Identification of analogue compounds of P-glycoprotein associated anthelmintics resistance reversal agents through cheminformatics approaches

Om Prakash, Shankarlingam Gomathinayagam, Tirunelveli Jeyagopal Harikrishnan, Muthusamy Raman, Velayudham Pandiyan and Samapika Sahoo

Abstract
The use of anthelmintics in intensive farming has followed swiftly by the emergence of anthelmintics resistance (AR) which is now an emerging phenomenon in parasitic nematodes of sheep, goats, horses, and cattle. AR is defined as a genetically transmissible trait in which the sensitivity to a particular drug is lost in a population of worms over time. Cheminformatics studies were carried out to find out the P-glycoprotein modulators/inhibitors from plant compounds/chemicals sources. Three dimensional structure of Pgp was designed using the sequences from NCBI. Five models of Pgp were received. Based on I-TASSER modeling and RAMPAGE score Pgp model 1 was selected and molecular docking was done to interact with 36 plant compounds/chemicals using Accelrys Discovery Studio client 2.5. Based on the best molecular docking score (more than 90 interactions), Curcumin, Quercetin, Kaempferol, Phloretin, Verapamil and Loperamide with molecular docking scores of 98.324, 96.073, 95.47, 94.895,93.453 and 92.807 were selected.

Keywords: anthelmintic resistance, cheminformatics, haemonchus, P-glycoprotein, docking

Introduction
In the continued absence of commercial vaccines, the use of broad-spectrum anthelmintics has been the primary method to control the pathogens in cattle and sheep for over 50 years [1, 2]. The use of anthelmintics in intensive farming has followed swiftly by the emergence of anthelmintic resistance (AR) which is now an emerging phenomenon in parasitic nematodes of sheep, goats, horses, and cattle [3]. AR is defined as a genetically transmissible trait in which the sensitivity to a particular drug is lost in a population of worms over time [4]. Resistance to the majority of the anthelmintics including the Benzimidazole [5, 6], Salicylanilides [6], Macrocyclic lactones [5, 7] were reported. The key to control anthelmintic resistance in H. contortus is to understand various mechanisms that may be involved, for each class of anthelmintics has a known different target. There are three main groups of mechanisms: those that change the binding sites of drugs, those that detoxify, and those that involve the active efflux of drugs by membrane transporters [8, 9]. The mechanism that is primarily considered to be involved in resistance to macrocyclic lactones is the detoxification process of P-glycoproteins. P-glycoproteins (Pgps) are efflux transporters which actively transport compounds, including drugs, across membranes [10]. The primary function of Pgp is to protect the organism by actively pumping toxic substances out of its cells [11]. P-glycoproteins have been identified in H. contortus and the full cDNA sequence has been obtained [12]. The mechanism believed to be associated with anthelmintic resistance in H. contortus is the overexpression of Pgp. Both benzimidazole and ivermectin-resistant strains of H. contortus have been found to possess Pgp alleles in higher frequency than susceptible strains. Pgp may modulate benzimidazole concentration at the target site [9]. A relationship between Pgp and benzimidazole resistance was indirectly demonstrated through the use of the Pgp inhibitor Verapamil [13, 14]. Verapamil is a calcium channel blocker, which actively inhibits the Pgp drug-binding domain. In the presence of Verapamil, the toxicity of the drug increased and the benzimidazole resistance could be partially reversed [12]. A role for P-glycoprotein (P-gp)
drug efflux pumps in ML resistance in *H. contortus* and other parasitic nematodes [15]. The expression of P-gps has been increased in IVM resistant isolates [11, 16, 14]. A number of P-gp inhibitors/modulators have been shown to reverse BZ and ML resistance in *H. contortus*, both *in vitro* and *in vivo*. Valspodar increased the sensitivity of resistant and susceptible strains of *H. contortus* and Teladorsagia circumcincta to IVM [17]. A potentiated efficacy of IVM against field IVM resistant isolate of *H. placei* by multidrug resistant inhibitors (MDRIs), Verapamil and Cyclosporine had been demonstrated resulting in higher efficacy and lower IVM EC50 [18]. Third generation Pgp inhibitors including tariquidar, zosuquidar and elacridar increased the efficacy of IVM, levamisole (LEV) and thiabendazole [19].

Cheminformatics is a field of information technology that uses computers and computer programs to facilitate the collection, storage, analysis, and manipulation of large quantities of chemical data. Cheminformatics approaches had been attempted to identify the MDRIs analogue compounds. The herbal compounds curcumin, baicalin and dronabinol have been identified as novel monoamine oxidase inhibitors for treatment of Parkinson disease in human [20]. The specificity and strong binding affinity of curcumin to major inflammatory mediators such as, cytokines/chemokines, signaling proteins and transcription factors using molecular docking [21]. *In silico* study of enzyme-inhibitor binding simulation between eight phytochemicals and Janus kinase enzymes (JAK 1, 2 and 3) using the Patchdock docking server [22]. The modelling of Pgp2 of *H. contortus*. The retrieved P glycoprotein constituted with 1275 amino acids. The modelling of Pgp2 of *H. placei* was online submitted to RAMPAGE [23].

**Materials and Methods**

The Permissible glycoprotein (Pgp), a member of group of integral membrane proteins that contain the ATP-binding cassette, widely represented in animal kingdom. In nematodes, possible functions include transport of lipophilic peptides and hormone as well as exclusion of toxin across cell membranes [11]. A Pgp mediated modulation of drug concentration at target site, is another potential mechanism of anthelmintic resistance [11, 9, 18, 8]. Identification of analogue compounds of anthelmintic resistance reversal agents through cheminformatics approach was performed as follows:

**Retention of P-glycoprotein**

The sequences of P glycoprotein (Pgp A) m RNA of *H. contortus* was retrieved from the National Centre for Biotechnology Information (NCBI, USA, 1988) as FASTA format files. P glycoprotein Gen Bank accession number was AF003908 [12].

**Modelling of P-glycoprotein**

Iterative Threading Assembly Refinement (I-TASSER) was a bioinformatic tool for predicting three-dimensional structure model of protein molecules, developed at Yang Zhang Lab, University of Michigan, Ann Arbor, USA. The retrieved P glycoprotein sequences were submitted to the “I-TASSER” database, for modelling of the P glycoprotein 3D Structures.

**Evaluation and analysis of residues**

The modelled 3D structure of P glycoprotein of *H. contortus* was online submitted to RAMPAGE [25].

**Ligand preparation**

Plantcompounds and certain chemicals had been identified as Pgp modulators/inhibitors in multidrug resistance [25]. The 3D structure of 36 plant compounds /chemicals known to be Pgp modulator/inhibitors in cancer drug resistance, anti-diabetics resistance and antibiotics resistance was downloaded from the Chemical Database “PubChem” (NCBI, USA, 2004) which was the repositary of the chemicals. Using these compounds, identification of compounds in the reversal of Pgp associated anthelmintics resistance was carried out in *silico* using software Accelrys Discovery Studio Client 2.5 (Biovia, USA, 2009).

**Molecular docking**

Molecular docking is a key tool in structural molecular biology and computer-assisted drug design. The goal of ligand-protein docking was to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. The molecular docking of 36 plant compounds/chemicals (Ligands) with P-glycoprotein (Protein) was carried out with Accelrys Discovery Studio client 2.5 (Biovia, USA, 2009) to predict the interaction of ligands with various sites of P glycoprotein of *H. contortus*.

**Selection of plant compounds/chemical for reversal of anthelmintic resistance**

Based on highest molecular docking score, plant compounds/chemicals were selected to find out the efficacy of these compounds in the reversal of anthelmintics resistance.

**Results**

Identification of analogue compounds of anthelmintic resistance reversal agents (Chemosensitizers) through cheminformatics approach is performed as follows:

**Retrieval of P-glycoprotein**

The retrieved P glycoprotein constituted with 1275 amino acids. The modelling of Pgp2 of *H. contortus* was done to get the three dimensional structure of Pgp2.

**Modelling of P-glycoprotein**

Five three dimensional Pgp2 models were received from I-TASSER (Plate 1). 1 – TASSER P gp model score for all five models were presented in Table.1 [27]. Confidence score (C score) of the model-1 was 2 and Topology score (TM score) was more than 0.5; hence model 1 was selected for further studies.
Evaluation and analysis of residues

The modelled 3D structure of Pgp2 of *H. contortus* was validated by submitting them to RAMPAGE [22] to visualize energetically allowed regions and amino acid residues in protein structure. Number of residues in favoured region (~98.0% expected), number of residues in allowed region (~2.0% expected) and number of residues in outlier region for all the five models were depicted (Table 2.0). Besides this ray diagrams for validation of all the five models were presented in Plate 2. Based on the RAMPAGE, (Ramachandran Plot) assessment of the Pgp2, model I was selected for molecular docking interaction with ligands.

Ligand preparation

The 3 D structure of 36 compounds (both chemicals as well as compounds of plant origin) known to be Pgp modulator/inhibitors in cancer drug resistance, anti-diabetics resistance and antibiotics resistance was downloaded and compounds are listed in Table 3.0. Majority of the compounds were from plant origin. The three dimensional structure of the downloaded compounds were presented in Plate 3a and Plate 3b. These compounds were individually interacted with all the binding sites of Pgp2.

Molecular docking

P-glycoprotein 2 of *H. contortus* had overall 47 binding sites. In this study, out of 47 sites, 13 binding sites viz., 1, 2, 3, 9, 15, 16, 28, 32, 33, 35, 38, 41 and 45 did not interact with any compounds. A total of 2151 interactions with all the thirty six compounds were observed in 34 sites of Pgp2. At the binding site 26, the highest interaction between ligands and protein i.e., 240 posses was found. Molecular docking score of some compounds (Ligands) are shown in Table 4.0.

Selection of plant compounds/chemicals for reversal of anthelmintic resistance

Based on highest molecular docking score (more than 90 interactions), Curcumin, Quercetin, Kaempferol, Phloretin, Verapamil and Loperamide with molecular docking scores of 98.324, 96.073, 95.472, 94.895, 93.453 and 92.807 were selected. Molecular docking (interaction between ligands and Pgp) of the selected compounds are depicted in Plate 4.

Discussion

P-glycoproteins (Pgp) are efflux transporters which actively transport compounds, including drugs across membranes [10]. The primary role of Pgp is to protect the organism by actively pumping toxic substances out of its cells [11]. Many researchers have reported higher levels of Pgp in resistant parasites [28, 9, 30, 31], and being over expressed in response to chemotherapy in tumor cells [32, 33]. But increased Pgp did not appear to be a primary mechanism of drug resistance in L3 of *H. contortus* [34]. Many findings after 2007 confirmed the involvement of several Pgps in the drug resistance [32, 35] found enhancement of Pgp2 and had shown The ability of Pgp2 and Pgp-11 to modulate ivermectin susceptibility by its expression [29]. All these results have clearly shown that there was involvement of Pgps in MDR. Hence, the objective of the study was to find out compounds that could alter or reduce the efflux of the drugs by modulating or inhibiting Pgp. Many researchers used in *silo* approaches or cheminformatics approaches to find out new compounds/drugs that can interact with the target sites/compound to bring about the desired effect in human diseases [20, 22, 36], and to find out anti-inflammatory drug [37]. In this study, cheminformatics has been used to identify compounds which will interact with Pgp to modulate or inhibit Pgp, thereby decreasing the efflux of the anthelmintics drug from cell that is attributed to be the main cause of drug resistance. Hence, toxicity of the drug is increased or potentiated. Otherwise resistance to the drug is reversed or reduced. There are many computer software used to identify compounds/design new drugs like AUTODOCK 4 Programme [21, 37, 38, 39, 40], Patchdock docking server [22], and Hex 6.3 tool. In this present, Accelrys Discovery Studio client 2.5 (Biovia, USA, 2009) was used to carry out docking study. One of the foremost requirements for the cheminformatics studies is the availability of three dimensional structures of the target as well as binding compounds. In present study the three dimensional structure of Pgp 2 or Pgp A from the database was not obtained and it had to be modelled from the protein sequence of Pgp downloaded from the PDB. P-glycoprotein A/Pgp2 was modeled through I- TASSER and five models were received. Model-I was selected for further study based on the highest C-score (2 for model-I) and TM score (0.99±0.04). C-score is a confidence score to evaluate the quality of predicted models by I-TASSER. It was based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. The range of C-score is -5 to 2, where a higher value of C-score signifies a model with a high confidence and vice-versa. TM-score and RMSD are known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling when the native structure is known. In case where the native structure is not known, it becomes necessary to predict the quality of the modelling prediction. C-score is highly correlated with TM-score and RMSD. In the table, the quality prediction (TM-score and RMSD) for the first model is presented, because the correlation between C-score and TM-score was weak for other models. TM-score is a recently proposed scale for measuring the structural similarity between two structures. The purpose of proposing TM-score is to solve the problem of RMSD which is sensitive to the local error. A TM-score more than 0.5 indicates a model of correct topology and a TM-score less than 0.17 means a random similarity [41]. In this study the model-I has a C score and TM score of 2 and 0.91 respectively. Hence, with high C and TM scores, model-1 was selected.

The modelled 3D structure of P glycoprotein of *H. contortus* was validated by RAMPAGE, Ramachandran Plot and based on less number of residues in outlier region model 1 was selected for molecular docking with legends [42]. Molecular docking is a tool in structural molecular biology and computer-assisted drug design. The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. In present study the molecular docking was performed with 36 plant compounds/chemicals (Ligands) having Pgp modulation and/or inhibitory properties as listed [29]. P-glycoprotein of *H. contortus* by using Accelrys Discovery Studio client 2.5 (Biovia, USA); Based on highest dock score (>90) Verapamil, Loperamide, Quercetin, Kaempferol, Phloretin and Curcumin were identified.

Previously many scientists used cheminformatics approaches like molecular docking to identify compounds such as curcumin for use in obesity [44]; Curcumin, Baicalin and Dronabinol in Parkinson’s disease [20]; Curcumin on MRPI in retinoblastoma cells [45]; Curcumin as natural JAK inhibitors [22]; Stigmasterol β – D glucoside isolated from the methanol
extract of rhizomes of *Hedychium spicatum* as anthelmintic against adult Indian earthworm [23]; interaction of ML and other anthelmintic drugs to Cel-Pgp-1 [40] and Thymoquinone, Curcumin in inhibiting the antioxidant enzymes of *F. gigantica* and Cathepsin L to prevent virulence [24].

Perusal of literature indicated that no or limited researchers have so far used cheminformatics approaches to find out interaction of Pgp modulators/chemosentizers with Pgps of *H. contortus* and application of this in silico technology for identification of compounds useful for reversal of anthelmintic resistance. In this study, all the 36 compounds already listed have been individually docked with modeled Pgp and finally four plant compounds and two chemicals had been shortlisted.

**Conclusion**

Cheminformatics studies were carried out to find out the P glycoprotein modulators/inhibitors from plant compounds/chemicals sources. Three dimensional structure of Pgp is not known and it was designed using the sequences from NCBI. Five models of Pgp were received. Based on 1-TASSER modeling and RAMPAGE score Pgp model 1 was selected and molecular docking was done to interact with 36 plant compounds/chemicals using Accelrys Discovery Studio client 2.5. Based on the best molecular docking score (more than 90 interactions), Curcumin, Quercetin, Kaempferol, Phloretin, Verapamil and Loperamide with molecular docking scores of 98.324, 96.073, 95.47, 94.895, 93.453 and 92.807 were selected.

Plate 1.0: Five 3D Models of P-Glycoprotein of *H. Contortus* by I-TASSER
Plate 2.0: Ray Diagrams for Validation of P-gp Models

Palmitic acid (CID 985)
Caffeine (CID 2519)
Curcumin (CID 2889)
Plate 3a: Three dimensional structure of PGP inhibitors/modulators as ligands
Plate 3b: Three dimensional structure of PGP inhibitors/modulators as ligands

Curcumin (CID 969516)  
Kaempferol (CID 5280863)
Plate 4.0: Molecular docking (interaction between ligands and Pgp) of the selected compounds

Table 1: I-TASSER modelling scores of Pgp of *H. contortus*

<table>
<thead>
<tr>
<th>Model</th>
<th>C-score</th>
<th>Exp. TM-score</th>
<th>EXP. RMS</th>
<th>No. of decays</th>
<th>Cluster density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model-1</td>
<td>2.00</td>
<td>0.99±0.04</td>
<td>4.6±3.0</td>
<td>2307</td>
<td>0.8576</td>
</tr>
<tr>
<td>Model-2</td>
<td>-0.05</td>
<td>-</td>
<td>-</td>
<td>202</td>
<td>0.0901</td>
</tr>
<tr>
<td>Model-3</td>
<td>-1.16</td>
<td>-</td>
<td>-</td>
<td>133</td>
<td>0.0299</td>
</tr>
<tr>
<td>Model-4</td>
<td>-1.45</td>
<td>-</td>
<td>-</td>
<td>84</td>
<td>0.0224</td>
</tr>
<tr>
<td>Model-5</td>
<td>-2.16</td>
<td>-</td>
<td>-</td>
<td>61</td>
<td>0.0110</td>
</tr>
</tbody>
</table>

Table 2: Evaluation and analysis of residues by RAMPAGE

<table>
<thead>
<tr>
<th>Pgp Model</th>
<th>Residues in favored region</th>
<th>Residues in allowed region</th>
<th>Residues in outer region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model-1</td>
<td>1192 (93.6%)</td>
<td>63 (4.9%)</td>
<td>18 (1.4%)</td>
</tr>
<tr>
<td>Model-2</td>
<td>1159 (91.0%)</td>
<td>81 (6.4%)</td>
<td>33 (2.6%)</td>
</tr>
<tr>
<td>Model-3</td>
<td>1157 (90.7%)</td>
<td>86 (6.8%)</td>
<td>30 (2.4%)</td>
</tr>
<tr>
<td>Model-4</td>
<td>1191 (93.6%)</td>
<td>59 (4.6%)</td>
<td>23 (1.8%)</td>
</tr>
<tr>
<td>Model-5</td>
<td>1123 (88.07%)</td>
<td>103 (8.1%)</td>
<td>47 (3.7%)</td>
</tr>
</tbody>
</table>

Table 3: Compounds (both chemicals as well as compounds of plant origin) known to be Pgp modulator/inhibitors in cancer drug resistance, anti-diabetics resistance and antibiotics resistance

<table>
<thead>
<tr>
<th>S.No</th>
<th>Pub Chem ID</th>
<th>Active compound</th>
<th>Molecular formula</th>
<th>Molecular weight/gmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CID 985</td>
<td>Palmitic acid</td>
<td>C16H32O2</td>
<td>256.42</td>
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<tr>
<td>2</td>
<td>CID 2519</td>
<td>Caffeine</td>
<td>C8H10N4O2</td>
<td>194.19</td>
</tr>
<tr>
<td>3</td>
<td>CID2889</td>
<td>Curcumin</td>
<td>C21H2O6</td>
<td>368.37</td>
</tr>
<tr>
<td>4</td>
<td>CID 4788</td>
<td>Phloretin</td>
<td>C15H15O5</td>
<td>274.26</td>
</tr>
<tr>
<td>5</td>
<td>CID 6241</td>
<td>caffeine citrate</td>
<td>C14H15N4O9</td>
<td>386.31</td>
</tr>
<tr>
<td>6</td>
<td>CID 3042554</td>
<td>9,10-anthraquinone</td>
<td>C14H8O2</td>
<td>208.21</td>
</tr>
<tr>
<td>7</td>
<td>CID 62969</td>
<td>Verapamil</td>
<td>C27H39CIN2O4</td>
<td>491.06</td>
</tr>
<tr>
<td>8</td>
<td>CID65036</td>
<td>Allicin</td>
<td>C6H10O2</td>
<td>162.27</td>
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</table>
Table 4: Molecular docking (interaction between ligands and Pgp of H. contorus) scores of compounds (ligands)

<table>
<thead>
<tr>
<th>Site</th>
<th>PubChem ID</th>
<th>Ligands</th>
<th>Dock Score</th>
<th>Ligands interaction energy</th>
<th>PMF</th>
</tr>
</thead>
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<tr>
<td>21</td>
<td>4788</td>
<td>Phloretin</td>
<td>98.324</td>
<td>207.07</td>
<td>75.83</td>
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<tr>
<td>21</td>
<td>969516</td>
<td>Curcumin</td>
<td>96.073</td>
<td>98.324</td>
<td>12.93</td>
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<tr>
<td>31</td>
<td>22227427</td>
<td>Quercetin</td>
<td>95.47</td>
<td>19.483</td>
<td>37.97</td>
</tr>
<tr>
<td>34</td>
<td>5280863</td>
<td>Kaempferol</td>
<td>94.895</td>
<td>60.005</td>
<td>56.38</td>
</tr>
<tr>
<td>21</td>
<td>71420</td>
<td>Loperamide</td>
<td>93.453</td>
<td>56.338</td>
<td>18.09</td>
</tr>
<tr>
<td>21</td>
<td>62969</td>
<td>Verapamil</td>
<td>92.807</td>
<td>543.325</td>
<td>70.25</td>
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<tr>
<td>34</td>
<td>CID 5281794</td>
<td>Shogaol</td>
<td>84.031</td>
<td>352.121</td>
<td>68.09</td>
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<td>17</td>
<td>CID 168115</td>
<td>10-Gingerol</td>
<td>77.251</td>
<td>208.102</td>
<td>78.781</td>
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