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Research Article

Forte of Bioinformatic Computational Tools in Identification of Targets of Digitalis in Unicellular Eukaryotes: Featuring *Acanthamoeba Castellanii*

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Abstract

Membrane Na^+- K^+- ATPase is a known target of the drug digoxin, which is a cardiotropic agent prescribed for congestive heart failure and certain arrhythmias in clinical practice. The drug in humans binds and inhibits the cell membrane Na^+- K^+- ATPase and a mitochondrial enzyme cytochrome P450-11A1. Recently, digoxin has been shown to exert anti-amoebic effects against pathogenic strains of *Acanthamoeba castellanii*, which we have selected as a model unicellular eukaryote to show the homology of the targets of digoxin in this pathogen compared with humans. The presence of human cellular target receptors and the pattern of cell death induced by digoxin in this unicellular eukaryote remains to be established. In this study, we performed a bioinformatics search, 3D structural modelling, ligand binding predictions, and experimental assays that show the target proteins in *Acanthamoeba* are similar to the cellular targets of digoxin in humans. Amino acid sequence identity scores, 3D modeling and ligand binding predictions show that *A. castellanii* do express a similar P-type ATPase. The cytochromes of amoeba did not show a amino acid sequence homology to Cytochrome P450-11A1, but has a few closely related cytochromes with similar ligand binding attributes. Growth assays show amoebicidal and amoebistatic effects while flow cytometry showed apoptosis, as a form of death in *A. castellanii* at a dose of 40 µg/ml and necrosis at higher doses of 80 µg/ml. Bioinformatic tools, 3D structural modelling and ligand binding attributes offer an precise method to explore drug targets and facilitates the study the evolution of these cardinal ion transporters and enzymes.

Keywords: *Target receptors of Digoxin; Acanthamoeba castellanii; Digoxin; Amoebicidal; Unicellular Apoptosis; Model unicellular eukaryotes*

Introduction

The use of bioinformatic computational tools, 3D structural modelling and ligand binding attributes have been used in the past to identify cellular targets of drugs used in humans to identify targets in model unicellular eukaryotes like *Acanthamoeba castellanii, Balamuthia mandrillaris* and *Naegleria fowleri*. Among the above-mentioned approaches, structural homology modelling and seeking ligand binding attribute homology in particular makes it possible to identify diverse group of evolutionarily conserved proteins, their function and the possibility of targeting them with drugs. Though the finding of an identical or similar amino acid sequence homology is ideal for establishing drug target similarity, this in reality is difficult in most of the cases because of the alterations in the amino acid number and the structure of non-docking portions that occur in these proteins during evolution as gene regulation and expressions are occasionally alike between different species [1]. The only entity that remains conserved in a ligand binding receptor is the constellation of amino acids in the docking site of the protein molecule [2,3]. Structural homology, 3D modelling and ligand binding attributes that use stringent pa-

rameters to detect the docking pits of these proteins has emerged as the latest protein homology validating modality. Recently, by using latter methods there have been reports of the evidence of a homolog of human muscarinic M1 receptor in a model eukaryote, *Acanthamoeba castellanii*, which is believed to be a possible target of anticholinergic drugs [4]. In the present study, we use 3D modeling, structural bioinformatic computational tools and ligand binding attribute detecting in-silico methods that are supplemented by experimental assays to show the evidence of presence of target receptors of digoxin in *Acanthamoeba castellanii*.

One of these targets is the Na^+- K^+-ATPase ion exchange protein pump that is inhibited by digoxin, ouabain and other digitalis like compounds. This cell membrane protein utilizes ATP to exchange three Na^+ ions to the extracellular space for two K^+ ions that it transports to the intracellular space [5]. By utilizing ATP, the Na^+- K^+-ATPase generates a transmembrane electrical gradient that maintains the resting potential, which is important for the function of cells in general and excitable tissues in particular [6,7]. The Na^+- K^+-ATPase is composed of different subunits; α, β, and a family of 35-amino acid signature sequence domains, namely the FXYD family [5,7]. There are 4 isoforms of the α subunit and 3 isoforms of the β subunit [6]. The α1 subunit is responsible for the catalytic function of the enzyme, while the β subunit not only helps in cleavage of ATP, but also determines the integration of the Na^+- K^+-ATPase molecule in the cell membrane (Figure 1). Digitalis is the name of a group of cardiotropic glycosides like digoxin, ouabain and related compounds that recognize the Na^+- K^+-ATPase as a receptor and inhibit it to exert a strong mechanical (ionotropic) effect on the heart [7,6]. Digoxin is a glycoside obtained mainly from the plant *Digoxin Lanata*; it consists of 3sugars and a single aglycone ring, digoxigenin [8]. Digoxin acts as an antagonist (Figure 1) of Na^+- K^+-ATPase, resulting in an increase of intracellular Ca^{+2} ion concentrations [5,6], the latter action being due to a secondary inhibition of a Na^{+1}- Ca^{+2} exchanging protein located over the cell membrane, which exchanges Na^{+1} for Ca^{+2} (Figure1) [8].

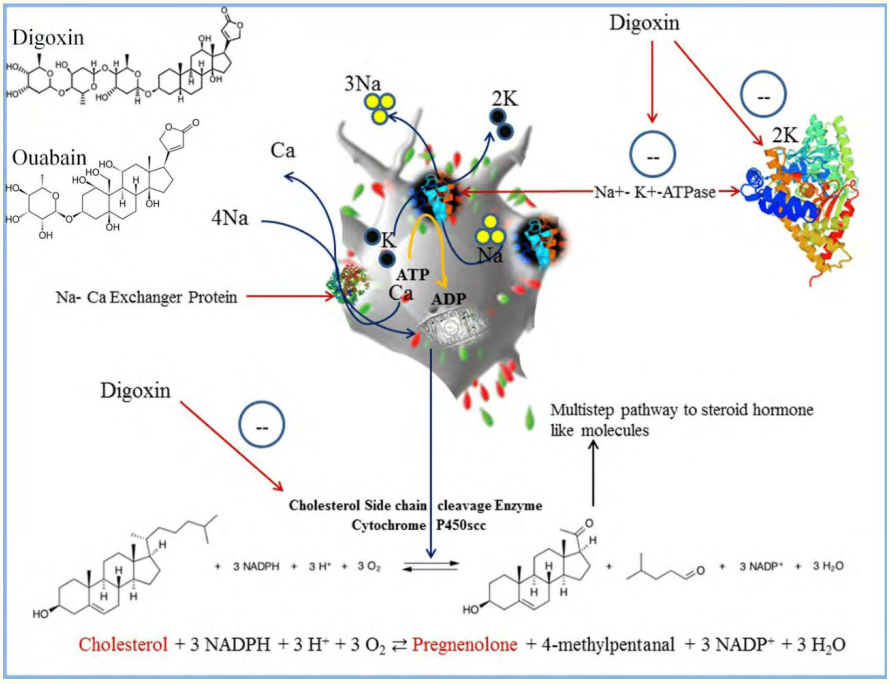

Figure 1: *This figure shows human targets of digitalis like drugs (top-left). Digoxin targets to inhibit the Na-K-ATPase and cholesterol side chain cleavage enzyme. The Na-K-ATPase is located on the cell membrane and functions as a ion transport protein. This protein pump 3Na ions to the extracellular space in exchange for 2K that are transported into the cell. The ATP provides the energy for this transport. The cholesterol side-chain cleavage enzyme (bottom) catalyzes the first step in the steroid hormone biosynthesis by converting cholesterol into pregnenolone is also a target of digoxin.*

Clinically digoxin has been used in the past for the management of congestive heart failure. The margin of safety of this drug is low, it is therefore used with caution when prescribed [9]. For this reason, its use in the treatment of congestive heart failure and cardiac arrhythmias is preferred mostly in hospitalized patients where its plasma levels could be monitored [10].

The Na^+- K^+-ATPase has been reported to be linked to Src, which is bound to Na^+- K^+-ATPase at the pocket of docking of ouabain [13]. The binding of the ouabain has been reported to stimulate a downstream pathway via Src [14], but other reports show that there is no concrete evidence for a direct molecular interface of Src with Na-K-ATPase under physiological conditions [15]. The digoxin binds to the extracellular aspect of the Na^+- K^+-ATPase, which is a physiological binding receptor site for K^{+1} ions and differs from the docking site of ouabain [6,8].

The function of the Na^+- K^+-ATPase is fundamental to both electrically excitable tissues as well as non-excitable tissues that it has remained conserved for a considerable period of evolution [16]. Recent studies have shown anti-amoebic activity of digoxin in protist pathogens [17,18], but no studies have reported the existence of any homology between the human targets of digoxin like Na^+- K^+-ATPase, the enzyme cytochrome P450 and sodium/calcium exchanger protein [19], with their counterparts in *Acanthamoeba* spp.

Additionally, digoxin also targets and inhibits a group of enzymes called cytochrome P450, of which the cholesterol side-chain cleavage mitochondrial enzyme and 11β-hydroxylase [11,15] are known targets of digoxin. These 2 cytochromes are known to be present in a diverse group of mitochondrial eukaryotes, but any homology of them with their human counterpart and if they are being targeted by digoxin in *Acanthamoeba* spp has not been reported.

The model eukaryote that we selected was *Acanthamoeba castellanii*, which is a protist pathogen and a free-living amoeba. This unicellular eukaryote has a space of around 2.3 billion years with humans and is one of the most primitive eukaryote known to exist on earth. Pathogenic genotypes like T4 are known to infect traumatized corneas in humans and cause *Acanthamoeba keratitis* [AK] [20]. This microbe is a free-living amoeba that has also been reported to enter the human body via infections of the skin and cause a fatal form of a central nervous system disease called granulomatous amoebic encephalitis [21] [GAE]. *Acanthamoeba* spp of the T4 genotype is notoriously known to resist conventional anti-parasitic drugs like Amphotericin-B and the Azole group of drugs and cause blindness in patients with AK and death in GAE in more than 97% of cases.

Although recent *in-vitro* studies [17,18] have shown anti-amoebic activity of digoxin in *Acanthamoeba castellanii*, there are neither reports of treatment successes in animals nor any definitive validations of target proteins of digoxin in *Acanthamoeba* spp. There are also no studies to show the extent to which the target of digoxin in human like cellular ion transporting Na^+- K^+-ATPase and cytochrome P450 resemble their counterparts in *Acanthamoeba* spp., which this study attempts to show. While the mechanism of action of digoxin is well established in humans, the existence of this receptor model of an ion transport system, the enzyme inhibition and the type of cell death, it induces in unicellular eukaryotes like *Acanthamoeba* still need to be established by using bioinformatics, 3D modelling, ligand binding predictions and experimental assays, which this study aims to do.

Materials and Methods

Digoxin [Lanoxin] was obtained by Glaxo-SmithKline. Acridine orange, Etoposide and Loperamide were purchased from Sigma AldrichR. Annexin-V FITC and PI kit were purchased from Merck-Millipore and 7AAD was purchased from InvitrogenR. To search for the amino acid sequence homology of the Na^+- K^+-ATPase and cytochrome P450 enzyme, queries were made at the *Acanthamoeba* nucleotide and protein databases at NCBI, Uniprot and Amoebadb.org. A BLASTn was selected for the nucleotide queries. To look for protein homology search, the above-mentioned databases were explored by using a BLASTp and data was downloaded. The amoebal and human amino acids FASTA sequences were submitted to the SWISS MODEL, Phyre2 database for structural homology and model building, and for amino

acid alignments, databases of uniprot KB and EMBL were used. The Swiss Model database and 3DLigandsite were used to search for 3D structure homology modelling and ligand binding predictions.

Microorganisms and cell cultures

Ocular isolates of keratitis causing *Acanthamoeba* trophozoites -T4 genotype, were grown in nutrient (PYG) medium in flasks at 37°C as in our previous studies [17]. The *A. castellanii* trophozoites cell lines are normally maintained in our lab and the medium is routinely refreshed to maintain their healthy state. *A. castellanii* that adhered to the floor of the flasks were healthy trophozoites forms, and were used in all assays. The Human Brain Microvascular Endothelial cells (HBMEC) were grown in RPMI _1640 containing 10% heat inactivated FBS-(fetal bovine serum), 2 mM glutamine, 100 µg/ml and 100U streptomycin and penicillin respectively. Nutritionally non-essential amino acids and vitamins like B_6, B_{12} and folic acid were added to HBMEC and maintained at 37°C in with a 5% CO_2. For experiments, HBMEC 10^5 cells per ml/well were inoculated in 6-well plates for 24 h and only healthy cells that were attached to the floor of the flasks were used.

Bioinformatics

Sequence Analysis

For nucleotide and protein homology, FASTA sequences of known targets of digoxin in humans, like Na^+- K^+-ATPase and cytochrome P450 were submitted to NCBI and amoeba database (amoebadb.org) by selecting the *Acanthamoeba castellanii* Neff strain. BLASTn and BLASTp were selected for nucleotide and protein searches respectively. Identities, similarities and E-values were obtained for each of the above-mentioned proteins and compared by pairwise alignment of nucleotide and amino acid sequences at EMBL database [22] and UniprotKB [23].

Structural Bioinformatics and Ligand binding predictions

Structural Homology and Ligand binding structure activity relationship

The 3D structure and Ligand binding homology was obtained by submitting the amino acid sequence of amoebal proteins identified at NCBI and Amoebadb.org to the Phyre^2 [24] database. Swiss Model database [25] was also used to develop a template-based model by submitting the P-type ATPase and cytochrome P450 of *Acanthamoeba*. The identities, coverage and confidence level of structural homology were recorded as a percentage. A website that establishes the ligand binding attributes of proteins and receptors (3DLigandsite) [26] and Protein database (PDB) was used for determining ligand clusters and predicting the amino acid residues for binding.

Experimental Assays

Amoebistatic and amoebicidal assays

For observing the growth, inhibitory and amoebicidal activity of digoxin and other drugs, healthy trophozoites 1 x 10^5 were grown in nutrient media (PYG) were exposed to different doses (40 µg/ml – 80 µg/ml) of digoxin at 37°C for 12 - 24 hours. High resolution images were captured by an inverted microscope at variable magnifications (20x, 40x) at the 6^{th}, 12^{th}, 18^{th} and 24^{th} hour to determine the effects of digoxin on *A. castellanii* growth and proliferation.

Effects of digoxin on HBMEC

The healthy human brain microvascular endothelial cells (HBMEC) 1 x 10^6 were incubated with 40 µg/ml digoxin to observe the safety of this drug on human cells. For controls HBMEC were exposed to 20 µM Etoposide and 150 µg/ml of loperamide. Etoposide is a known apoptotic agent and loperamide, like digoxin, causes Ca^{+2}-dysregulations in the cytosol.

FACS Analysis

Acanthamoeba castellanii (1 x 10^6 cells/100 µL) were loaded into FACS tubes. The amoebae were washed twice with 2 mL PBS, and centrifuged at 1500 x g for 5min, and then poured out of the buffer from pellets containing trophozoites. *A. castellanii* trophozoites were then added to 100 µL of flow cytometry staining buffer. Following this, 10 µL of 7AAD was added to the staining solution to a control tube of trophozoites in order to adjust flow cytometer settings for 7AAD. After mixing for 30 minutes at 4°C in the dark, 7AAD fluorescence was determined using the FL-2, as staining with 7AAD alone was intended. The data were acquired for unstained trophozoites and the positive control. 10 µL of the 7AAD staining solution was added to the digoxin treated samples and incubated for 30 minutes at 4°C in the dark before the analysis. Trophozoites were not washed with PBS, as a loss of the dye takes place in doing so. The viable trophozoite counting was optimized from a dot-plot of forward scatter versus 7AAD.

Results

Bioinformatics: Sequence homology, 3D Modelling and Ligand Binding Predictions

***Acanthamoeba* P-type ATPase and human Na^+- K^+-ATPase show nucleotide sequence mRNA homology**

The nucleotide (m-RNA) FASTA sequence of Homo sapiens ATPase Na^+/K^+ transporting subunit alpha 1 (ATP1A1), transcript variant 1, mRNA was compared with the P-type ATPase, PMR1-type, putative (ACA1_313610) mRNA. A pairwise alignment of the mRNA of both the proteins showed the homology between the mRNA of both species with 296 identities, 296 similarities and a score of 532 at EMBL database alignment query (Figure 2A).

Figure 2: *(A) Showing the pairwise nucleotide sequence and amino acid sequence alignment of the protein human ATPase Na+-K+ ATPase subunit alpha 1 and P-type ATPase, putative protein of Acanthamoeba. A total 57% identities and the same percentage of similarity scored 532 at Emboss-EMBL database for nucleotides.*

(B) Amino acid sequence homology of both human and Acanthamoeba P-type Na^+-K^+-ATPase showed an E-value of $2e^{-120}$ for Acanthamoeba P-type. Human sequence of amino acids is shown in the top row (red-star) and the Acanthamoeba sequence in the bottom row (yellow-star) in both, A and B.

Acanthamoeba spp, like humans expresses a P-type ATPase that is has a homology with human Na^+- K^+-ATPase

A search for amino acid sequence homology of human Na^+-K^+-ATPase subunit alpha-1 on the *Acanthamoeba* protein database at the NCBI and amoeba database.org revealed a protein named P-type ATPase (Figure 2), putative (*Acanthamoeba castellanii str*. Neff). With a substantial E-value of $2e^{-120}$ and near similar identities and similarities (Figure 2B) of the BLASTp results show the homology between both the proteins. The sequence of amino acids of human protein is shown in the top row (red-star) and the *Acanthamoeba* protein sequence in the bottom row (yellow-star). (Figure 2A-2B).

Acanthamoeba spp, P-type ATPase has a structural homology to human Na^+- K^+-ATPase

The amino acid FASTA sequence of P-type ATPase of *Acanthamoeba* was submitted to Phyre[2] database for model building and structural homology. The amino acid sequence of this protein generated a template that was found to have a homology to the crystal structure of the human sodium-potassium pump (Figure 3A). 872 residues (95% of the sequence) were modelled with 100.0% confidence by the single highest scoring template. This reflects the identical structural features of this *Acanthamoeba* protein to human Na^+-K^+-ATPase.

An 3D model built by Swiss Model database also showed identical structural homology of the amoebal P-type ATPase to human Na^+-K^+ ATPase (Figure 3A) with 37% identities. The receptor site for ouabain-ligand-binding pocket (Figure 3 black circle) was clearly developed with the details of the model by both structural bioinformatic sites (Figure 3).

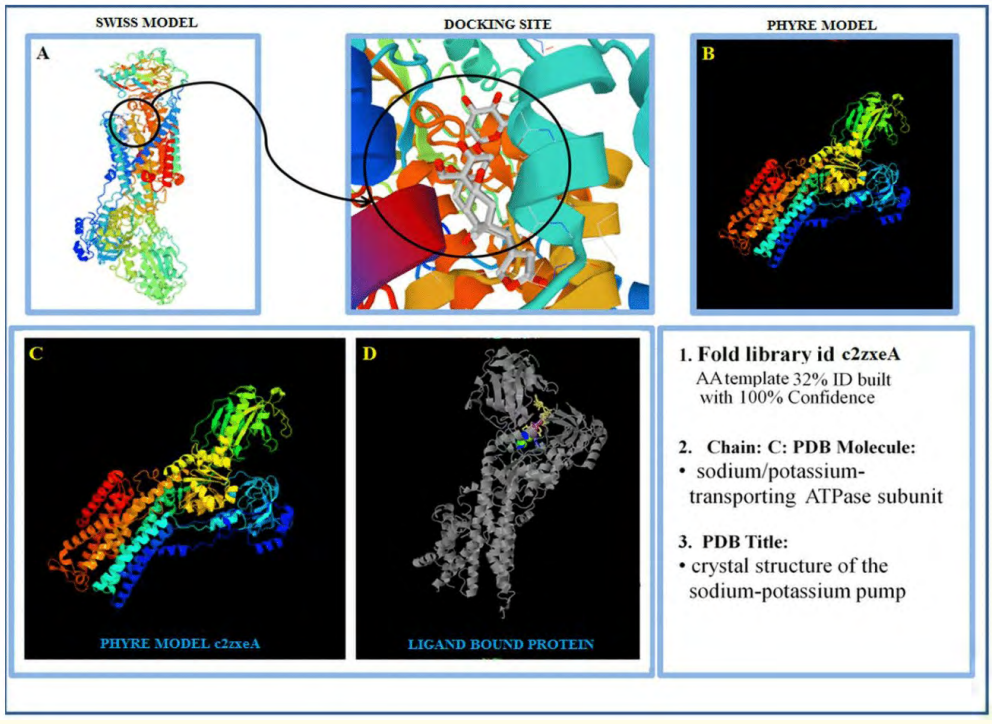

Figure 3: *This figure shows the models build by SWISS MODEL and Phyre2 for amino acid FASTA sequence of P-type ATPase of Acanthamoeba. The amino acid sequence of this protein produced templates at SWISS MODEL that generated models of the human sodium-potassium pump. The model shows the digitalis-binding pocket (black circle) (top-left). Phyre2 generated a model c2zxeA with 100% confidence (B-C) that binds the digitalis compound digoxin (D). This template is recognized by protein data base (PDB) as sodium-posasium ATPase.*

Acanthamoeba P-type ATPase and human Na^+- K^+-ATPase have an similar Ligand binding prediction

The 3DLigandsite and protein database (PDB) was used to predict the ligand for the model build by Phyre[2] and Swiss Model database. The ligand binding predictions to amino acid residues and the receptor site location in the structure of the modelled protein for binding digitalis like compounds are shown (Figure 3, Figure 4). The model showed a docking site for Ouabain, and digoxin, which are well-known for binding to human Na^+-K^+-ATPase (Figure 4C). The amino acids predicted for the docking of the ligand on human Na^+-K^+-ATPase and the heterogens that they engage (Figure 4B), when compared, showed to be similar to the model developed for the *Acanthamoeba* P-type ATPase (Figure 4A).

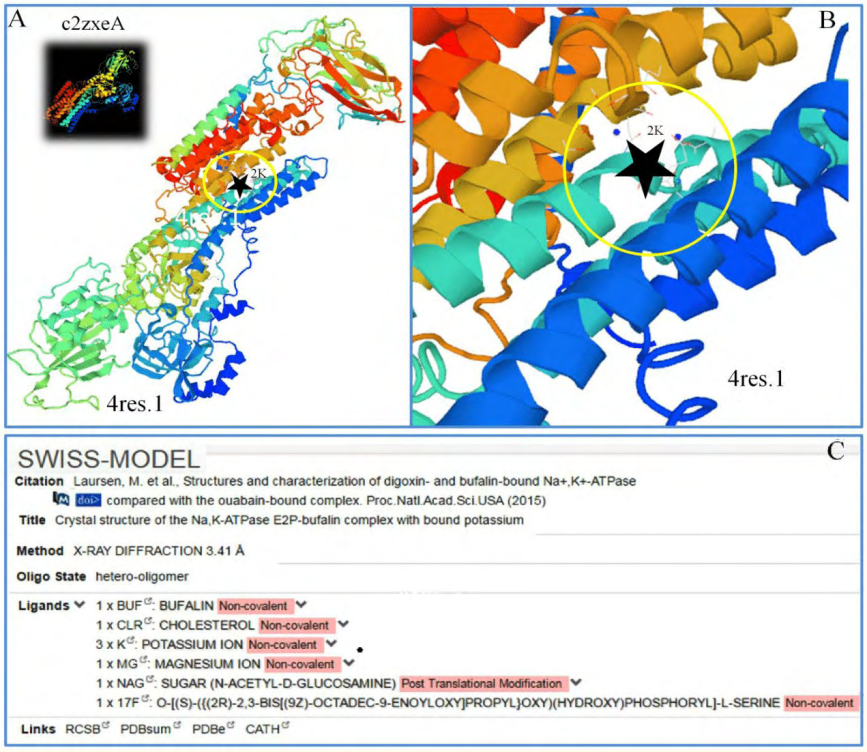

Figure 4: *Shows the ligand binding attributes of the model generated for Acanthamoeba protein P-type ATPase. The template generated was 4res.1 that resembles the model developed by Phyre[2](A). Zooming the ligand site showed K^+ ion binding docking pit of bufalin and digoxin (B) in the crystal structure of the developed model.*

Acanthamoeba does not express sodium/calcium exchanger protein

This exchanger is expressed by human tissues and is indirectly inhibited by digoxin. However, *Acanthamoeba* does not express a similar Na^{+1}/ Ca^{+2} exchanger protein, (Supplementary File -S1).

Acanthamoeba, like humans expresses a similar cholesterol side-chain cleavage enzyme with identical ligand binding sites

Bioinformatics search results on the *Acanthamoeba* protein database at NCBI and amoebadb.org for human cholesterol side-chain cleavage enzyme cytochrome P450, (CYP450- CYP11A1) revealed a similar cytochrome p450 superfamily protein *Acanthamoeba castellanii* Neff strain. An alignment of nucleotides (mRNA) sequence (Supplementary File-S2) of this protein with human cholesterol side-chain cleavage enzyme Cytochrome P450 showed a substantial similarity with significant. For the development of a 3D model of cytochrome

P450 superfamily protein of *Acanthamoeba castellanii*, *SWISS MODEL database was used*. The model developed was not identified as a model of human cholesterol side chain cleavage enzyme CYP11A1, rather we found other isoforms of CYPs as CYP7A1, CYP1A1 and CYP2B4 (Figure 5) of which the latter 2 show similar hetrogen and ligand binding as CYP11A1 (Figure 5).

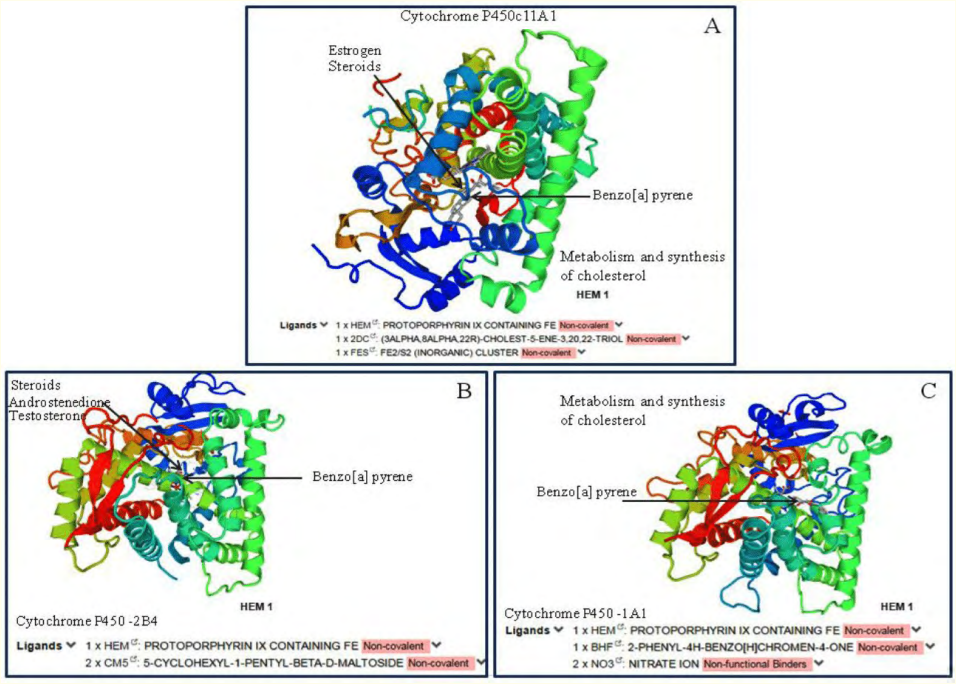

Figure 5: *(A). Bioinformatics search in database on Swiss Model for Cytochrome P450 superfamily protein Acanthamoeba castellanii developed 2 structurally related models of the enzyme Cytochrome P450 (B-C). Protein database revealed a docking site similar, but not identical to human cytochrome P450-CYP11A1 (B-C). Note the similarities in the ligands and functions of the human enzyme (A) and Amoebal Cytochrome P450 (B-C).*

Imaging Assays

Digoxin showed growth inhibition in *Acanthamoeba* spp

To observe the effects of digoxin on *Acanthamoeba castellanii*, 1x 10^6 trophozoites grown in PYG with different concentrations of digoxin were used (Figure 6) and the cell proliferation was done by using a hemocytometer. Digoxin was dissolved in ethanol and a dose of 40µg/ml was seen to marginally inhibit the proliferation of trophozoites (Figure 6B1, 6B2), as compared to the solvent controls (Figure 6A1), At doses of 10, 20 and 30 µg/ml, no considerable effects on the viability of the trophozoites were observed (Supplementary File-S3). The PYG controls, showed a sustained proliferation and growth of *Acanthamoeba* trophozoites (Figure 6A1, 6A2).

Digoxin showed significant amoebicidal effects on *Acanthamoeba* at 80 µg/ml

A dose range of 75 – 80 µg/ml digoxin showed significant amoebicidal effects at the 12th hour (Figure 6C1-6C2). The cell proliferation was calculated by using a hemocytometer. A difference that was observed was a sharp reduction in the numbers of viable trophozoites at 80 µg/ml of digoxin at the 12th hour, which is in contrast to the effects of 40 µg/ml that showed the death of the trophozoites at the 18th hour (Figure 6B1-6B2). The cell that went into necrotic death at 80 µg/ml were seen to be Trypan blue positive (data not shown) in contrast to the cell that died at 40 µg/ml.

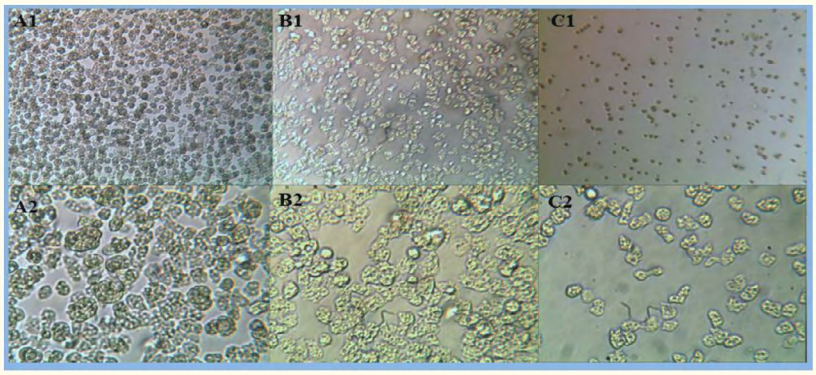

Figure 6: *Images showing amoebicidal effects of digoxin on 1x 10^6 Acanthamoebal trophozoites at different doses.*
(A) A. castellanii in solvent (ethanol) without digoxin at a magnification of 20x (A1) 40x (A2)
(B) The effects of 40 µg/ml of digoxin on A. castellanii at the18^{th} hour shown at 20x (B1) and 40x (B2) magnifications
(C) The effects of 80µg/ml of digoxin on at 12^{th} hour on A. castellanii at shown 20x (C1) and 40x (C2) magnifications. Note the substantial amoebicidal effects at 80µg/ml of digoxin after 12 hours of exposure to the drug.

Digoxin showed HBMEC damage

Digoxin was tested for its cytotoxic effects on HBMEC cells, with Etoposide (known cytotoxic/apoptotic agent) used as the control. HBMEC that were not exposed to any drug showed an intact monolayer after 12 hours (Figure 7A). HBMEC exposed to Etoposide 20µM showed clear areas of damage to monolayer (Figure 7B). HBMEC that were grown in growth medium for 24 hours without digoxin remained intact (Figure 7C). HBMEC exposed to digoxin at a dose of 40 µg/ml for 24h showed a limited damage to HBMEC (Figure 7D). Damage to HBMEC layer was not observed until 12h of its exposure to the digoxin, but an 18-hour incubation showed isolated areas of the discontinued monolayer (Figure 7D).

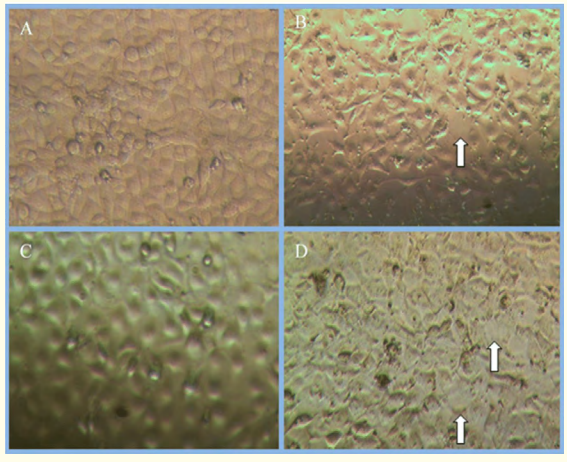

Figure 7: *Effects of Etoposide and Digoxin on HBMEC at 40x.*
(A) Appearance of normal HBMEC monolayer in growth medium at 12^{th} hour.
(B) Effects of 20 µM of Etoposide after 12 hours of exposure
(C) Normal HBMEC monolayer in growth medium after 18hours.
(D) Appearance of HBMEC monolayer 18 hours after exposure to 40 µg/ml of digoxin.
Note the Etoposide (B-white arrow) and digoxin (D-white arrows) damage the layer in patches.

Flow cytometry findings

Flow cytometry was performed to determine whether *A. castellanii* exhibited apoptosis or necrosis at 40 µg/ml of digoxin by using 7AAD staining of drug-treated and untreated cells. In controls, trophozoites were incubated with RPMI alone. The typical FACS scatter grams showed the three classical regions, i.e., 7AAD–negative (Figure 8-A1 – Q3- live cells), 7AAD stained cells that were treated with drugs for 16h (Figure 8-B2 - early apoptotic cells), and 7AAD stained cells that were treated with drugs for 18h (Figure 8-B3 - late-apoptotic and dead cells).

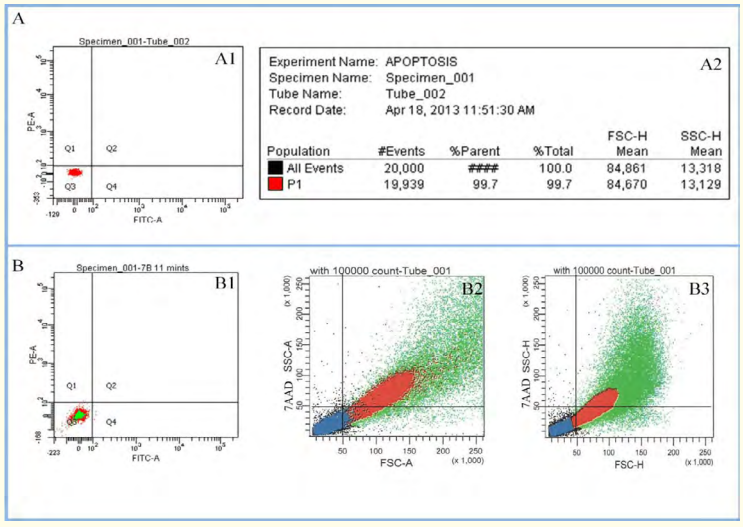

Figure 8: *Shows flow cytometry results of digoxin at a dose of 40µg/ml on A. castellanii.*

(A) Showing 7AAD control with 1x10^{6} trophozoites without digoxin. The trophozoites mostly are in (A1) viable zone Q3. The experiment is representative of 20,000 events (A1, A2).

(B) Represents the effects of 40µg/ml of digoxin on A. castellanii with 7AAD. The cells at 13th hours start staining and show movement towards other zones (B1). The trophozoites start drifting towards early apoptotic- Q4- (B2) and towards the late apoptotic zone -Q2- (B3) at 16th and 18th hours after respectively, after exposure to digoxin.

Discussion

Phylogenetic studies have classified the ATPase proteins independent of the organism from which they are isolated and showed that the evolutionary extension of the P-type ATPase family took place prior to the partitioning of the eubacteria, archaea, and eukaryota [27]. There are a range of ways to establish the cellular targets of a drug, which includes methods like the use of gene knockin, protein expression and bioinformatic computational tools. In this study, we provide the bioinformatics based evidence for the homology of targets of digoxin in humans with similar proteins in *Acanthamoeba* spp. By the use of nucleotide/amino acid sequence identity rates, structural similarity, ligand binding attributes and experimental assays, we show that the targets of digitalis in both the species are near similar. These target proteins in humans include, Na^{+}- K^{+}-ATPase, cholesterol side-chain cleavage enzyme and sodium/calcium exchanger, which it affects indirectly [6].

The search for a protein resembling human Na^{+}- K^{+}-ATPase, revealed *Acanthamoeba* P-Type ATPase as the closest match in *A. castellanii*. Sequence and pairwise homology of nucleotides and amino acids of human Na^{+}- K^{+}-ATPase and *Acanthamoeba* P-Type ATPase shows a high score on nucleotide homology (Figure 2A). An E-value of 2e-10 $^{-120}$ was found on amino acid homology pairing (Figure 2B). As gene

regulation and expressions are sporadically alike [1] between 2 diverse species separated by over ~ 2 billion years, we did not find an identical match. As previous studies, have focused more on finding of a consistent constellation of amino acids in docking pits of ligand binding receptors, we adopted a similar approach to build 3D models in order to seek a structural homology.

With the evidence of existence of many types of ATPase in eukaryotes, we used softwares and online structural homology building web-engines like Phyre[2] and Swiss model to seek the structural homology of *Acanthamoeba* P-Type ATPase with its human counterpart Na^+- K^+-ATPase. The FASTA sequence of amino acids of the P-Type ATPase in *Acanthamoeba* was submitted to 3D structure building databases to build a template-based model. The results from two different databases showed a homology to human Na^+- K^+-ATPase with confidence of 100% (Figure 3). In order to further validate the models that were built for P-Type ATPase in *Acanthamoeba*, for similarities in ligand binding attributes, we used ligand binding prediction of the models developed by the SWISS MODEL, protein database (PDB) and 3DLigandsite database. Swiss Model database identified the template-based model 4res.1 to have a docking cleft for Digitalis (Figure 4-B, 4C) and Oubain. 3DLigandsite tool showed identical heterogens in the predicted amino acid binding sites of human and *Acanthamoeba* P-Type ATPase and human Na^+- K^+-ATPase. The SWISS MODEL predicted the digoxin binding site (K^+ ion site) ligand for the developed model 4res.1 (Figure 4A,4B). Phyre[2] developed a similar model based on template C2zxeA (Figure 3B,3C). Taken collectively, our results show an interesting structural homology and ligand binding attribute similarities of *Acanthamoeba* P-Type ATPase with human Na^+- K^+-ATPase. To further confirm that the action of digoxin occurred at a site of docking as shown in Figure 4B, we added K+ ion to antagonize the action of digoxin on amoeba trophozoites. This antagonism is well known for the human Na^+- K^+-ATPase (Supplementary File -4) in cases of digoxin intoxication [6]. We observed these ions to prevent the amoebicidal action [18] of digitalis (data not shown).

Taking into account the fact that digoxin exposure resulted in the death of the trophozoites and cysts of *Acanthamoeba* spp in our past experiments, it appears that the P-Type -ATPase is where digoxin docks to cause the amoebicidal and cysticidal effects. Digitalis has been reported to cause death in prostate cancer cell lines [28]. Digoxin has shown to be antiproliferative for liver cancer cell lines. It has been suggested that digoxin exerts its anti-cancer properties through affecting cell division and apoptosis. We were also able to obtain FACS findings of apoptosis (Figure 7), which is akin to finding of the reports in the past [28,29]. The exact cascade of the apoptosis induced by digoxin in *Acanthamoeba* is not known, but an endoplasmic/mitochondrial driven intrinsic mechanism secondary to calcium dysregulation might be responsible.

As digitalis is a known inhibitor of mitochondrial protein, cholesterol side-chain cleavage enzyme cytochrome P450-11A1, we were curious to answer two questions; is there a similar enzyme in *Acanthamoeba*? Are there any reports of the effects of the inhibition of this enzyme on the viability of the trophozoites of *Acanthamoeba castellanii*?: For the first part, we were unable to find a similar enzyme in *Acanthamoeba* spp. This human enzyme cytochrome P450-11A1) had a limited sequence homology to few amoebal cytochromes (Figure 5), but shared many ligand binding features to the cytochromes in *Acanthamoeba*. In the search for the second query, we found that there were no reports of *Acanthamoeba* death by inhibiting these cytochromes.

Although, there have been reports of amoebicidal effects of inhibiting steroid synthesis in *Acanthamoeba* by HMG-CoA synthase inhibition [30], the significance of inhibition of other cytochromes in the biology of *Acanthamoeba* is not certain. There are reports in humans of mutations in CYP11A1 gene that codes for this enzyme that have resulted in 'Lipoid congenital adrenal hyperplasia', a steroid hormone deficiency that usually results in death [31]. Given the fact that digoxin is a known inhibitor of this enzyme, we could not find its contribution in the observed amoebicidal effects of digoxin on *Acanthamoeba* spp.

Using the similar approaches of building models, 3D structures and ligand binding site homology, some recent studies have shown the evidence of expression of a homolog of human muscarinic M1 receptor in *Acanthamoeba* [28], and *Naegleria fowleri* [29].

Though there have been reports for the amoebicidal effects of digoxin in the past [18,32] the present study attempts to find homologous targets of digoxin in *Acanthamoeba*.

We ruled out the contributions of a sodium/calcium exchanger in causing calcium dysregulation in *Acanthamoeba*, as there was no bioinformatic evidence of its expression in this protist pathogen. New findings in our experimental assays, are the effects of digoxin on *Acanthamoeba* at different doses. It was observed to be rapidly amoebicidal for the trophozoites at dose 80 µg/ml and flow cytometry showed it to be an apoptotic agent at a dose range of 35 - 40 µg/ml (Figure 8B). Digoxin showed a substantial killing of *Acanthamoeba* at a dose range of 70 - 80 µg/ml (Figure 6C2). It is possible that the extended effects of digoxin on a an as yet unidentified target located in the cytosol that gets affected at the dose of 80 µg/ml.

We thought of using siRNA and miRNA and other methods of knocking out the P-Type ATPase in *A. castellanii*, but doing so could have resulted in the death of the trophozoites, and hamper further insights into our target of interest, which is the reason we favoured bioinformatics more than experimental assays in this study.

Conclusion

The bioinformatic tools and 3D modelling with ligand binding predictions of proteins and receptors offers an effective comparison between the evolutionarily conserved molecular targets of drugs in humans with similar target in primitive unicellular pathogenic microbes. The databanks of the genomes and proteomes of the free-living amoeba are fortune repositories that could be explored to select drug targets. Finding a target protein requires the comparison of the known targets of drugs in human with the protein deposited in the databanks of pathogenic microbes using sequence and structural homology softwares and experimentation. Future studies with fluorophore tagging of target proteins and digoxin is expected to validate the target of this drug in *Acanthamoeba* spp and other free-living amoeba.

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Source of Downloaded Data

NCBI, UniportKB, Phyre^2, Swiss model, 3DLigandsite and EMBL were used for bioinformatic search, structural model development and ligand predictions.

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