doi.org/10.36721/PJPS.2020.33.3.SUP.1183-1190.1

Appraisal of *in vitro*, *in vivo* and multi-targeted molecular docking analysis of atorvastatin to elucidate its anti-arthritic potential

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Abstract: Atorvastatin, an HMG-CoA reductase inhibitor, has been employed in the current study with animal models. *In vitro* models comprised of thermally induced Bovine serum albumin denaturation and egg albumin denaturation along with membrane stabilization assay while *in vivo* study involves formaldehyde induced arthritis. Paw swelling, arthritic score and body weight was evaluated for a period of 10 days after induction with formaldehyde. Histological analysis of ankle joint was also performed on 10th day. Molecular docking was done by targeting BSA (PDB id 4JK4), Histamine H1 receptor (PDB id 3RZE) and Prostaglandin E2 (PDB id 5YWY) to explore binding interactions of atorvastatin with BSA, Histamine H1 receptor and Prostaglandin E2. Atorvastatin revealed protein denaturation inhibition and stabilization of RBC membrane in a concentration dependent manner, with maximal effect at the concentration of 6400 µg/ml. Likewise, at the dose of 40mg/kg maximum attenuation of paw edema was being observed. Significant reduction in arthritic indices with body weight increment was observed in atorvastatin and standard drug treated groups. Histological investigation unveiled no substantive structural alterations in ankle joints of treated rats in comparison to arthritic control group. Molecular docking analysis of atorvastatin with the selected three targets revealed that the drug displayed strong binding interactions with BSA, Histamine H1 receptor and PGE2. Thus it can be concluded on the basis of aforementioned results that atorvastatin provided significant protection against arthritis by multiple mechanism and should be considered for further pharmacological evaluation to point out the exact mechanism of action.

Keywords: Atorvastatin, *in-vitro*, *in-vivo*, arthritis, molecular docking.

INTRODUCTION

Rheumatoid arthritis, a disease thought to originate due to immune system dysfunctioning, has turned out to be the most prevalent inflammatory disorder affecting a large percentage of population. Exact mechanism responsible for inducing this autoimmune malady is still unclear (Nair *et al*, 2011). Most commonly employed treatment strategies to encounter this disorder include NSAIDs, anti-TNF-α drugs, IL-1 receptor antagonist, corticosteroids and DMARDs. Nonetheless, surplus side effects including immunosuppression along with opportunistic infections are being associated with available drug therapies (Qureshi *et al*, 2006). Consequently, there is an utmost need to introduce some potent, long acting anti-arthritic agent possessing minimal side effect profile.

Statins, along with their lipid lowering attributes, are being reported to possess several pleiotropic attributes such as anti-inflammatory and immune-modulatory (Niwa *et al*., 1996; Li *et al*., 2000; Kobashigawa *et al*., 1995), plaque stability (Tandon *et al*., 2005), endothelial dysfunction improvement (Wong *et al*., 2002; Laufs *et al*., 1998; Tanaka *et al*., 2001). Various studies conducted on acute inflammatory model with simvastatin (Jick *et al*., 2003) and rosuvastatin (Sparrow *et al*., 2001) also demonstrated anti-inflammatory attributes of above mentioned statins. Similarly, atorvastatin and lovastatin revealed interferon-α (IFN-α) provoked stimulation of MHC class II molecules on a variety of cell types (Naito *et al*., 2006). It has also been demonstrated by some studies that lovastatin (Kwak *et al*., 2000; Schimdt *et al*., 2002) and atorvastatin provoked various pro inflammatory actions both *in-vitro* and *in-vivo*.

Clinical trials on atorvastatin also confront that atorvastatin reduces inflammation in patients affected from Crohn's disease and Type 2 diabetes mellitus (Kiener *et al*., 2001; Grip *et al*., 2008). So, keeping in mind the beneficial effects of statins particularly atorvastatin in alleviating the process of inflammation it was thought worthwhile to evaluate atorvastatin for its anti-rheumatic potential by utilizing some *in vitro* and *in vivo* experimental models. Selection of atorvastatin for current study resides on effectiveness of atorvastatin in various chronic inflammatory rat models (Hogue *et al*., 2008) and also on available preliminary data revealing beneficial effects of atorvastatin in humans (Youssef *et al*., 2002).

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MATERIALS AND METHODS

Chemicals

Chemicals being employed in the current study include bovine serum albumin (Sigma-Aldrich, USA), disodium hydrogen phosphate (Merck, Germany), atorvastatin (Sigma-Aldrich, USA), piroxicam (Sigma-Aldrich, USA), sodium chloride (Sigma-Aldrich, USA), formaldehyde (VWR, England), sodium hydroxide (Sigma-Aldrich, USA), potassium dihydrogen phosphate (Riedel-de-Haën, USA).

Experimental animals

Sprague Dawley rats of either sex, weighing between 160-220g, young and healthy were used. Animals were kept at animal house of Faculty of Pharmacy, University of Sargodha and provided with standard pellet diet and water ad libitum . All experiments were conducted with prior approval from Institutional Animal Ethical Committee of college of pharmacy, University of Sargodha, Pakistan (Approval No. 56B17 IEC/UOS).

Egg albumin denaturation inhibition

A test reaction mixture of 5ml was prepared by adding hen's egg albumin (0.2ml) in 2.8ml of phosphate buffer (pH 6.4) along with 2mL of various concentrations (100-6400 µg/mL) of atorvastatin and piroxicam, respectively. Similar quantity of distilled water worked as control. Incubation of reaction mix was done for 15 min at 37 ± 2°C later on for 5 min at 70°C. Spectrophotometric absorbance was measured at 660 nm, after cooling. Percent inhibition of albumin denaturation was estimated by using below mentioned formula (McCarey *et al*., 2004).

$$\text{Percentage inhibition} = 100 \times \left[\frac{(\text{Absorbance of test sample})}{(\text{Absorbance of control})} - 1 \right]$$

BSA denaturation inhibition

In vitro anti-arthritic efficacy of atorvastatin was estimated through BSA protein denaturation inhibition assay (McCarey *et al*., 2004). Various concentrations (100, 200, 400, 800, 1600, 3200, 6400 µg/mL) of atorvastatin and piroxicam (reference drug) were prepared, accordingly. Test control solution (0.5mL) comprised of 5% w/v aqueous solution of bovine serum albumin BSA (0.45 mL) and distilled water (0.05 mL). Likewise, 0.5 mL of product control comprised of test solution (0.05 mL) and distilled water (0.45 mL). Test solution of 0.5mL contains BSA (0.45 mL) along with 0.05mL of test solution. Standard solution (0.5mL) was made up of piroxicam (0.05 mL) and BSA (0.45 mL). Aforementioned solutions were maintained at a pH of 6.3. Afterwards incubation of all solutions was done at 37°C for 20 minutes followed by heating at 57°C for 3 minutes. All the mixtures thereafter were provided with 2.5ml of phosphate buffer. Spectrophotometric absorbance was estimated at 660 nm and percentage protection of BSA denaturation was computed as under:

$$\text{Inhibition \% age} = 100 - \frac{\text{Absorbance test solution} - \text{Absorbance product control}}{\text{Absorbance test control}} \times 100$$

Human red blood cell (HRBC) membrane stabilization assay

About 5ml of blood from healthy human volunteer was taken and mixed with equal quantity of Alsever's solution. Mixture was then centrifuged at 3,000 rpm. Packed cells at the bottom were taken out and a 10% suspension was made using normal saline. 4mL of test solution comprised of hypotonic saline (2mL), 1mL of phosphate buffer, 0.5 mL of test samples at several concentrations (100, 200, 400, 800, 1600, 3200, 6400 µg/mL) and 10% v/v HRBCs (0.5mL). Likewise, test control contains the same ingredients as of test solution except for different concentrations of test samples. Standard solution is also of same composition as test solution except that it contains piroxicam instead of test samples. All solutions were incubated for 30 min at 37°C followed by centrifugation at 3000 rpm. Hemoglobin content was then estimated via spectrophotometer by taking absorbance at 560 nm. Percent protection from lysis was computed through following formula (Alamgeer *et al*., 2015):

$$\text{Percentage protection} = 100 - \left[\left(\frac{\text{Optical density sample}}{\text{Optical density control}} \right) \times 100 \right]$$

Formaldehyde-provoked arthritis

Animals were allocated into various groups with 6 animals in each group. Normal control group (Group I) was administered 3 mL/kg of distilled water only, Group II was provided with 10 mg/kg of piroxicam while remaining three groups were given 10, 20, 40 mg/kg of atorvastatin respectively. Oral route was employed for administration to rats. 30 min subsequent to oral administration on day first, non-immunological acute arthritis was provoked by sub planter formaldehyde 2% (0.1 mL) injection, followed by repetition on day 3. Drug treatment was administered continuously for 10 days. Appraisal of arthritis was done by estimating paw volume with digital plethysmometer (Narayani *et al*., 2014). Suppression of paw edema achieved with atorvastatin and piroxicam was equated with arthritic control. Body weight and arthritic indices were calculated on the start and end of study. Histopathological examination of the ankle joint was also performed according to the method described by Akhtar *et al*. (2011).

Molecular docking procedure

MOE-Dock of Chemical Computing Group Inc was employed to conduct docking procedure. Crystal structure of Bovine serum albumin (BSA), Histamine H1 receptor and Prostaglandin E2 was retrieved from Protein Data

Bank (PDB id 4JK4, 3RZE, 5YWY). Ensuing docked complex was employed for energy parameters calculation and for the prediction of docked interactions at protein's active site.

STATISTICAL ANALYSIS

Results were represented as mean ± S.E.M. Statistical analysis was accomplished through two-way analysis of variance (ANOVA) test, followed by Bonferroni posttest, using GraphPad Prism 5. The value of significant difference was considered at $p<0.05$.

RESULTS

Inhibitory impact of atorvastatin against thermally induced egg albumin denaturation

Inhibitory consequences of atorvastatin on protein denaturation induced in egg albumin by providing high temperature are depicted in fig. 1. Concentration dependent inhibition of protein denaturation was being displayed by atorvastatin and standard drug piroxicam. Atorvastatin at the concentration of 6400µg/ml showed percentage inhibition of 1860 % which is higher than the percentage inhibition displayed by standard drug piroxicam (1088%) at the same concentration.

Inhibitory impact of atorvastatin against thermally induced BSA denaturation

Atorvastatin at several different concentrations provides considerable protection against thermally induced protein denaturation in bovine serum as shown in fig. 1. Results depicted a maximum percentage protection of 94.50% at the maximum concentration of 6400µ/ml which is significantly higher than the protection provided by the standard drug piroxicam (87.33%). Thus, atorvastatin proved to be an efficacious agent in inhibiting protein denaturation.

Protective aspect of atorvastatin on membrane of RBCs

Atorvastatin remarkably protected erythrocytic membrane from lysis instigated by hypotonic solution in a

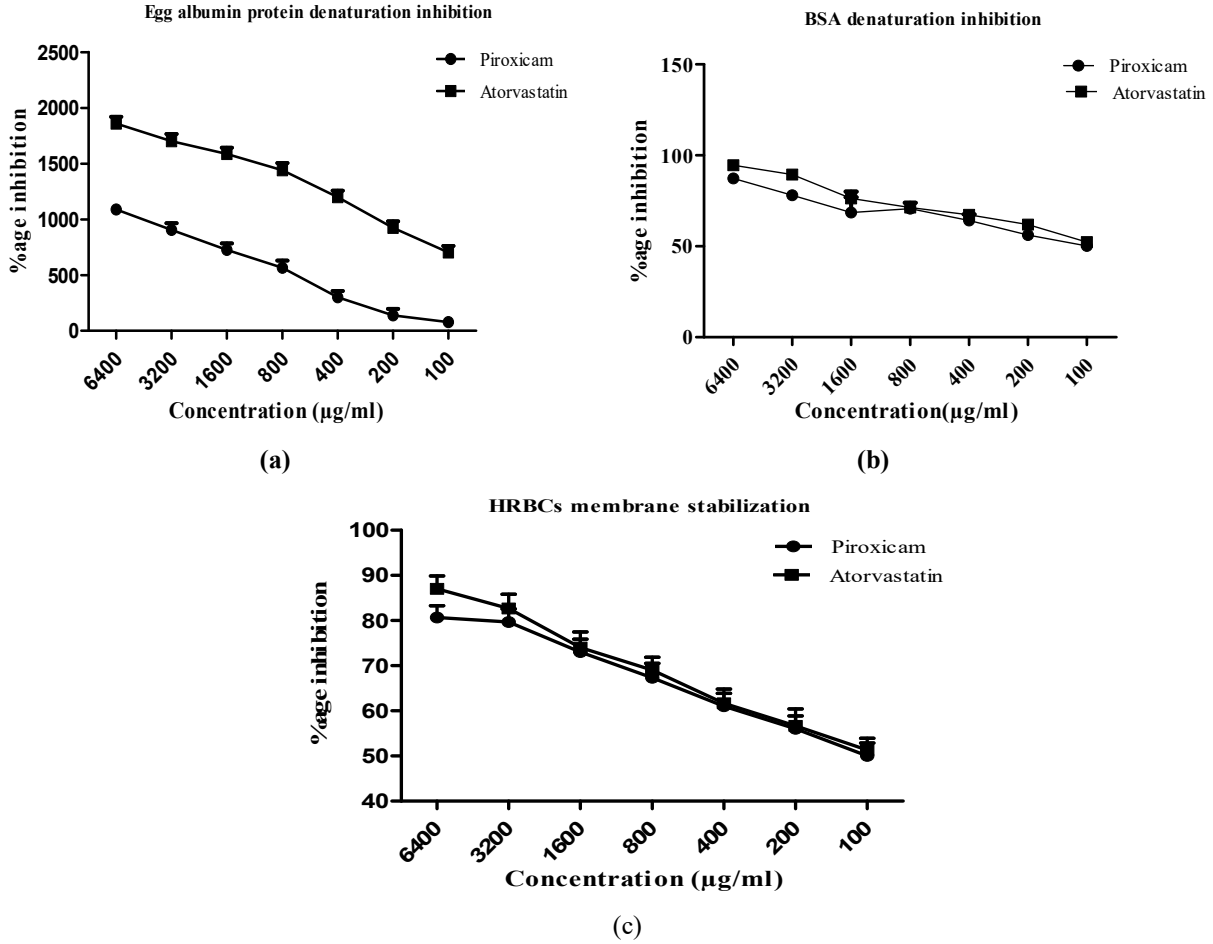


Fig. 1: Effect of atorvastatin on *in vitro* anti-arthritic experimental models. (a)Egg albumin denaturation assay (b) Bovine serum denaturation assay (c) Membrane stabilization assay.

Values are expressed as Mean ± SEM (n=3), *p < 0.05, **p < 0.01, ***p < 0.001 compared with arthritic control by two way ANOVA followed by Bonferroni multiple comparison test.

Table 1: Effect of atorvastatin on right hind paw volume of rats in formaldehyde induced arthritis

Treatment Groups	Effect on Rat's Paw volume (ml)				
	2nd Day	4th Day	6th Day	8th Day	10th Day
Arthritic Control (1ml/kg)	1.55±0.045	1.78±0.026	1.72±0.022	1.66±0.055	1.67±0.055
Piroxicam (10mg/kg)	1.15±0.032*** (25.80%)	0.99±0.076*** (44.38%)	0.79±0.052*** (54.06%)	0.65±0.051*** (60.84%)	0.48±0.031*** (71.25%)
Atorvastatin (10mg/kg)	0.97±0.054*** (37.41%)	1.26±0.146*** (29.21%	0.87±0.030*** (49.41%)	0.70±0.094*** (57.83%)	0.54±0.058*** (67.66%)
Atorvastatin (20mg/kg)	0.92±0.166*** (40.64%)	1.11±0.061*** (37.64%)	0.77±0.074*** (55.23%)	0.62±0.076*** (62.65%)	0.46±0.042*** (72.45%)
Atorvastatin (40mg/kg)	0.85±0.031*** (45.16%)	1.050±0.033*** (41.01%)	0.65±0.028*** (62.20%)	0.53±0.057*** (68.07%)	0.34±0.079*** (79.64%)

Data is expressed as Mean± SEM, n=6, percentage inhibition from edema is shown in parenthesis.. *** = $P < 0.001$ when compared to arthritic control.

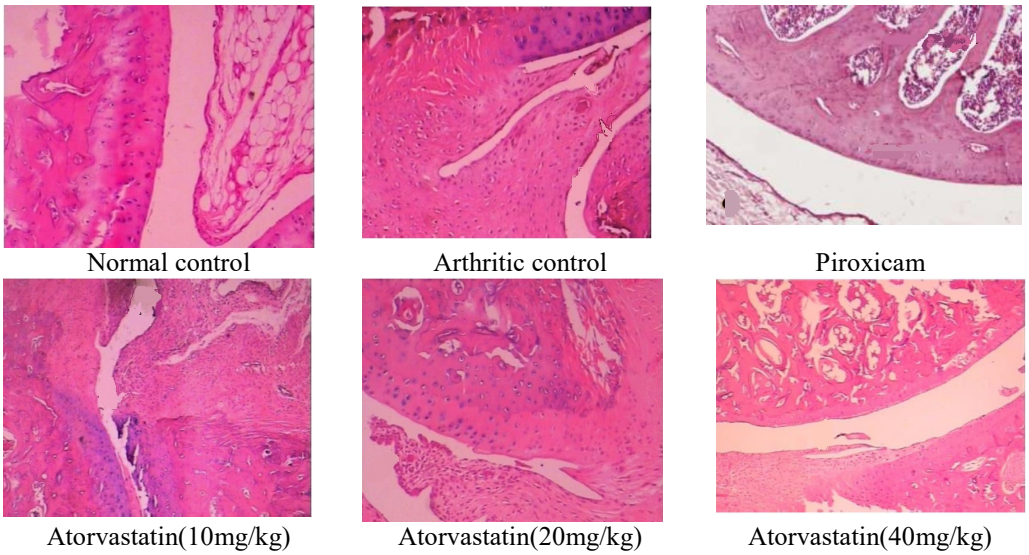


Fig. 2: Histopathological investigation of treated rat's ankle joint.

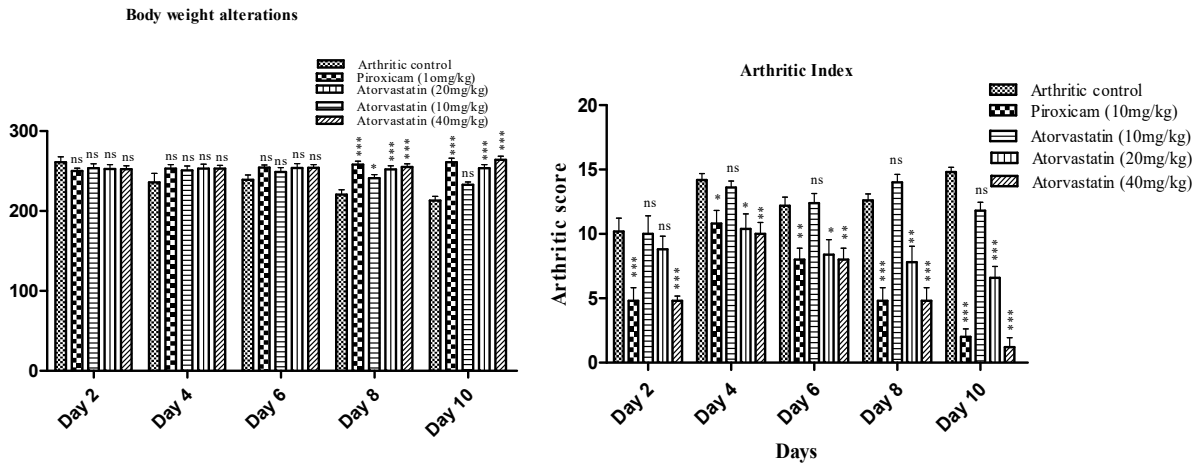
concentration gradient manner (100-6400 µg/mL) (fig. 1). The maximum effect (87%) was obtained at the maximum concentration of 6400ug/ml and the findings of the current study are in accordance with the findings of standard drug piroxicam which showed 80.66% of membrane protection from lysis. Thus, this proves another way by which atorvastatin can exert its anti-arthritic effect by stabilizing erythrocyte membrane

Impact of Atorvastatin on formaldehyde induced arthritis

Anti-arthritic attribute of atorvastatin was also assessed *in vivo* via formaldehyde provoked arthritis in rats. Formaldehyde injection in arthritic control group demonstrated substantial increment in paw volume. The rat hind paw evaluation done on 10th day revealed that treatment with 40 mg/kg of atorvastatin significantly reduced paw volume (79.64 %) as compared to the arthritic control group while the standard drug piroxicam provides a percentage inhibition of paw edema of 71.25% respectively as shown in table 1. Also atorvastatin treated group displayed marked increase in body weight relative to arthritic control rats which failed to achieve a gain in their weight recorded at the end of study (fig. 3) Arthritic indices were also reduced in atorvastatin treated group as shown in fig. 3. Histopathological examination of ankle joint of test drug treated animals displayed significant reduction in inflammation, pannus formation and bone formation as compared to untreated arthritic group especially at the dose of 20mg/kg and 40mg/kg as shown in fig. 2.

Molecular docking analysis

Atorvastatin was being docked at the active site of 4JK4, 3RZE and 5WYW respectively. Various positions of Atorvastatin-Target complex were evaluated for prediction of preference for binding site and mode of ligand-target interaction. Best conformation based on lowest binding energy was selected. Binding free energy data obtained after docking procedure showed that atorvastatin exhibit favorable docked complex with the targets (fig. 4, table 2). The calculations were also used to



(a) (b)

Fig. 3: Effect of atorvastatin on clinical parameters after induction with formaldehyde (a) body weight alterations (b) arthritic index of treated rats.

Values are expressed as Mean ± SEM (n=6), *p < 0.05, **p < 0.01, ***p < 0.001 compared with arthritic control by two way ANOVA followed by Bonferroni multiple comparison test.

Table 2: Binding Interactions observed among atorvastatin-target complexes

Atorvastatin-Target Complex	H-bonding			π-π interactions	Arene-cation interactions
	Distance (°A)	Score (%)	Amino acids		
Atorvastatin-BSA Complex	2.81 2.39	56% 29%	Lys116 Glu 515	---	Arg 141 Arg 135
Atorvastatin-H1 Receptor complex	2.42	43%	Tyr 431	---	Lys179 Lys179
Atorvastatin-PGE2 Complex	3.04 1.52 2.67 2.14	29% 41% 52% 25%	Thr A168 Thr A168 Tyr A80 Ser A319	---	---

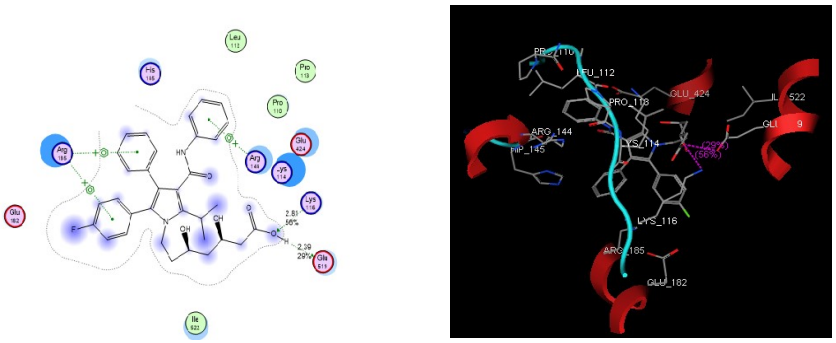
predict the atorvastatin-target complex structures and to study possible interactions, based on H-bonding, π-π, and arene-π interactions, within 5 Å range. Atorvastatin was found to bind in the same pocket of active site as by actual ligands of BSA, Histamine H1 receptor and PGE2.

DISCUSSION

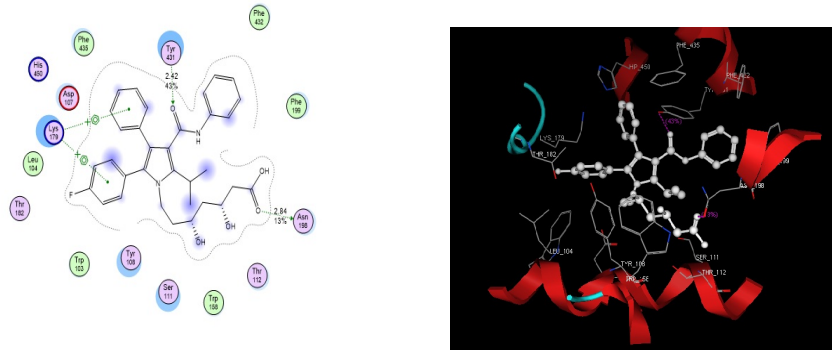
The outcomes obtained from current study revealed a dose dependent anti-arthritic potential of atorvastatin in all models used for assessing anti-arthritic potential. *In vitro* methods employed to assess the anti-rheumatic activity of atorvastatin comprised of inhibition of denaturation of BSA and egg albumin along with membrane stabilization assay, whereas *in vivo* anti-arthritic potential of atorvastatin was appraised with formaldehyde induced arthritis.

Protein denaturation has been documented to be the primary reason of inflammatory and arthritic disorders via production of auto-antigens. Protein denaturation leads to alteration in disulphide, hydrogen, hydrophobic and electrostatic bonds (Alamgeer *et al*., 2017). Several anti-inflammatory drugs inhibit protein denaturation evoked by heat in a dose dependent manner (Gautam *et al*., 2013). Thereby, agents which can deter denaturation of protein would be thought as promising candidate for anti-arthritic drug development. Current analysis revealed a marked inhibitory effect of atorvastatin on protein denaturation even more prominent than piroxicam (reference drug). Thus atorvastatin holds the potential of reducing auto-antigens generation thereby capable of relieving arthritis.

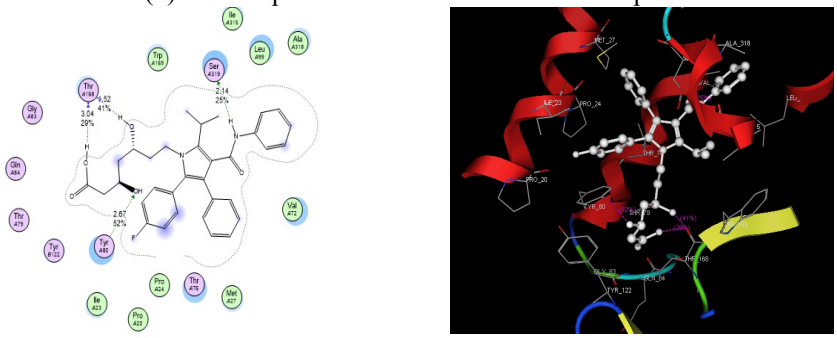
Erythrocytic membrane has been thought to be similar to lysosomal membrane thereby protection of human RBC from lysis instigated by hypo tonicity can be associated with anti-arthritic attribute of substance. Hypotonic solution leads to lysosomal membrane rupturing with subsequent liberation of lysosomal content (bacterial enzymes and proteases) along with phospholipase A2 and thus inflammatory intermediaries' generation. Therefore RBCs membrane stabilization averts inflammatory mediators' liberation with subsequent inhibition of cell rupture and tissue injury (Alamgeer *et al*., 2015). Some glucocorticoids and NSAIDs have been documented to



(a) Docked pose of atorvastatin with BSA



(b) Docked pose of atorvastatin with H-1 receptor



(c) Docked pose of atorvastatin with PGE2

Fig. 4: 2D and 3D ligand interaction diagram of atorvastatin with target proteins (a) atorvastatin-BSA complex (b) Atorvastatin-H1 receptor complex (c) Atorvastatin-PGE2 complex

stabilize lysosomes in tissue cells thus impedes the liberation of lysosomal enzymes (Menon *et al*., 2011). A remarkable suppression of RBCs membrane lysis induced by hypotonic solution has been observed with atorvastatin in a concentration gradient manner comparative to piroxicam. Current work findings recommend stabilization of membrane as additional anti-arthritic mechanism of atorvastatin.

Formaldehyde-provoked arthritis has been documented to be the most reliable *in vivo* model for anti-arthritic potential screening due to resemblance with human arthritis (Udavant *et al*., 2012). Formaldehyde instigates arthritis via protein denaturation at injection site thus provokes an inflammatory response against denatured proteins (Ben *et al*., 2016). Additionally, formaldehyde prompted arthritis has been documented to be a biphasic response comprising of neurogenic phase followed by tissue intervened reaction phase (Nair *et al*., 2012). The first stage of edema has been associated with release of kinins, histamine and serotonin whereas second stage of edema occurrence might be linked to release of prostaglandin like substances (Owoyele *et al*., 2011; Shaikh, 2011). Former studies proved that centrally acting agents can deter both stages of arthritis provoked by formaldehyde though peripherally functioning substances can inhibit later stage only (Narayani *et al*., 2014). Alleviation in paw volume delineated by atorvastatin in formaldehyde induced arthritis model can be thought to be due to inhibition of these inflammatory mediators. Maximum decline in paw volume was presented at the dose of 40mg/kg. Moreover, as atorvastatin inhibited both phases of inflammation it can be conceivable that it acts centrally to produce anti-arthritic effect.

Weight loss has been concluded to be an extra-articular attribute associated with RA possibly due to muscle wasting induced by protein denaturation or reduced food intake due to high leptin level after arthritis induction with formaldehyde (Qasim *et al*., 2020). Atorvastatin averted this weight loss as observed in arthritic control group thus prevented cachexia associated with RA. Histological analysis of all the rats revealed that the arthritic control group manifested some hallmark features of RA such as cartilage and bone erosion with inflammatory cells infilteration (Zhu *et al*., 2014). Atorvastatin (40mg/kg) and piroxicam treated group successfully preserved these histological alterations relative to arthritic control group. However atorvastatin treated group at the dose of 10 mg/kg also displayed synovial infilteration and bone erosion.

Molecular docking has been proved to be a reliable tool for predicting various binding poses for protein ligand interactions. Thereby, this facile strategy could be utilized so as to assess molecular interaction along with the most suitable binding site. Additionally, different types of interactions on the basis of distance between the atoms in the amino acid and ligand could also be determined. Current study was conducted with an attempt to demonstrate the interaction of atorvastatin with BSA (PDB id 4JK4), Hitamine H1 receptor and PGE2. Atorvastatin revealed strong binding interaction with all these selected targets thus by this way inhibit thermally induced protein denaturation in BSA along with inhibition of histamine and prostaglandin release observed in formaldehyde induced arthritis model. Thus atorvastatin provide its anti-arthritic effect by targeting multiple parameters involved in pathophysiology of RA.

CONCLUSION

Atorvastatin may serve as an effective anti-arthritic agent; however detailed mechanistic studies are required to point out the exact mechanism of action before introducing the drug to clinical trials. Results obtained from current study point out towards the repurposing of atorvastatin for rheumatoid arthritis as this strategy of drug discovery will help to save time and cost and also have high success rate.

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