CHAPTER 3: STRUCTURAL ANALYSIS OF MOLT INHIBITING HORMONE AND COMPUTATIONAL APPROACH TO DESIGN NOVEL CLASS OF GROWTH ENHANCERS

3.1 Introduction

In crustaceans, molting, growth, and development is governed by ecdysteroids synthesized by paired endocrine glands, Y-organs that are located in the anterior cephalothorax [1-4]. CHH family neuropeptides are potential modulators of various regulatory processes in the crustacean physiological system: CHH stimulate during stress conditions such as hypoxia, hyper or hypothermia, Molt inhibiting hormone [MIH] inhibits the synthesis and/or secretion of ecdysteroids from Y-organ and vitellogenin inhibitory peptide [VIH] inhibits the reproduction. The CHH family neuropeptides reveal pleotropic properties, i.e. these neuropeptides having more than two diverse biological activities have been described [5]. The most extensively acknowledged paradigm proposes that synthesis of ecdysteroids, polyhydroxylated C-27 steroid derived from cholesterol is negatively regulated by molt inhibiting hormone synthesized in the medullar terminalis of X-organ and then transported to the sinus gland [6, 7].

Various quantification studies have revealed that the quantity or the number of receptors on the Y-organ remains constant and during intermolt period, the synthesis of ecdysteroids favorably inactivated by MIH [8, 9]. Contemporary reports from
radioreceptor binding assays also intend a relation between the rate of ecdysteroid production and the receptiveness of the Y-organ to MIH, but the MIH receptor has not been fully characterized for any species [10-14]. Inhibition of facultative synthesis of ecdysteroid by MIH is facilitated by activation of the specific transcription factor that impedes phantom expression [15]. It has been reported that MIH predominantly binds to Guanylyl cyclase (GC-II) and/or G protein coupled receptor on the Y-organ cells and suppresses the ecdysteroid biosynthesis by a cAMP-dependent activation of nitric oxide synthase (NOS) and NO-dependent guanylyl cyclase (GC-I), both of which are articulated in YOs [16-21]. The existence of MIH, one of the most critical neuropeptide in the family of crustacean hyperglycemic hormone (CHH) was suggested by the fact that eye-stalk ablation leads to prompt upsurge in ecdysteroid titer in hemolymph and hence, persuades precocious molting [22]. However, as crustacean hyperglycemic hormone (CHH), gonad inhibiting hormone (GIH) and mandibular organ inhibiting hormone (MOIH) are also synthesized in X-organ and secreted from sinus gland, eye-stalk ablation cause imbalances in other physiological processes [23, 24].

3.1.1. Structure of Molt Inhibiting Hormone (MIH)

MIH has been sequestered and characterized from decapod crustaceans of several taxa and it was apparent that the size of mature MIH ranges from 8 to 11 kDa and are 72 to 78 amino acid residues in length. Reports strongly indicate the presence of six highly conserved cysteine residues in all CHH family neuropeptides that are responsible for the formation of three disulfide bridges to stabilize their structure [25]. Where pertinent data exist, most MIH comprise a non-amidated C-terminus whereas CHH have an amidated
C-terminus substantial for conferring hyperglycemic activity [26]. The solution structure of MIH from the kuruma prawn, *Marsupenaeus japonicus* reveals the existence of five α-helices located within the N-terminal motif in the MIH structure and was considered a part of the determinants of the functional specificity [27-29]. Mutation analysis has validated that the N-terminal N13 and C-terminal S71 and I72 residues are especially significant for conferring molt inhibiting activity [Figure 3.1] and these two motifs were close to each other in the 3-dimensional spatial arrangement [30].

Since the binding of MIH with receptors in the Y-organ suppresses the growth and development in crustaceans, hence a transient interference with binding of MIH to its receptor can promote the growth [31-33]. Most of the CHH family peptides exhibit biological and functional specificity and the homology model of CHH evidently indicates the absence of the N-terminal α-helix and C-terminal tail.
Figure 3.1: Multiple sequence alignment and characteristic features depiction of molt inhibiting hormone peptides (MIH). The crystal structure of *Marsupenaeus japonicus* MIH was used to derive the structural properties and subsequently the sequences for four α-helices are represented. Six cysteine residues and the three respective disulfide bridges are indicated by numbers. Three residues (Asn13, Ser71 and Ile72) critical for MIH activity is shown below the sequences as asterisk (*). The following crustacean MIH are analyzed: MJ- *Marsupenaeus japonicus*, CM- *Carcinus maenas*; CS- *Callinectes sapidus*; CP- *Cancer pagurus*; CF- *Charybdis feriatus*; OL- *Orconectes limosus*; PC- *Procambarus clarkii*; PJ- *Penaeus japonicus*; ME- *Metapenaeus ensis*; LV- *Litopenaeus vannamei*; PM- *Penaeus monodon*.
3.1.2. Pharmacophore Based Approach

To date, the 3D structure of receptor guanylyl cyclase has not been obtained by the experiment and the homology module packages were unsuccessful to build the initial model of rGC due to very little sequence and structural resemblances in the database. Additional structural information, notably of the receptor guanylyl cyclase in either free or MIH-bound state, will be very helpful for the optimization of inhibitors, such as to increase frequency of molting to achieve the better growth in crustaceans.

In the absence of three-dimensional structure of receptor guanylyl cyclase and data from crystal structure and binding site of molt inhibiting hormone, pharmacophore-based design of competitive inhibitors is one of the ubiquitous approaches for drug discovery and lead optimization. The discovery of the three dimensional structure of molt inhibiting hormone by X-ray crystallography initiated the way for design and synthesis of competitive inhibitors. MIH as a therapeutically relevant drug target with undetermined active site geometries, HipHop based pharmacophore modeling provides an effective mechanism for virtual screening. In recent years, the pharmacophore-based approach has become very commanding for the discovery of novel lead compounds and for lead optimization. In this study, by employing various molecular recognition techniques including HipHop, several possible drug candidates were screened in silico and manually modified to produce novel drug like compounds. Pharmacophore modeling was adopted to rapidly identify new potential drugs to overcome dawdling growth by interfering with the interaction of MIH with its receptor. Pharmacophore models for the MIH-antagonists were generated using HipHop algorithm entailing identification and overlay common
features. These models were anticipated to provide a rational hypothetical picture of the primary chemical features responsible for binding of MIH with its cognate receptor, and thus to supply useful knowledge for developing new active candidates targeting the MIH receptor.

3.2 Materials and Methods

3.2.1. Feature Delineations and Pharmacophore Representation

To logically design and identify inhibitors as growth enhancers via a pharmacophore-based design method a precise pharmacophore is essential. The absence of published pharmacophore for competitive inhibitors of the molt inhibiting hormone is a fundamental problem to the design of novel inhibitors. Using the data from various published data regarding MIH concentration required for conferring biological activity and the CATALYST common pharmacophore feature generation program, pharmacophore was developed for the dipeptide of MIH. When validated this pharmacophore features was then used for virtual screening and in-silico validation.

3.2.2. Structural Analysis and Selection of Training Sets

The co-ordinates and the spatial arrangement of residues of MIH were obtained from refined X-ray structure of Marsupenaeus japonicus [MJ-MIH] which was retrieved from the Protein Data Bank (accession code: 1J0T). The most critical task in the drug discovery process is developing an appropriate model to predict the activity of given molecules. The dipeptide from position 71 and 72 substantial for deliberating molt
inhibiting activity were chosen as a training set and were used in common feature pharmacophore generation using HipHop module from Accelrys Discovery Studio 2.5 [Figure 3.2].

Only those residues accountable for binding with receptor were included because they could alone deliver critical information on pharmacophore requirements. The molt inhibiting hormone from *Carcinus maenas*, *Callinectes sapidus* and *Charybdis feriatus* were homology-modeled using the solution structure of MIH [PDB: 1J0T] from *Marsupenaeus japonicus* [data unpublished]. The pharmacophore features of dipeptides at 71 and 72 positions critical for molt inhibiting activity from these MIH structures were generated and analyzed. It was observed that the isoleucine at 72 position in MIH is highly conserved while 71 position is variable. Hence, the four possible combinations which include glutamate, tyrosine, arginine and serine at 71 position along with isoleucine at 72 position were used to generate pharmacophore features. All the four dipeptide combinations were extracted from MIH structure along with their spatial arrangements and the bond orders were verified by applying CHARMm force field.
Figure 3.2: Pharmacophore mapping of Ser71 and Ile72. Two amino acid residues, Ser71 and Ile72, substantial for deliberating molt inhibiting activity were chosen as a training set and were used in common feature pharmacophore generation using Accelrys Discovery Studio 2.5. Both the residues were clustered and a pharmacophore feature map was generated and the best map (Hypo-I) was used to virtually screen the 3D database.

3.2.3. Pharmacophore Feature Generation and Validation

HipHop module of CATALYST which was popularly known for its common feature pharmacophore generation was used as search queries to virtually screen 3D-
structural libraries [34]. CATALYST identified 3D spatial arrangements of chemical features that are common to active molecule in a training set and retrieve structures that fit the hypotheses or as models to predict the activities of novel compounds [35]. These four dipeptide combinations of modeled MIH were selected as a training set to generate a common feature pharmacophore (Hip-Hop) model for virtually screening competitive inhibitors. Two pharmacophore-modeling modules, namely HypoGen [36] and HipHop [37] were incorporated in CATALYST where HypoGen allows programmed pharmacophore construction by using an assembly of molecules with bioactivities spanning over four orders of magnitude.

Alternatively, HipHop creates common feature pharmacophores, an ensemble of steric and electronic features irrespective to the activities of the training compounds and engender three dimensional pharmacophores that can be used as probes to virtually screen 3D-structural libraries. Therefore, HipHop was employed to generate common feature pharmacophores for competitive inhibitors using dipeptide combinations as training set along with their molar concentrations required for exhibiting the inhibitory activity to ensure the optimal intermolecular interactions with guanylyl cyclase. Active training set members were then assessed on the basis of the varieties of chemical features they encompass, in association with the ability to adopt a conformation that permits those features to be superimposed on a specific configuration.

The chemical features were analyzed and the high-ranking pharmacophores associated with their conformation models were then conceded to CATALYST
hypotheses generation. Hypotheses were clustered for developing more selective models by calculating the distance between residues and this distance was used as a function of the number of common pharmacophore features and the root-mean-squared displacement between the matching features. Hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and hydrophobic (HY) chemical functions were selected on the basis of the chemical features of the clustered molecule in the training set that are considered to be responsible for a desired biological activity [Figure 3.3].

Figure 3.3: Feature delineations and pharmacophore representation. Hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and hydrophobic (HY) chemical functions were selected on the basis of the chemical features of the clustered molecule in the training set that are considered to be responsible for a desired biological activity.
The pharmacophore features of residues critical for conferring molt inhibiting activity were generated from various other decapod crustacean species to virtually screen compounds which can exhibit the better fit value for all the different pharmacophores [Figure 3.4].

Figure 3.4: Protein modeling & pharmacophores were generated from (a) *Charybdis feriatus* (b) *Carcinus maenas* (c) *Marsupenaeus japonicus* (d) *Callinectes sapidus*. Green color indicates hydrogen bond acceptor (HBA); brown hydrogen bond donor (HBD); cyan indicates hydrophobic (Hy) features.
3.2.4. Database Virtual Screening and Manual alterations

The objective of the virtual screening is to reduce of the vast virtual chemical space of small organic compounds, to screen against a specific target protein, to a wieldy amount of compound that impede a highest chance to lead to a drug candidate [38]. The validated hypothesis was used as a query in the screening of 3D conformational molecular structure MiniMayBridge database. The best and fast searching along with flexible or rigid mapping method in CATALYST was used for the screening of database which calculates and stores multiple conformations for each structure during the database generation process. A large hit list was retrieved due to implementation of flexible screening method. Therefore, downstream filters were used to reduce the hit list to a manageable size containing the most promising hits for \textit{in vitro} testing.

The first filter applied was to cluster hits according to their fit score and molecular weight which allows selecting representative compounds from each structure classes. The top predicted compound then underwent a series of manual modifications to improve chemical features and the modified compound was reanalyzed with the same parameters that were used previously for virtual screening. The hits obtained were further filtered for drug-likeness, which is expressed as physicochemical properties that contribute to favorable ADME/Tox profiles to eliminate toxicity and poor pharmacokinetics [39, 40].

The ADMET descriptors were then calculated in Discovery studio 2.5 which evaluates aqueous solubility, plasma protein binding (PPB), percent human intestinal absorption (HIA), ADMET AlogP98, cytochrome P450 (CYP450) 2D6 inhibition, and
hepatotoxicity. The predicted compounds from virtual screening and arbitrated by
toxicity and pharmacokinetics was selected for further analysis.

3.3 Results and Discussion

3.3.1. Pharmacophore Feature Generation and Validation

Pharmacophore models were developed in HipHop module in CATALYST
software and these models were considered for further analysis. Molecule in the training
set was predicted as active and ten statically best pharmacophore models were generated.
In the training set, active compound maps the features of hydrophobic point (Hy),
hydrogen bond acceptor (HBA) and donor (HBD). The residues in the training set map
the three hydrogen bond donor feature, which divulges that this feature could be
essentially accountable for the high molecular bioactivity, and thus should be taken into
account in discovering and manually modifying novel competitive inhibitors for MIH
receptor [41, 42]. The common features from all the pharmacophore hypothesizes were
analyzed, clustered and ranked.

Interestingly all the pharmacophore hypothesis comprises of very similar
chemical features. The best hypothesis (Hypo-1) was comprises of six features: two
hydrophobic (Hy), three hydrogen bond donor (HBD), and one hydrogen bond acceptor
(HBA) [Figure 3.5]. HipHop attempts to construct the simplest hypotheses where
tolerance sphere describes the expanse in space that should be engaged by a specific kind
of chemical functionality. Among the ten hypotheses generated automatically by
CATALYST, the first ranked molecule has been shown to have the acceptor–donor feature in proper spacing. Therefore, this pharmacophore can elicit most of the compounds having acceptor, donor and hydrophobic features together from the database which would enable the binding with receptor. These compounds were mapped to the pharmacophore map and had a fit value of 3.6 which mean that a minimum of four out of six features can map well with training compound. A low fit value shows that the centers of the functional groups of the compounds are displaced from the centers of the corresponding pharmacophore features even though the chemical functions of the individual hits overlay with the corresponding pharmacophore. Subsequently, eliminating poor overlapping compounds to the pharmacophore model advances the success rate of the retained bio-active compounds exhibiting comparable biological effects. The highly active compound should possess chemical assemblies that perfectly overlap (map) with corresponding features.

Hence, the same criteria were applied in database searching; the fits were successively fitted against hypo-I and those having a fit value below 3.6 were not allowed to pass the second filter of the virtual screening. The filters ensure the reliability of the pharmacophore hypotheses in virtual screening of the commercial databases. However, acquaint with an extra filter to reduce the hits and rank the compounds to be screened in vitro is essential as the pharmacophore searching produces too many compounds in the specified fit value standards.
3.3.2. Virtual Screening, Alteration and Ranking of Molecules

A library of commercially available chemical compounds supplied by Accelrys was screened for MIH receptor inhibitory activity by a virtual screening [43]. A database search in CATALYST comprises of two algorithms in which the “Fast Flexible Search Database” calculates already existing conformers of the database, and the Best Flexible Search Databases is capable of changing the conformation of a molecule during computation. 56 diverse and drug-like inhibitors were screened with the pharmacophore model by means of “Best Flexible Search Database” selection, and a maximum of 100 conformers per compound were generated. Despite the use of various filters, the screened compounds were not particularly drug like. Consequently, the top ranked molecules, CGE-1, CGE-6 and CGE-8 retrieved from virtual screening were carefully chosen for further analysis and manual modification. Manual alterations were guided by pharmacophore features, fit score, pharmacodynamics and pharmacokinetic properties. Ten drug-like compounds were successively created by making manual modifications in which only CGE-6 and CGE-8 demonstrated better result [Figure 3.5].

For both the procedures, only those structures that map at least three features of the pharmacophore template were retrieved. Subsequently, fourteen ligands were selected that passes the second filter of high throughput screening which eliminates molecules with unfavorable physicochemical and pharmacokinetic properties based on the Lipinski’s rule of five [Figure 3.6]. Out of the fourteen ligands analyzed, eight [Figure 3.5] were predicted to be free from toxicity using toxicological endpoints such as
Daphnia magna EC50, mutagenicity, carcinogenicity, chromosome damage and skin sensitization [44].

Figure 3.5: Representative structures of competitive inhibitors for MIH receptor.

The CGE-2, 3, 4, 5 and 7 were acquired from high throughput screening from the database, whereas CGE-1 and 8 were designed by manual modifications of CGE-6. CGE-1 exhibited a high fit value (5.8), a lower relative energy and conceded the Lipinski’s rule of five after manual alteration.
Figure 3.6: Optimal prediction space attributes of ADMET for CGE-1. Each elliptical ring represents the absorption, distribution, metabolism, excretion and toxicity for the designed compound CGE-1 was predicted using Accelrys Discovery Studio 2.5.

The selection of best competitive inhibitor depends upon the better fit values, various molecular properties, toxicity. The CGE-5 exhibits the highest fit value but possess higher number rotatable bonds while CGE-2 has higher number of acceptors and rotatable bonds which is contrary to the Lipinski’s rule of five. The variation in the fit values of CGE-6 and CGE-8 is due to the difference in the position of halides. In CGE-6, chlorine atom is attached to the ortho-position whereas bromine is attached to the meta-position in the CGE-8. Bromine being less electronegative than chlorine may provide
appreciable more hydrophobic character to the nucleus. In CGE-8, presence of three nitrogen atom in the alternate position of the chain viz. nitrogen from thiourea and the adjacent amide group benefit the molecule to map with the pharmacophore features. Evidently, competitive inhibitors such as the CGE-1 described here epitomize powerful research tools and can be used to assess the possible regulatory roles of MIH in various physiological processes. Moreover, the homology models of CHH has also suggested that the design of a new series of inhibitors for MIH receptor is highly specific and had no measurable affinity for other CHH family neurohormones.

3.4 Conclusion

One of the key therapeutic strategies is to impede the biological activity of MIH, and hence, to upsurge the ecdysteroid level in the hemolymph. The findings obtained from the present study are practically helpful to increase the production by controlling molt in crustaceans. The results from the study of chemical features of dipeptide of MIH reveal that Hypo1 has a remarkable capability to design novel competitive inhibitors and we have found that hydrogen bong donors and hydrophobic pockets play a key role in MIH selectivity. With the intention to validate the key pharmacophore feature, we generated several homology models from different species of decapod crustaceans and validated. The Hypo-2 where the HBD features were removed failed to produce the result as Hypo1. Therefore, we concluded that the HBD group in the binding site of MIH (dipeptide) plays a key role in MIH selectivity. Hence, Maybridge and the database of manually altered molecules was screened using Hypo1 and the active hits from these databases were subsequently ranked based on the fit value, ADMET scores (drug like
properties). Finally, seven compounds display noteworthy similarities with the critical amino acids. Therefore, Hypo1 can be used as a precious tool for screening structurally dissimilar, novel and highly selective compounds. These molecules will be subjected to \textit{in-vitro} and \textit{in-vitro} studies to validate them as a potent lead for the inhibition of binding of MIH with its receptor in the Y-organs.

3.5 References


