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RESEARCH ARTICLE

STUDY OF ANTI-DIABETIC ACTIVITY OF ARTESUNATE AS AN AGONIST TO GLP-1 BY MOLECULAR DOCKING AND *IN-VITRO* ANALYSIS

Sridharan, K., Vimal Anand, S., Dr. Saravanan, N., *Dr. Ilamathi, M. and Dr. N. G. Ramesh Babu

Adhiyaman College of Engineering, Hosur, V S Clinical Research and Hospital (P) Ltd., TICEL, Taramani, Chennai

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ABSTRACT

The prevalence of diabetes mellitus (DM) is increasing with ageing of the population and life style changes associated with rapid urbanization and westernization. Though there are many drugs available in market to address this issue, the drug resistance is a real challenge to the pharmaceutical industry. To address this problem, we have selected a well-known anti-malarial drug, Artesunate (AS). With strong earlier reports on anti-inflammatory, anti-oxidant, anti-pyretic activities of this compound, we aim to study the anti-diabetic activity of AS. AS act as an agonist to Glucagon like protein (GLP) and can bind to GLP-1 Receptor, thus facilitating the glucose uptake by cells. Initially, a docking study was performed to check the affinity of AS with GLP-1R followed by prediction of ADME/Tox analysis. From the docking results, we were able to find that AS have good binding activity to GLP-1R and also have good drug likeliness properties. The computational result was then evaluated using biochemical assays and cell based assays in MIN6. α-amylase inhibitory effect and glucose uptake by yeast cells were studied. AS inhibits α-amylase and improve glucose uptake by cells at the concentration of 80μM. A cytotoxic analysis was performed using MTT assay. Upto 100μM, AS was non-toxic to cells. AS also induce the cell to produce insulin which was confirmed by measuring the Insulin secreted by the cells. This clearly proves that AS by binding to GLP-1R can provoke the cells to uptake glucose and secrete insulin as well. Thus, AS can be a potential anti-diabetic drug with high efficacy and low cytotoxicity compared to other drugs.

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INTRODUCTION

Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. In low- and middle-income countries including Asia and Africa has the greatest increase in prevalence, where most patients be found by 2030. The increase in incidence in developing countries follows the trend of urbanization and lifestyle changes, including increasingly sedentary lifestyles, less physically demanding work and the global nutrition transition, marked by increased intake of foods that are high energy-dense but nutrient-poor (often high in sugar and saturated fats, sometimes referred to as the western pattern diet). The risk of getting type 2 diabetes has been widely found to be associated with lower socio-economic position across countries(Thomas et al., 2012). Until 2016, 422 million people have diabetes worldwide, up from an estimated 382 million people in 2013 and from 108 million in 1980. Accounting for the shifting age structure of the global population, the prevalence of diabetes is 8.5% among adults, nearly double the rate of 4.7% in 1980.

Corresponding author: **Dr. Ilamathi, M.**
Adhiyaman college of Engineering, Hosur, V S Clinical Research and Hospital (P) Ltd., TICEL, Taramani, Chennai.

In such case type 2 makes up about 90%. Where some data indicate rates are roughly equal in women and men, but male excess in diabetes has been found in many populations with higher type 2 incidence, possibly due to sex-related differences in insulin sensitivity, consequences of obesity and regional body fat deposition, and other contributing factors such as high blood pressure, tobacco smoking, and alcohol intake Controlling blood sugar (glucose) levels is the major goal of diabetes treatment, in order to prevent complications of the disease. Type 1 diabetes is managed with insulin as well as dietary changes and exercise. Type 2 diabetes may be managed with non-insulin medications, insulin, weight reduction, or dietary changes. Traditional herbal medicine is well known for their hypoglycaemic activities, and the available literature indicate that there are more than 1300 plant species showing hypoglycaemic activity. Currently it has been increasing demand for the use of plant products with anti-diabetic activity due to low cost, easy availability and lesser side effects. Therefore, plant materials are continuously scrutinized and explored for their effect as hypoglycaemic agents(Viseshni, 2017) (Moukette et al., 2017) (Kumar Sharma, Kumar, Patel, and Hugar, n.d.). This made us to look for an herbal based compound for our study.

On search of these compounds, we thought of repurposing a drug which will reduce our time and cost in clinical research. We have chosen Artesunate (AS) which is the first line treatment for children or adults with severe malaria. AS is also used to treat less severe forms of malaria when it can be given orally. Artemisinin is a drug which was extracted from the plant *Artemisia annua*, that belongs to plant family of Asteraceae. AS is a semisynthetic derivative of artemisinin and has a sesquiterpine lactone structure with a peroxide bridge. The presence of the peroxide bridge gave the medicinal properties to the compound. In this research, we aim at targeting a protein which plays a major role in diabetes – GLP1R. Glucagon-like peptide 1 receptor (GLP1R) is a receptor protein found on beta cells of the pancreas. It is involved in the control of blood sugar level by enhancing insulin secretion (Drucker and Nauck, 2006). In humans it is synthesised by the gene GLP1R, which is present on chromosome 6. It is a member of the glucagon receptor family of G protein-coupled receptors. GLP1R is composed of two domains, one extracellular (ECD) that binds the C-terminal helix of GLP-1, and one transmembrane (TMD) domain that binds the N-terminal region of GLP-1. In the TMD domain there is fulcrum of polar residues that regulates the biased signalling of the receptor (Athauda and Foltynie, 2016; Zhao, Liang, Yang, Yu, and Qu, 2017).

While the transmembrane helical boundaries and extracellular surface are a trigger for biased agonism. GLP1R is known to be expressed in pancreatic beta cells. Activated GLP1R stimulates the adenylyl cyclase pathway which results in increased insulin synthesis and release of insulin. Consequently, GLP1R has been a target for developing drugs usually referred to as GLP1R agonists to treat diabetes. Exendin-4 is the peptide used therapeutically to treat diabetes, and its biological binding mode to the GLP-1R has been demonstrated using genetically engineered amino acids. GLP1R is also expressed in the brain where it is involved in the control of appetite. Furthermore, mice which over express GLP1R display improved memory and learning. Where the activated GLP1R stimulates the adenylyl cyclase pathway which results in increased insulin synthesis and release of insulin (Meloni, DeYoung, Lowe, and Parkes, 2013). This protein is an important drug target for the treatment of type 2 diabetes and stroke.

Insulin influences the intracellular utilization of glucose in a number of ways. It increases hepatic glycolysis by increasing the activity and amount of several key enzymes including glucokinase, phosphofructokinase, and pyruvate kinase. With this background, we hypothesised in our project that, if AS binds to GLP-1R mimicking GLP1, the cells can uptake glucose very effectively. AS is also reported to have many properties and indexed in many literatures and proven to have the negligible side effects. We can repurpose the anti-malarial drug to have a good anti-diabetic potential.

MATERIALS AND METHODS

Cell lines and culture media

MIN6 cell lines, which are insulinoma cell line derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic beta cells. MIN-6 produce insulin and T antigen and have morphological characteristics of pancreatic β cells. MIN-6 cells exhibits glucose-inducible insulin secretion comparable with cultured normal mouse islet cells. The media used for study is Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% antibiotics.

Retrieval of Protein and Ligand 3D structures

The three dimensional structure of GLP-1R protein was retrieved from PDB database. It was then energy minimized using swiss-pdb viewer. The modified residues were removed and optimized (Guex and Peitsch, 1997). The ligand ATS structure was retrieved from pubchem compounds. The energy minimization of ligand was performed using Frog2 tool(Leite et al., 2007).

Molecular docking

Docking was performed using Autodock in Linux platform. This is most probably used to find binding site of protein and ligand, binding energy, binding affinity etc., The computer simulated docking work was performed using MGL Tools software 1.5.6 and Autodock 1.5.6 program (Leite et al., 2007). The initial step was preparing protein by adding/editing colour, hydrogen (merge non-polar), charges (kollman charges), and atoms (assign AD4 type) etc., and saved it in pdbqt format. Ligand preparation was also done and grid was set with active site residues. The autogrid and autodock was run. The docking score and the docking interactions were then studied.

ADME/Tox analysis

To predict the pharmokinetic properties, preADMET tool was used online. DruLito was also checked to evaluate the drug likeliness properties of the compound ATS (Lee et al., 2002).

α-amylase inhibition assay

This assay is used to study the effect compound that inhibits the activity of the α-amylase. α-amylase is the enzyme which hydrolyses the alpha-linked polysaccharides such as starch, glycogen yielding the glucose and maltose (Bernfeld, 1955). This inhibition assay was performed by preparing the stock solutions. 0.5mg of α-amylase is mixed with 1mL of PBS. 1% starch solution is prepared by dissolving 100mg of starch in 10mL PBS. Then DNS reagent is prepared by adding 1g of DNS in 20mL of 2M NaOH and mix with 30g sodium potassium tartarate and makeup to 100ml with distilled water. The different concentration of ATS (20µm, 40µm, 60µm, 80µm, 100µm) was prepared. 500µl of α-amylase was added to the different concentration of ATS and incubated at 25˚C for 10 min. Then add 500µl of starch solution and again incubated at 25˚C for 10 min. 1ml of DNS reagent was added and incubated at 100˚C for 5 min. After the incubation it is cooled down to room temperature and madeup to 10ml with distilled water. The spectrophotometer reading was taken at 540nm and the percentage inhibition was calculated by using the formula,

% inhibition = {absorbance (control)- absorbance (sample)} ÷ absorbance (control) × 100

Glucose uptake by yeast cells

This assay was performed to study the effect of ATS on uptake of glucose by the yeast cells (Gonçalves and Loureiro-Dias, 1994). Yeast suspension was grown on YPD broth and centrifuged at 3000*g for 5 mins.

This was continued until the supernatant fluids were clear. 10% suspension was performed with the supernatant fluid. Three different concentration (5mM, 10mM , 25Mm) of glucose solution was prepared and 1ml glucose solution was added to different concentration (20µm, 40µm, 60µm, 80µm, 100µm) of ATS and incubated for 10 min at 37˚C. Then 100µl of yeast suspension was added and vortexed and incubated for 60 min at 37˚C. After the incubation the mixture was centrifuged at 2,500*g for 5 min. Then the glucose content was estimated with the supernatant. The percentage increase in glucose uptake by yeast cells was calculated using the following formula

%uptake = {absorbance (control)-absorbance (sample)}÷ absorbance (control)×100

Cytotoxicity assay

MIN6 cell lines were used for the study and is cultured in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% FBS and 1% antibiotic solution. Cytotoxicity of ATS was determined by MTT assay. MTT (3-(4, 5- dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide), proliferation assay was utilized in MIN6 cell lines to assess the dose-dependent effect of ATS. The culture was incubated with the test solution in DMEM at 37^{O}C for 18hrs in a 5% CO2 atmosphere. After an overnight incubation, cells were treated with varying concentrations (0µM, 20µM, 40µM, 60µM, 80µM, 100µM, 120µM, 140µM) and incubated for 18hrs. After incubation the readings were taken by ELISA at 570nm (Gonçalves and Loureiro-Dias, 1994).

Insulin Secretion assay using ELISA method

Media used for insulin secretion was serum free DMEM with no glucose, supplemented with 2 mM L-glutamine and 25 mM Hepes, pH 7.4. Glucose was added to prepare basal (1 mM) and other glucose concentrations (2 mM and 5 mM) and warmed to 37°C prior to use. MIN6 cells were washed twice with PBS and placed in basal serum free media for 2 hours, washed with fresh basal media and placed in the stated media for 15 minutes. Media was then replaced with media containing higher concentrations of glucose for 15 minutes. 15 minutes was the minimum time necessary for the cells and culture media to reach 37°C (approx. 5 minutes) and insulin secretion (approx. 10 minutes) to occur.

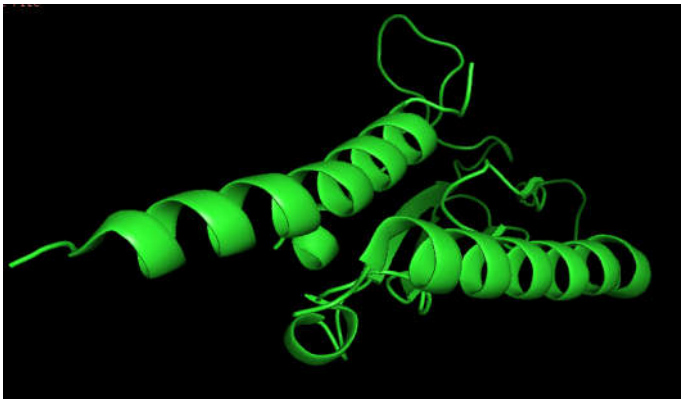

Fig.1. Represent the structure of the GLP-1R protein which is viewed in PYMOL. Ligand

After completion of the incubations, the cells were lysed with acid/ethanol for measurement of total insulin content using ELISA kit (Konstantinova et al., 2007). The procedure followed for ELISA was given as manufacturer's protocol.

RESULTS AND DISCUSSION

Retrieval of Protein and Ligand 3D structures

The protein GLP-1R was retrieved from PDB and the protein was found to be 4ZGM which was the structure of Homo sapiens GLP-1R protein (Fig 1). The active site residues of GLP-1R were Ser31, Leu32, Thr35, Val36, Typ88, Trp39. The ligand AS structure was taken from the PubChem database (Fig 2). The pubchem ID of AS is 6712286.

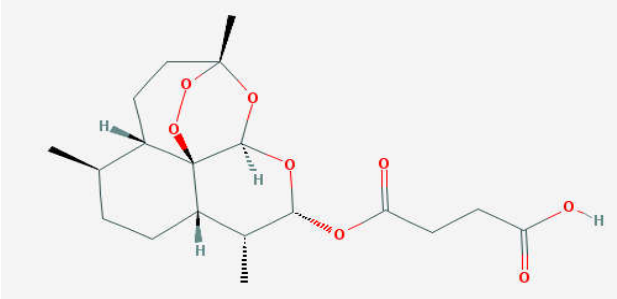

Fig.2. Represent the structure of the ligand AS

ADME/Tox analysis

It was performed to study the pharmacokinetic property and toxicity of compound AS. From ADME/Tox we determined three properties Drug likeness, ADME prediction, Toxicity. AS was Non-Mutagen to the cells. It obeyed the Lipinski rule of five.The results are tabulated in Table 1, 2 and 3

Table 1. Represents the Drug likeness prediction

S.No	ID	Acceptable Value	Obtained Value
1.	Molecular Weight <=	500	384.18
2.	LogP <=	5	2.925
3.	H-Bond donor	5	1
4.	H-Bond acceptor	10	8

Table 2. Represent the ADME prediction

S.No	ID	VALUE
1.	Caco2	13.2193
2.	MDCK	0.154021
3.	BBB	0.0192715
4.	Plasma_protein_binding	90.610666
5,	Skin_permeability	-3.13133

Table 3. represent the Toxicity prediction

S.No	ID	VALUE
1.	Ames_test	Non-mutagen
2.	Carcino_mouse	Negative

Molecular docking

Docking was performed to bind the ligand with receptor. It was done to determine the total interaction energy for a ligand with a macromolecule. Docking was done to find the interaction of ligand with receptor at their active site. The docking score was determined from auto dock tool is -5.31 kcal/mol. (Fig 6 and Table 4 shows the result of molecular docking analysis)

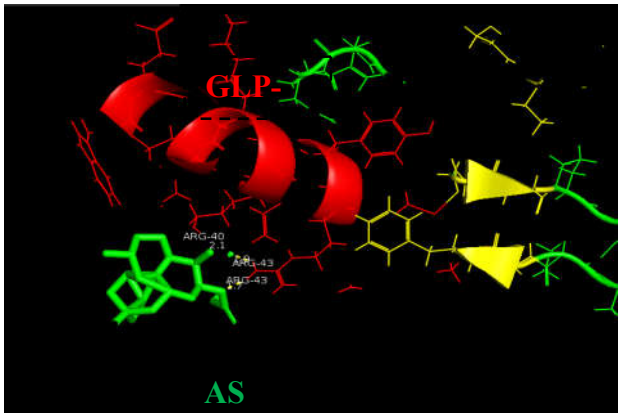

Fig.6. Represent the AS bound to the GLP-1R protein at active sites. Green colour stick represent the ligand AS, red colour represent the GLP-1R protei

Table 4. Represent the docking score, interacting residues and bond length.

Name	Score	Interacting residues	H-bond length
AS	-5.31 kcal/mol	ARG-43	1.9
ID_6712286		ARG-43	1.7
		ARG-40	1.8
		ARG-40	2.1

α-amylase inhibition assay

α-amylase inhibition assay was performed with different concentration (20µM, 40µM, 60µM, 80µM and 100µM) of AS. At the concentration of 80µM, higher inhibition of the α-amylase was observed (Fig 7).

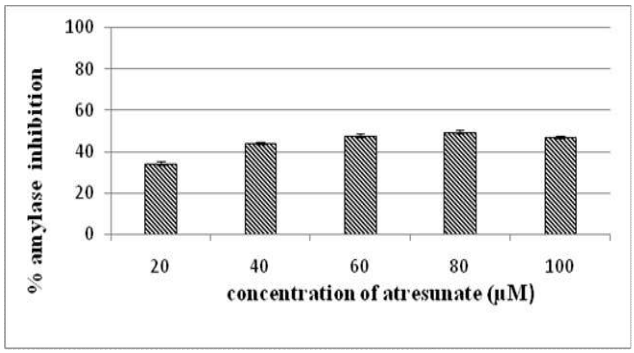

Fig 7. Represents the α-amylase inhibition with increasing concentration of AS

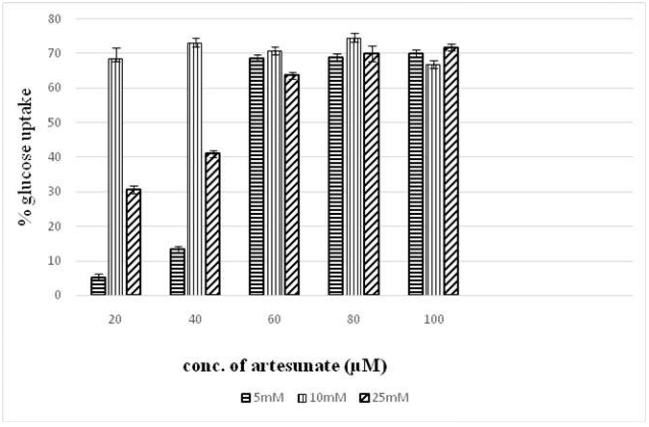

Fig 8: Represents the glucose uptake by yeast cells at different concentrations of glucose and AS

Glucose uptake by yeast cells

Glucose uptake assay was performed with different concentration (20µM, 40µM, 60µM, 80µM and 100µM) of AS with three concentration (5mM, 10mM and 25mM) of glucose. In this 80µM had higher uptake of glucose of all the three concentration of glucose by the yeast cells (Fig 8).

Cytotoxicity assay

MTT assay was performed with MIN-6 cells against the different concentration of AS. The result shows that the AS is non-toxic to the MIN-6 cells (Fig 9).

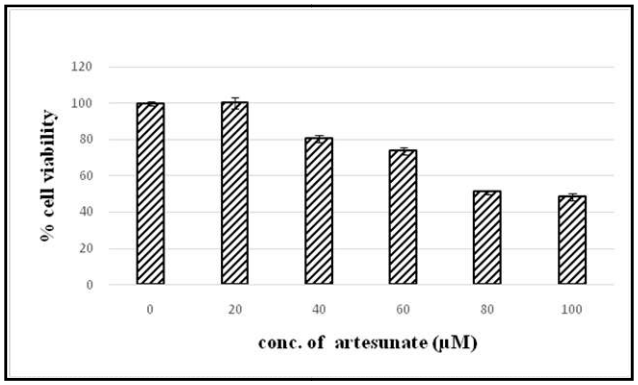

Fig 9. Cell viability of MIN6 cells when treated with AS

Insulin Secretion assay using ELISA method

Insulin secretion assay was performed with the MIN-6 cells. The AS treated cells secretes more insulin than the normal cells (Fig 10).

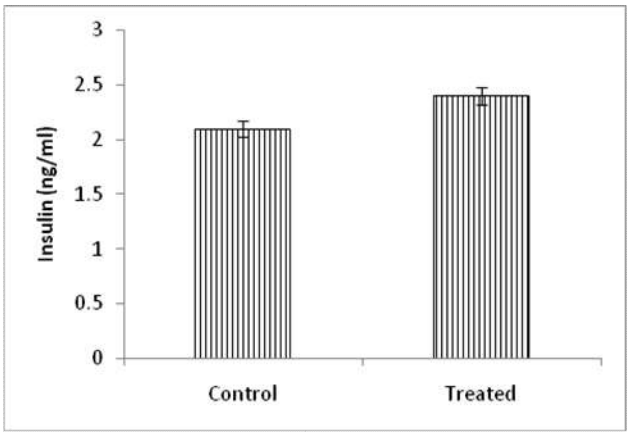

Fig. 10. Amount of Insulin secreted from MIN6 cells

AS act as an agonist to Glucagon like protein (GLP) and can bind to GLP-1 Receptor, thus facilitating the glucose uptake by cells. Initially, a docking study was performed to check the affinity of AS with GLP-1R followed by prediction of ADME/Tox analysis. From the docking results, we were able to find that AS have good binding activity to GLP-1R and also have good drug likeliness properties. The computational result was then evaluated using biochemical assays and cell based assays in MIN6. α-amylase inhibitory effect and glucose uptake by yeast cells were studied. AS inhibits α-amylase and improve glucose uptake by cells at the concentration of 80µM. A cytotoxic analysis was performed using MTT assay. Upto 100µM, AS was non-toxic to cells.

AS also induce the cell to produce insulin which was confirmed by measuring the Insulin secreted by the cells.

Conclusion

This study clearly proves that Artesunate by binding to GLP-1R can provoke the cells to uptake glucose and secrete insulin as well. Thus, AS can be a potential anti-diabetic drug with high efficacy and low cytotoxicity compared to other drugs. Further, molecular experiments should be carried out to provide strong evidence on the mechanism of action of the compound.

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