left(1 - \left({}^{Vt}/_{Vc}\right) \times 100\right)$

Where *Vt* and *Vc* are the volume of oedema of, treated and control group respectively.

Statistics

Analysis was done by ANOVA followed by Bonferroni's multiple comparison test. $P<0.05$ was considered significant. Data expressed as Mean ± SE. The analysis was performed using Graph Pad Prism Software.

RESULTS

In-silico ligand-protein interaction

COX2 Protein, Ligand Preparation, and Minimization Results

Protein structure of COX2 was minimized using Smart Minimizer Algorithm (hybrid algorithm of steepest descent and conjugate gradient algorithm) and CHARMm force field was applied to proteins and minimized using Smart Minimizer Algorithm. COX2 was verified by ADS with an Initial Potential Energy (-2140.9996), Potential Energy (-37195.135), Van der Waals Energy (-3877.1845), Electrostatic Energy (-38342.14073) and RMS Gradient was reduced from (initial 657.36431kcal/(mol x Angstrom), to final (0.16771 kcal/(mol x Angstrom), Power of Hydrogen (4) 3.20, iso-electric Point (pI) 8.89. Potential energy was stabilized up to 1000 steps. Six binding siteswere found in COX2 protein **(Figure 1 and figure 2)**. The minimized ligand complex energies, histograms and minimized ligands drugs complex and dock score histograms to predict the protein energies and docking energies.

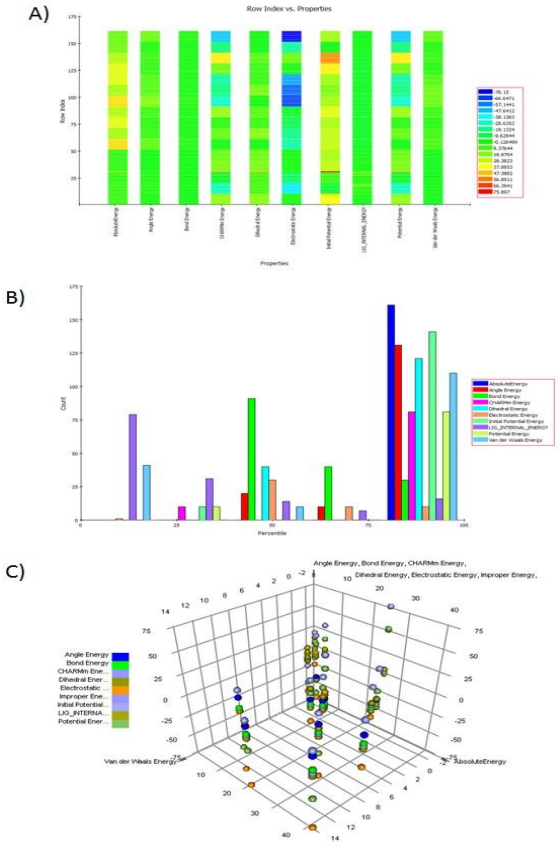


Figure 1: Minimized Ligands Drugs Complex.A) Energies Heat Plot, B) Energies Histogram; C) Energies 3D Point Plot

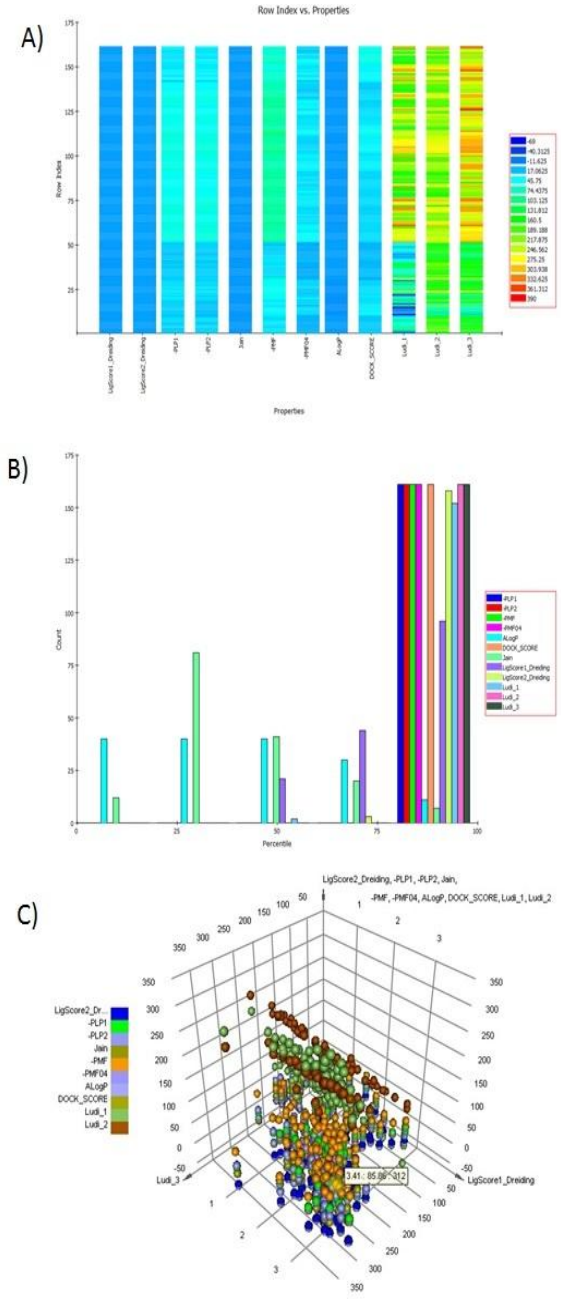
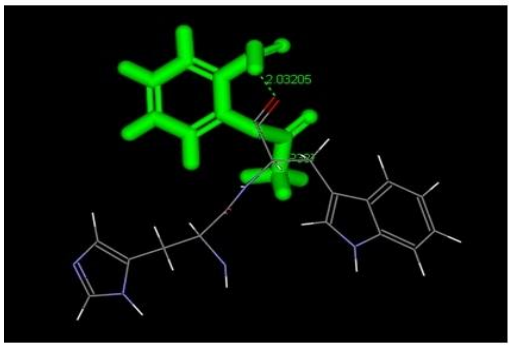


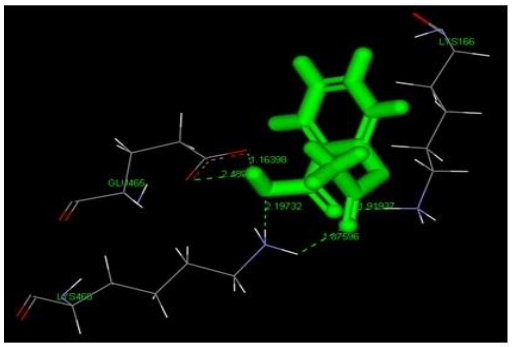
Figure 2: Minimized Ligands Drugs Complex –Dock Score.A) Heat Plot; B) Dock Score Histogram; C) 3D Point Plot

Docking results

Aspirin was found to be docked at 3rd and 4th active binding site of COX2 protein with a dock score 36.346 and 49.90. Aspirin form one hydrogen bond interaction at 3rd ABS with TRP^{387} with 2.032 Aºof COX2 enzyme protein. Aspirin formed four hydrogen bonds interactions with Lys^{166}, Lys^{468}, and Glu^{465} of distance 1.919 Aº, 1.876 Aº, 2.1s97 Aº and 1.164 Aº 4th ABS **(Figure 3 and Table 1).**



COX2-ABS : 3
Ligand : Aspirin
Dock Sore : 36.34
H-bond distance and interaction
2.032 Aº- Aspirin:H21 - A:TRP387:O



COX2-ABS : 4
Ligand : Aspirin
Dock Sore : 49.905
H-bond distance and interactions
1.919 Aº -A:LYS166:HZ3 - Aspirin:O3
1.876 Aº -A:LYS468:HZ1 - Aspirin:O4
2.197 Aº -A:LYS468:HZ3 - Aspirin:O2
1.164 Aº -Aspirin:H21 - A:GLU465:OE2

Figure 3: Intermolecular interactions of Aspirin with COX2 protein. Aspirin docked with COX2 protein at active binding site 3rd and 4th. At 3rd ABS aspirin formed one hydrogen bond while in 4th ABS aspirin formed 4 hydrogen bonds. Number of hydrogen bonds is directly proportional with dock score and inversely proportional to hydrogen bond length. Four hydrogen bonds with a lesser bond length increased dock score at 4th ABS than 3rd ABS of COX2.

Table 1: Docking functions of the Aspirin with COX2 protein

3rd Active Binding Site of COX2 protein (ABS3)											
Index	**DS**	**LS1**	**LS2**	**P1**	**P2**	**J**	**P3**	**P4**	**L1**	**L2**	**L3**
1	36.346	2.52	4.25	60.31	57.1	1	15.79	24.42	306	267	360
2	36.204	2.55	4.24	61.14	57.71	0.8	21.07	27.28	266	258	349
3	35.513	3.32	4.45	56.12	52.92	0.75	33.66	30.96	279	281	311
4	35.342	2.4	4.29	59.95	53.87	0.78	23.55	27.87	263	255	346
5	35.309	2.83	4.27	61.31	56.08	1.24	23.37	30.04	314	285	377
4th Active Binding Site of COX2 protein (ABS4)											
1	49.905	3.52	3	33.78	36.2	1.08	61.22	12.33	355	336	307
2	49.904	3.54	3.04	34.03	36.55	1.03	61.55	12.64	354	343	314
3	48.598	3.36	2.92	42	44.1	2.31	57.69	17.31	368	342	311
4	47.262	3.65	3.62	37.34	35.54	-0.06	61.47	14.63	325	334	298
5	47.232	3.64	3.57	36.08	34.88	0.08	59.37	13.15	341	321	288

ABS4: Active Binding Site of COX2 protein; DS: Dock Score (affinity and stability of ligand at receptor protein); Empirical fitting functions like LS1 and LS2: LigScore1 and 2_Dreiding; J: Jain score; L1, L2 and L3: Ludi score 1, 2, and 3 (Binding affinity between protein and ligand complexes); Docking Functions like P1 and P2: PLP1 and 2 (binding affinity, internal energy (Van der Waals energy and electrostatic energy) and interaction energy). Stochastic analysis was calculated by P3: PMF; P4 PMF04 (binding free energy).

Mouse paw oedema

C57/BL6 mice of 6 to 7 weeks old, and 30–35 g of weight, produced biphasic paw oedema, being maximum at 6 to 7 h 125.0±35.10µL (acute / immediate Phase) and again at 48th to 72nd h (166.7±19.66µL) (late Phase). There was decrease in oedema after 72nd to 96th h. The treatment of animals with a single dose of the aspirin (91 mg/Kg P.O, 30 min before) significantly reduced the early, and phase of carrageenan-evoked oedema, with inhibition of 15.2±17.61 % and 27.4±13.66 % at 2nd and 4th h, respectively **(Figure 4)**. 3rd h after the injection aspirin was able to markedly reduce the oedematogenic response, an effect that was observed till 96th h. The percentage inhibition of paw edema with respect to time was 25.6±16.02%, 37.5±14.72%, 66.6±23.38%, 79.0±16.43% and 92.7±5.16%, at 6th, 24th, 48th, 72nd and 96th h, respectively.

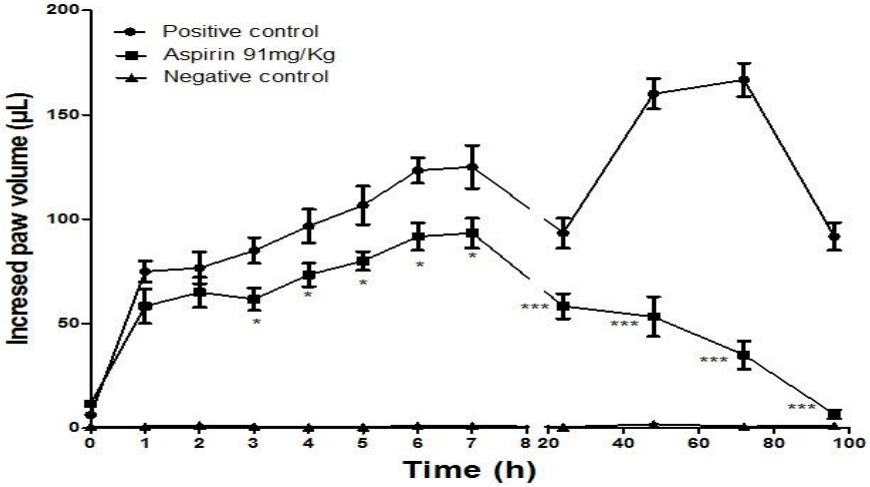


Figure 4: Carrageenan induced mice paw oedema formation. Mouse paw oedema formation induced by carrageenan (300 µg/paw) in C57/Bl6 wild-type mice. Paw volume was gradually increase and peaked at 6th to 7th h (initial phase) followed by decreased up to 24h and again second phase was peaked at 48th to 72nd h (late phase). Each point represents the mean of 6 animals and vertical lines show the S.E.M. Asterisks denote the significance levels when the values obtained for C57/BL6 wild type were compared to those for Aspirin 91mg/Kg P.O dosed mice. Values are mean ± SEM, n=6 in each group. ANOVA followed by Bonferroni Multiple comparison test at each individual time points,* P<0.05, **P<0.01, ***P<0.001.

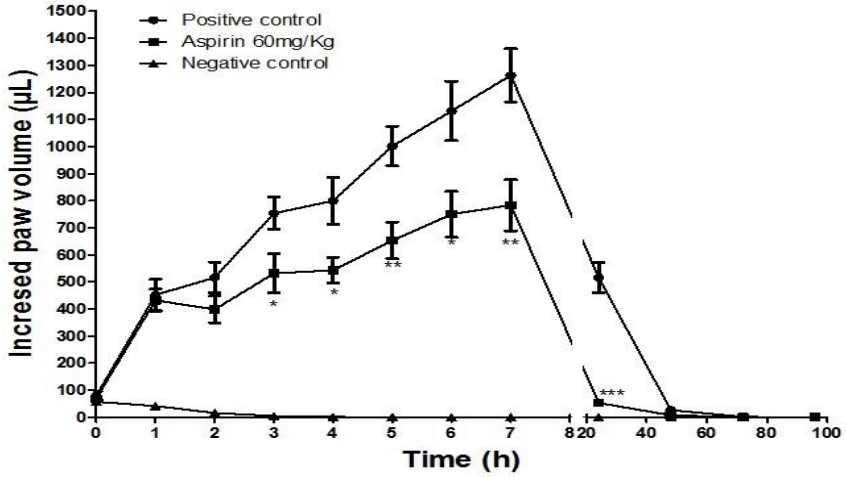


Figure 5: Carrageenan induced rat paw oedema. Rats paw oedema formation was observed induced by carrageenan (4000 µg/paw) in WNIN rat. Paw volume gradually increased and was maximum at 6th to 7th h (initial phase) and followed by a gradual decrease from 7th h to 96th h. Each point represents the mean of 6 animals and vertical lines show the S.E.M. Asterisks denote the significance levels when the values obtained for WNIN rats were compared to those for Aspirin 60mg/Kg P.O dosed rats: Values are mean ± SEM, n=6 in each group. ANOVA followed by Bonferroni Multiple comparison test at each individual time points,* P<0.05, **P<0.01, ***P<0.001

Rat paw oedema

In carrageenan induced rat paw oedema, immediate phase of inflammation was maximum at 7th h (1263±241.8µL) (acute / immediate Phase), but in late inflammatoryphase was absent **(Figure 5).** Aspirin significantly reduced odema from 3rd h onward in both the specieswhen compare with positive control. However, the level of significance increased in second phase of inflammation, which was observed in only mice.

DISCUSSION

In the present study, aspirin was selected as standard anti-inflammatory compound to establish *in-silico* and *in-vivo* correlation. Molecular docking of aspirin with COX2 protein was evaluated through Accelrys Discovery Studio v2.1. Initially, 3D structure of aspirin molecule as ligand was obtained from Pubchem, followed by energy minimization. Aspirin showed lower Van der Waals energy value denoting the impact of hydrogen bonding property of this compound during protein/enzyme interaction.

As 3rd Active Binding Site (ABS), docking function PLP1 and PLP2 of aspirin was highest at 5th and 2nd index pose while at 4th ABS 3rd index pose was highest representing higher binding affinity, internal energy (Van der Waals energy and electrostatic energy) and interaction energy with COX2 protein. Binding affinity between protein and ligand complexes, empirical fitting functions like Jain, LigScore and Ludi scores. Jain score at 3rd ABS of COX2 protein aspirin 5th index pose, was high which show lipophilic and polar attractive interaction with COX2 protein. LigScore 1 and 2, of aspirin at 3rd of ABS 5th and 3rd pose and at 4th ABS of 4th index pose reflects highest binding affinities towards receptors. Ludi score 1, 2, 3 predict binding affinities between ligand-protein complex which depends up on number and distance of hydrogen bonds, ionic and lipophilic interactions, translational and rotational entropy loss of the ligand and freezing of internal degrees of freedom of ligand. Number of hydrogen bonds is proportional to binding affinity and stability of ligand pose at ABS of protein and dock score. Aspirin formed one hydrogen bond at with 21st hydrogen of aspirin with Oxygen of TRP387 residue at 3rd ABS of COX2 protein with 2.031 Ao distance. While at 4th ABS of COX2 protein, aspirin formed four hydrogen bondsat 1stindexpose *viz*. with 3rd oxygen of aspirin with 3rd hydrogen of Lys166 with 1.919 Ao, 2nd oxygen and 4th oxygen form two bonds with 1st and 3rd hydrogen Lys468 2.197 Aoand 1.876 Ao, respectively and 21s hydrogen of aspirin form 2nd oxygen of Glu465 amino acid, with 1.164 Aodistance. Dock score is proportional to number of hydrogen bond interactions and inversely proportional to bond length that represents higher binding affinity and stability which lined with their Ludi score and dock score. Binding free energy (calculated by stochastic analysis: PMF and PMF04) of aspirin was stable at all index poses which predict the stability of ligand interaction with protein. High docking function, empirical function (J, LS 1 and 2, L1, 2, 3) and stochastic function (PMF and PMF04) of aspirin represents highest binding affinity, stability, formal charges, number of hydrogen bond interactions with lesser bond length and binding free energy which indicate a strong binding with 4th binding site of COX2 protein. Molecular docking analysis was evaluated by *in-vivo* anti-inflammatory animal model.

The conventional rat paw model is always preferred for evaluation of anti-inflammatory compounds. Carrageenan mouse paw biphasic oedema was characterized and described by many researchers (3, 9, 10, 12). Anti-inflammatory activity of new substance was studied by the rat or mouse paw oedema, and the protocol was described by Levy and Sugishita(9, 10). Several non-steroidal anti-inflammatory drugs have been shown to be active in the mouse model at the same extent as in the rat model (16).

Indeed, 7 or 8 week-old C57Bl/6 mice of 30–35 g weight, respond with a consistent inflammatory pattern to carrageenan, displaying a biphasic oedema that develops in the first 6th to 7th h, followed by a second phase that starts at 24th h and continue for 48th, 72nd to 96th h.(3, 8-11, 17). Many studies have shown that carrageenan-induced rat paw oedema is largely associated with the production of several

inflammatory mediators such as Prostaglandins (formed by the action of both COX-1 and COX-2) nitric oxide (NO) (derived from nNOS, eNOS and iNOS) histamine, Prostaglandins (PG), Kinins, and Cytokines (3, 6-8, 10, 12, 15, 17-20). According to Posadas (8) the initial phase may be due to secretion of PGE2 (via COX1), NO (via eNOS) and Myloperoxidase (15) and second phase may be due to PGE2 (via COX2), NO (via eNOS and iNOS) and MPO. Tumour Necrosis Factor-α (TNF-α) is a potent pro-inflammatory cytokine that possesses multiple effects, including the activation of inflammatory cells, the induction of several inflammatory proteins, cytotoxicity, etc. (19, 21, 22). Carrageenan-induced mouse paw oedema is also associated with paw nociceptive changes and the migration of inflammatory cells to the site of injection (8).

While in rat, only first phase of inflammation was observed up to 6th to 7th h. After 24thh there was no second phase of inflammation. Thus, the present results are in support to the earlier reports on mice and rat inflammation in response to carrageenan induced paw oedema related to the phase of the oedema formation (10, 12).

Aspirin was found to be active in both rat and mice from the time 3rd h. Nevertheless, in mice, aspirin was found to be prominently active in second phase (72nd h) as compared to first phase (7th h) of inflammation. This may be due to PGE2 inhibition, secreted by COX2 enzyme. As shown in *in-silico* study aspirin found to be docked at 4th binding site of COX2 protein and aspirin was found to be active in late phase of inflammation of mice which may be due to COX2 enzyme. The result showed a correlation in the inhibition of COX2 enzyme by aspirin in late phase of inflammation in the present study which predict provided molecular interaction. Aspirin found to be active in immediate phase of inflammation due to single-phase response in rat. However, at the same time due to the biphasic response in mice species, anti-inflammatory activity of aspirin can be separated phase and evaluated.

CONCLUSION

Our molecular docking scores and *in-vivo* anti-inflammatory activity of aspirin clearly showed that screening of new anti-inflammatory molecules can be initiate to from *in-silico* molecular docking with COX2 inflammatory proteins. Among them highest dock score molecules can evaluated for *in-vivo* anti-inflammatory activity in C57Bl/6 mice of 7 to 8 week-old and 30–35 g weight, which produces carrageenan induced paw oedema in two phases i.e immediate (acute) and late phase, as against the rat paw oedema that displays only an acute phase. Mice paw oedema model is better than rat paw model to evaluate anti-inflammatory activity of various compounds due to time wise statistically differentiable inflammatory phase.

ACKNOWLEDGEMENT

This study is financially supported by a research grant from Indian Council of Medical Research (ICMR), Department of Health Research, Ministry Health and family welfare, Govt. of India, India.

REFERENCES

1. Armstrong EJ, Morrow DA, Sabatine MS. 2006. Inflammatory biomarkers in acute coronary syndromes: part I: introduction and cytokines. Circulation 113:e72-5.
2. Castardo JC, Prudente AS, Ferreira J, Guimaraes CL, Monache FD, et al. 2008. Anti-inflammatory effects of hydroalcoholic extract and two biflavonoids from Garcinia gardneriana leaves in mouse paw oedema. J Ethnopharmacol 118:405-11.
3. Henriques MGMO, Silva, P.M.R., Martins, M.A., Flores, C.A., Cunha, F.Q., Assreuy-Filho, J., Cordeiro, R.S.B. 1987. Mouse paw oedema. A new model for inflammation? Brazilian Journal of Medical and Biological Research 20:243–9.
4. Neophytou N, Leonis G, Stavrinoudakis N, Simcic M, Grdadolnik SG, et al. 2011. Docking and Molecular Dynamics Calculations of Pyrrolidinone Analog MMK16 to COX and LOX Enzymes. Mol. Inf. 30:2 – 15.
5. Di Rosa M. 1972. Biological properties of carrageenan. J Pharm Pharmacol 24:89-102.
6. Di Rosa M, Giroud JP, Willoughby DA. 1971. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J Pathol 104:15-29.
7. Di Rosa M, Papadimitriou JM, Willoughby DA. 1971. A histopathological and

pharmacological analysis of the mode of action of nonsteroidal anti-inflammatory drugs. J Pathol 105:239-56.
8. Posadas I, Bucci M, Roviezzo F, Rossi A, Parente L, et al. 2004. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. Br J Pharmacol 142:331-8.
9. Levy L. 1969. Carrageenan paw edema in the mouse. Life Sciences 8:601–6.
10. Sugishita E, Amagaya S, Ogihara Y. 1981. Anti-inflammatory testing methods: comparative evaluation of mice and rats. J Pharmacobiodyn 4:565-75.
11. Henriques MG, Rae GA, Cordeiro RS, Williams TJ. 1992. Endothelin-1 inhibits PAF-induced paw oedema and pleurisy in the mouse. Br J Pharmacol 106:579-82.
12. Morris CJ. 2003. Carrageenan-induced paw edema in the rat and mouse. Methods Mol Biol 225:115-21.
13. Njamen D, Talla E, Mbafor JT, Fomum ZT, Kamanyi A, et al. 2003. Anti-inflammatory activity of erycristagallin, a pterocarpene from Erythrina mildbraedii. Eur J Pharmacol 468:67-74.
14. Wu WP, Hao, J.-X., Halldner-Henriksson, L., Xu, X.-J., Jacobson, M.A., Wiesenfeld-Hallin, Z., Fredholm, B.B. 2002. Decreased inflammatory pain due to reduced carrageenaninduced inflammation in mice lacking adenosine A3 receptors. Neuroscience 114:523–7.
15. Rocha AC, Fernandes ES, Quintao NL, Campos MM, Calixto JB. 2006. Relevance of tumour necrosis factor-alpha for the inflammatory and nociceptive responses evoked by carrageenan in the mouse paw. Br J Pharmacol 148:688-95.
16. Calhoun W, Chang J, Carlson RP. 1987. Effect of selected antiinflammatory agents and other drugs on zymosan, arachidonic acid, PAF and carrageenan induced paw edema in the mouse. Agents Actions 21:306-9.
17. Omote K, Hazama K, Kawamata T, Kawamata M, Nakayaka Y, et al. 2001. Peripheral nitric oxide in carrageenan-induced inflammation. Brain Res 912:171-5.
18. Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, et al. 2005. Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. Proc Natl Acad Sci U S A 102:904-8.
19. Hopkins SJ. 2003. The pathophysiological role of cytokines. Leg Med (Tokyo) 5 Suppl 1:S45-57.
20. Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, et al. 1996. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. Br J Pharmacol 118:829-38.
21. Fernandes D, Assreuy J. 2004. Involvement of guanylate cyclase and potassium channels on the delayed phase of mouse carrageenan-induced paw oedema. Eur J Pharmacol 501:209-14.
22. Haddad JJ. 2002. Cytokines and related receptor-mediated signaling pathways. Biochem Biophys Res Commun 297:700-13.