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Moult inhibiting hormone: a new approach to the discovery and design of growth promoters in crustaceans?

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Abstract

Several chemicals have been tested as growth promoters in aquaculture but they cannot be endorsed for commercial processes due to their residual effects in the body of prawns, lobsters and crabs. The concern over environmental hazard, human health and food safety have led to a search for alternative growth promoters significantly to improve the growth of crustaceans with no such effects. Neurohormones, regulatory signalling molecules of crustaceans that coordinate multiple developmental and physiological processes, are major determinants underlying phenotypic integration. Competitive inhibitors for the moult inhibiting hormone receptor are expected to have a direct growth promoting effect on crustaceans by direct involvement in interference with the receptor in the Y-organ, which can cause a surge the production of ecdysteroids. Competitive inhibitors therefore can be regarded as growth promoters in crustacean fishery in addition to various other benefits. This review emphasizes the manifold effects of moult inhibiting hormone, a most versatile animal hormone, with an emphasis on the target sites and competitive inhibition.

Key words: competitive inhibitor, crustaceans, ecdysteroid, growth promoter, moult inhibiting hormone, Y-organ.

Moult inhibiting hormone: a prominent member of the pleotropic hormone family

Crustaceans encounter circumstances that necessitate or favour coordinated responses from several autonomic systems. The sinus gland neuropeptides have a major role in regulating various behaviours that contribute to growth and reproduction by integrating external and internal stimuli (Keller 1992; De Kleijn & Herp 1995; Beltz 1998; Zmora *et al.* 2009; Chung 2010). The results from various studies clearly point to a crucial role of moult inhibiting hormone (MIH) in conjunction with its family of neuropeptides and associated signalling pathways in a biologically relevant response to different conditions (Nakatsuji & Sonobe 2004; Zheng *et al.* 2008). The moult inhibiting hormone synthesized in the X-organ, which is part of the medulla terminalis, is then transported to a neurohaemal organ, the sinus gland that lies between the medulla externa and medulla interna in the eyestalks (Skinner 1985; Lachaise *et al.* 1993; Shih *et al.* 1998). Physiological evidence favours the X-organ/sinus gland complex as the key site responsible for the production, storage and release of MIH and its family of chemical messengers in malacostracan crustaceans. In all cases, the hormones produced by the X-organ are conducted distally via axons regrouped in tracts that reach and compose the neurohaemal organ, to be secreted under appropriate stimuli and can serve as intermediates between neurological signalling and terminal hormone signalling (i.e. steroid hormones) (Bliss & Welsh 1952; Passano 1953; Skinner 1985; Dircksen *et al.* 1988; Watson *et al.* 2001). The MIH is released and transported by the circulating haemolymph and binds to its receptors on the cell surface of the endocrine glands where they stimulate intracellular signal transduction pathways leading to the release of the next neurohormone in the cascade or directly governing certain physiological actions (Soumoff & O'Connor 1982; Mattson & Spaziani 1985; Watson & Spaziani 1985; Webster 1986; Webster & Keller 1986; Schoettker & Gist 1990; Chung & Webster 2003; Asazuma *et al.* 2009; Nakatsuji *et al.* 2009).

Receptiveness to moult inhibiting hormone

In the case of crustaceans, ecdysone is peripherally transformed into the active hormone 20-hydroxyecdysone (Lachaise *et al.* 1993; Grieneisen 1994; Mykles 2011). The

moult inhibiting hormone exerts an inhibitory effect on the Y-organ and hence, partially accounts for the dramatic fluctuation of the haemolymph ecdysteroid concentration during the moult cycle (Webster 1986; Webster & Keller 1986). It is evident from various studies that the Y-organ is unresponsive to MIH during moulting and also the decreases of circulating MIH just prior to premoult elicits the secretion of ecdysone by the Y-organ, causing a precipitous increase in the circulating ecdysteroid levels (Sefiani *et al.* 1996; Nakatsuji & Sonobe 2004; Nakatsuji *et al.* 2006b). These alterations facilitate the variety of biochemical and physiological processes that occur during the moult cycle. The moult inhibiting hormone maintains ecdysteroid levels at low circulating levels during the intermoult period and therefore, removal of both eyestalks results in a shortening of the intermoult period and increases the number of moult cycles (Keller & Schmid 1979; Chang & Bruce 1980; Hopkins 1983).

The moulting hormone, ecdysteroids, are well characterized signalling molecules and their primary function is to mediate the moulting process (Lachaise *et al.* 1993; Lafont 1997). Ecdysis is triggered by the successive waning of ecdysteroids back to basal levels just after premoult. Exuviation of the old exoskeleton and an increased uptake of water results in the formation of a larger cuticle which is essential for the growth of the organism. Ecdysteroids, from the maternal source, are packed into the eggs for use during the early stage of embryonic growth and evidence also suggests that ecdysteroids are furthermore responsible for reproduction (Chang & Bruce 1980; Okazaki & Chang 1991; Chang *et al.* 1992; Subramoniam 2000).

Inhibition of the facultative synthesis of growth hormone by moult inhibiting hormone is assisted by activation of the specific transcription factor that impedes phantom gene expression in the Y-organ (Asazuma *et al.* 2009). The MIH predominantly trusses its transmembrane receptor (guanylyl cyclase) and activates a cAMP-dependent nitric oxide synthase (NOS) and NO-dependent guanylyl cyclase (GC-I), both of which are articulated in the Y-organs and hence, suppresses ecdysteroid biosynthesis (Sedlmeier & Fenrich 1993; Von Gliscynski & Sedlmeier 1993; Bocking & Sedlmeier 1994; Saidi *et al.* 1994; Baghdassarian *et al.* 1996; Nakatsuji *et al.* 2006b; Covi *et al.* 2009). The synthesis and release of MIH in the X-organ/sinus gland complex was suggested by the fact that eyestalk ablation leads to a prompt upsurge in the ecdysteroid titre in the haemolymph and hence, initiates precocious moulting. However, several other neurohormones such as crustacean hyperglycaemic hormone (CHH), gonad inhibiting hormone (GIH) and mandibular organ inhibiting hormone (MOIH) which play a critical role in the regulation of several other functions are also produced in the X-organ and hence, eyestalk ablation cause imbalances in other physiological processes. A recent finding also suggests that the moult inhibition does not conform strictly to predictions of the above model and compounds other than MIH have direct or indirect impacts on the regulation of Y-organs.

The structure of moult inhibiting hormone determined from the *Marsupenaeus japonicus* revealed the presence of five α-helices in which N13 at the *N*-terminal alpha-helix and S71 and I72 at the *C*-terminal tail are critical to binding with rGC in the Y-organ of crustaceans (Katayama *et al.* 2003). The cDNA sequence of the putative moult inhibiting hormone from *Metapenaeus ensis* consists of a 315 bp coding region that encodes a 77 amino acid residue mature peptide. Various analyses also confirm the presence of MeMIH in the eyestalks as well as in the brain of the sand shrimp.

Receptor guanylyl cyclase: a regulator of assorted physiological functions in crustaceans

Apposite functioning of the mature nervous system depends on the precise development of neuronal circuitry (Mandoki *et al.* 2004; Lee & Mykles 2006; Imayavaramban *et al.* 2007). Definition of the physiological roles and unravelling the functions of the receptors in crustaceans has often relied on the use of a radioreceptor binding assay, mutation analysis or in the use of relatively specific neuropeptides and inhibitors, but the regulation of the various functions is still not clearly known (Webster 1993; Han & Watson 2005; Chung & Webster 2006; Nakatsuji *et al.* 2006a). The structure and function of receptors for various other neuropeptides remain unclear, although the receptors have been strongly implicated as having a significant counter-regulatory role to a variety of growth factors in crustaceans. A limited number of animal models have been conceded to isolate and characterize the receptors for the CHH family of neuropeptides in various target tissues (Lee *et al.* 2007b; Katayama & Chung 2009).

Guanylyl cyclase is the receptor for small peptides such as MIH produced locally in various different tissues (Zheng *et al.* 2008). Expression of the receptor guanylyl cyclase in the Y-organ, hepatopancreas and female reproductive organs was evident, and significant phenotypes associated with each of these organs were apparent in crustaceans (Kuppert *et al.* 1978; Londershausen & Spindler 1985; Laverdure & Soyez 1988; Lee *et al.* 2007a,b). The MIH neuropeptide-dependent increases of guanylyl cyclase activity and the decrease in the production of ecdysteroid appear to be required for the progression of moult inhibition. It has been observed throughout the moult cycle that there is no variation in the quantity of receptor guanylyl cyclase in the Y-organ and that the Y-organ is favourably inactivated by MIH in the intermoult phase, and inhibits the production of 20-hydroxyecdysone (Zheng *et al.* 2006, 2008; Lee *et al.*

2007a). This result was due to receptiveness of the Y-organ to MIH and to some extent CHH that inhibits the facultative synthesis of ecdysteroid by activation of the specific transcription factor that impedes phantom expression (Webster 1991; Yasuda *et al.* 1994; Asazuma *et al.* 2009). Thus, the transmembrane guanylyl cyclase receptor is critical for the growth and development of crustaceans. Contemporary reports from various radioreceptor binding assays demonstrated that MIH predominantly binds to its receptor (guanylyl cyclase) on the Y-organ and suppresses ecdysteroid biosynthesis (Webster 1993; Asazuma *et al.* 2005; Chung & Webster 2006). Moulting in crustaceans is regulated by a cAMP-dependent activation of nitric oxide synthase (NOS) and NO-dependent guanylyl cyclase (GC-I), both of which are articulated in the Y-organs (Saidi *et al.* 1994; Kim *et al.* 2004). Additionally, MIH-activated protein kinase cascades and Ca^{2+} signalling pathways appear as targets after receptor guanylyl cyclase activation (Bocking & Sedlmeier 1994). The expression of three guanylyl cyclases (GC), NO-sensitive GC, a membrane receptor GC and a NO-insensitive soluble GC were observed in relation to the ecdysteroid level and it was found that MIH and CHH inhibit secretion through different GC-dependent pathways. The presence of guanylyl cyclase isoforms in various tissues including the Y-organ suggest that these tissues can modulate responses to neuropeptides by altering GC expression. The existence of several GC-receptor isoforms also suggests that diverse functions are facilitated by specific isoforms and raises the possibility of functional variability (Webster 1993; Zheng *et al.* 2006; Lee *et al.* 2007a,b).

A gold-conjugated vitellin labelling experiment demonstrated that a neuropeptide secreted from the sinus gland inhibits vitellogenin endocytosis in *M. rosenbergii*, possibly by binding to its cognate receptor (Jugan & Soyez 1985). A solid phase binding assay from *Orconectus limosus* revealed that a 30 kDa protein that binds specifically with vitellogenin, increased significantly at the start of vitellogenesis, but decreased in older oocytes (Laverdure & Soyez 1988). Studies on a purified transmembrane receptor (a 230 kDa protein) from the oocytes of the red mud crab, *Scylla serrate,* displayed a high binding affinity vitellogenin in the presence of Ca^{2+}, suggesting a similar manner of regulation of various physiological functions (Warrier & Subramoniam 2002). Similarly, a putative vitellogenin receptor has been described from the tiger prawn, *P. monodon*, which has a molecular weight of 21 kDa and consists of conserved domains such as cysteine-rich and epidermal growth factor-like domains. This 1943 amino acid protein has YWTD motifs similar to the LDL, VLDL and vitellogenin receptor of insects and vertebrates (Tiu *et al.* 2008). In *M. japonicus*, the expression of the ovarian low density lipoprotein receptor was seen to be explicit to the ovary and showed peak levels during the previtellogenic phase (Okumura 2006). Recent findings on mature *Callinectes sapidus* females indicated that MIH also regulates vitellogenesis in the hepatopancreas but the membrane binding affinity is 77 times lower than that of the transmembrane receptor guanylyl cyclase in the Y-organ and the molecular weight of the MIH receptor in the Y-organ and hepatopancreas is 61 kDa (Zmora *et al.* 2009).

In general, however, the target sites for these neuropeptides have remained unclear, in part since specific inhibitors of the receptor-signalling pathway have not been available. In conclusion, these investigations suggest that peptide-dependent augmentation of GC signalling is critical for normal growth. Signalling through this receptor may also be important for the physiological function and maturation of female reproductive organs.

Topology: guanylyl cyclase as a receptor for MIH

In the past few years, the purification, cloning and expression of various forms of crustacean guanylyl cyclase (GC) have revealed that besides soluble GC, there are at least four receptors for nitric oxide (NO) (Goy 1990, 2005; Scholz *et al.* 1996, 2002; Prabhakar *et al.* 1997; Scholz 2001; Aonuma 2002; McDonald *et al.* 2011). Though moderately homologous to soluble GC, the receptor GCs share a unique topology that comprises an extracellular ligand binding domain, a transmembrane region, an ATP binding domain and an intracellular domain that contains the catalytic (GC) region at its *C*-terminal end. In contrast to soluble GC activators, which are small gaseous molecules (NO and CO), activators of membrane GCs are peptides. The existence of the extracellular ligand binding domain suggests that all these isoforms of rGC function as receptors for moult inhibiting hormone.

The basic topology of *Gecarcinus lateralis* guanylyl cyclase receptor [Genebank accession number: ABC94532.1] consists of a ~399 amino acid extracellular ligand-binding domain, a 238 residue catalytic domain-spanning region and a 12 residue ATP binding site. The receptor guanylyl cyclase can be further divided into a receptor family ligand binding region that is similar to known protein kinases called the kinase homology domain (KHD), a three-amino acid chloride ion binding domain and a 9-residue putative dimerization interphase. In the absence of ligand, rGC exists as a homodimer or homotetramer, and ATP binding does not lead to further aggregation. Multiple domains that are located both outside and inside the plasma membrane mediate the oligomerization of guanylyl cyclase. The intracellular dimerization interface region has been mapped to the amphipathic sequence that bisects the kinase homology domain and cyclase domains. It was observed that the deletion of this region results in monomeric and inactive

intracellular constructs, suggesting that dimerization of the cyclase domains is required for guanylyl cyclase catalytic activity (Okazaki & Chang 1991; Pyriochou & Papapetropoulos 2005; Lee *et al.* 2007b).

Development of competitive inhibitors of MIH: a novel strategy to accelerate growth

There have been some intermittent methods for expansion of enhanced practices for rearing and grow-out of crustaceans including prawns, lobster and crabs, but reliable research towards the advancement of sustainable technologies have yet to be commenced (Rajinikanth *et al.* 2010; Kotiya *et al.* 2011). Most of the growth promoters used in aquaculture are uncertified with regard to their constituents as well as being very expensive, which has diverted farmers to look for inexpensive feeds, organic fertilizers and antibiotics etc. for feeding the crustaceans. For these hatchery inputs, the growth and quality of crustaceans along with the aquatic environment are frequently worsened, giving farmers a negative financial return. The evolution and expansion of antibiotic-resistant bacterial strains is another significant concern for the use of antimicrobial agents as growth stimulators in aquaculture. Numerous antibiotics have been in use as growth promoters and the mechanisms of growth stimulation are still not known precisely. Therefore, various countries recommend the sensible use of antimicrobial agents as therapeutics and for growth enhancement and most of the growth promoters have now been proscribed as feed additives because they could lead to a problem of increasing bacterial resistance of human and animal origin (Wegener *et al.* 1999; Butaye *et al.* 2003; Casewell *et al.* 2003). Lobster and prawn rearing centres often use eyestalk ablation to promote growth in crustaceans. Various *in vitro* and *in vivo* approaches have indicated that, in addition to eyestalks, some other neuroendocrine tissues including the pericardial organs, thoracic, abdominal and cerebral ganglia might be important in the neuroendocrine control of different physiological processes including moulting and that several other neuropeptides might be synthesized and secreted from these sites (Chang *et al.* 1999; Hsu *et al.* 2006). Hence, eyestalk ablation is not an appropriate key for enhancing the growth in crustaceans.

Various unilateral and bilateral eyestalk ablation studies have already been undertaken on crustaceans indicating high mortality due to a failure in recovery from moulting (Mauviot & Castell 1976; Santiago 1977; Hesni *et al.* 2008; Venkitraman *et al.* 2010).

It is well established that the MIH mediated signalling pathway is amenable to regulation of ecdysteroid synthesis and hence, a wide variety of functions. The moult inhibiting hormone has secondary endocrine glands as the target, for example MIH binds to its cognate receptor on the Y-organ which represses the production of ecdysteroids, and is explicitly responsible for inhibiting growth. However, studies on the specific mechanisms by which the endocrine system can be regulated are scarce, and this is a critical point to be developed for fully understanding the regulation of moulting and reproduction, as well as for predicting the potential cross activation of signals whose disruption effects on endocrine systems are not still evident. In fact, endocrine regulation of MIH can take place at different physiological levels such as varying the secretion of hormones in the X-organ or altering the structure of receptors on target tissues.

From this perspective, competitive inhibition of moult inhibiting hormone mediated signalling could furnish a promising new strategy to enhance growth in crustaceans (Fig. 1). Competitive inhibition could reduce the overall antibiotic use in animals and could also be a prudent strategy to overcome the problem of antibiotic resistance and environmental pollution. With the existence of data from mutation analysis and the structure of MIH (Katayama *et al.* 2003; Katayama & Nagasawa 2004; Chen *et al.* 2005), it seems apposite to employ methodology that is suitable in the early phase of drug discovery. The possible inhibiting or stimulating effect of MIH is related to mechanisms that control both the release and synthesis of moult inhibiting hormone in the sinus gland. Recent advances in crustacean neuroendocrinology and signalling mechanisms could lead to the identification of numerous growth promoters that interfere with MIH signalling in the Y-organ. In this sense, a competitive inhibitor could act as an agonist or antagonist by directly binding to a hormone receptor. Indirectly, though, a competitive inhibitor could interfere by several mechanisms at any step of the transduction pathway of a hormone, thereby altering its final effect. The physiological effects of MIH could also be imparted by modifying the metabolism of circulating hormones by decreasing their excretion rate and/or biotransformation in the neurosecretory centres.

Ligand-based pharmacophore feature generation and *in silico* screening

The structure of moult inhibiting hormone from *Marsupenaeus japonicus* was obtained from the Protein Data Bank [PDB ID: 1J0T]. The most vital task in the ligand based drug design process is developing appropriate pharmacophore features to predict the probable binding site of given molecules. The residues, serine and isoleucine at 71 and 72 positions, respectively, accountable for binding with receptor are substantial for deliberating moult inhibiting activity were chosen as a training set and were used to generate common pharmacophore features using Accelrys Discovery

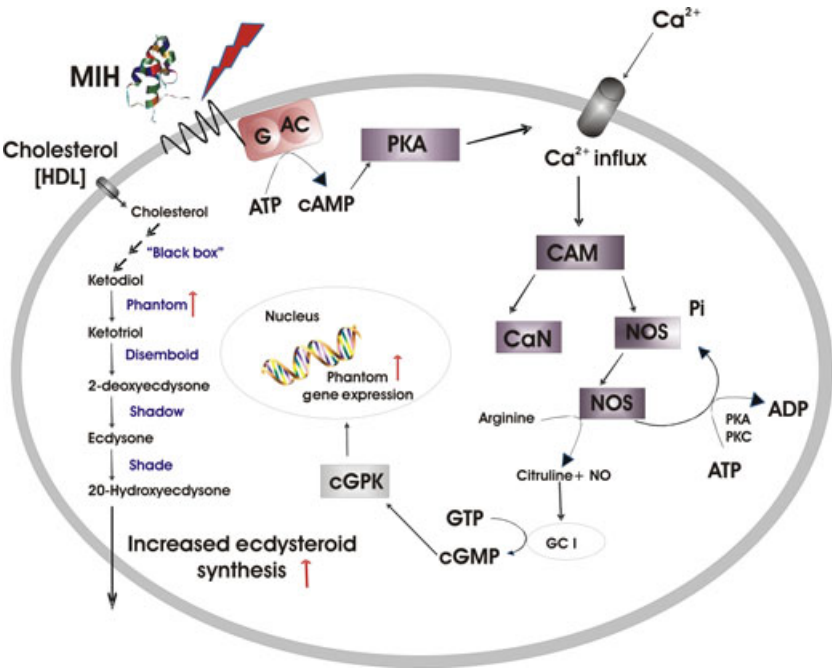

Figure 1 Interference with MIH binding to the transmembrane receptor guanylyl cyclase. The small molecule competitive inhibitors will bind to the receptor for moult inhibiting hormone resulting in the continuous expression of phantom gene in the Y-organ. The activation of gene expression, and dietary sterol uptake, transport and trafficking in the midgut will lead to a precipitous increase in the biosynthesis and hence, the circulating ecdysteroid levels. The high level of ecdysteroid in the haemolymph will lead to higher growth and reproduction rate in crustaceans.

Studio 2.5 (Accelrys Inc., San Diego, CA, USA) (Fig. 2). HipHop modules for pharmacophore modelling were used that create common feature pharmacophores, a collection of steric and electronic features regardless of the activities of the training compounds. These three dimensional pharmacophores were used as probes virtually to screen the 3D-structural libraries of molecules. Therefore, this method of screening competitive inhibitors ensures optimal intermolecular interactions with guanylyl cyclase to block its biological response. The distance between S71 and I72 was calculated and the hypotheses were clustered for developing more selective models. The distance between these two residues was used as a function of the number of common pharmacophore features and the root-mean-squared displacement between the matching features. The hydrogen bond acceptor (HBA), two hydrogen bond donor (HBD) and a hydrophobic (HY) chemical function were selected on the basis of the chemical features of both the clustered residues in the training set that are considered to be responsible for a moult inhibiting activity (Fig. 2). The common feature pharmacophore generation was used as search queries virtually to screen 3D-structural libraries of designed molecules using the HipHop module of Catalyst (Klebe 2006). This identified the 3D spatial arrangements of chemical topographies that are common to the active molecule in a training set and to retrieve structures from the database that fit the hypotheses (Martin 1992). The hits with high fit values were further filtered for drug-likeness, which are expressed as physicochemical properties that contribute to favourable adsorption, distribution, metabolism, excretion [ADME] profiles to eliminate toxicity and

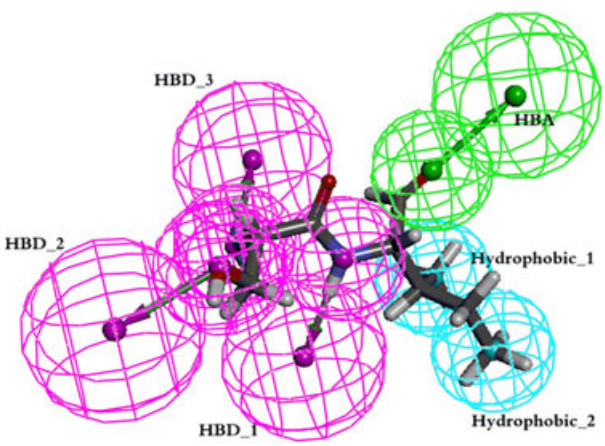

Figure 2 Clustered pharmacophore features for S71 and I72. The pharmacophore features of serine (71) and isoleucine (72) from MIH were clustered being responsible for binding with the receptor guanylyl cyclase in the Y-organ. The hypothesis comprised two hydrophobic (Hy), three hydrogen bond donor (HBD) and one hydrogen bond acceptor (HBA).

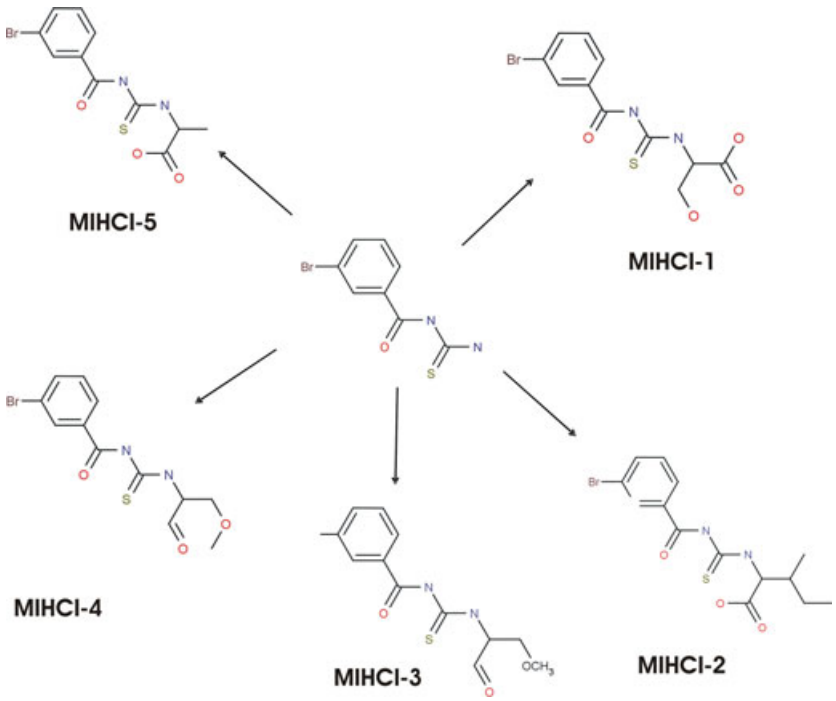

Figure 3 Representative structures of competitive inhibitors for MIH receptor. Six competitive inhibitors with best fit value were selected after feature mapping, pharmacokinetics and pharmacodynamics studies.

poor pharmacokinetics (Lipinski 2000; Waterbeemd & Gifford 2003). The ADMET descriptors were then calculated to evaluate the aqueous solubility, cytochrome P450 (CYP450) 2D6 inhibition, hepatotoxicity, plasma protein binding (PPB), percent human intestinal absorption (HIA) and AlogP98. The predicted compounds with a high fit value from the virtual screening and assessed by toxicity and pharmacokinetics can be used as potent competitive inhibitors to enhance growth in crustaceans (Fig. 3).

Future perceptions

Moult inhibiting hormone affects a remarkable number of processes in crustacean development and life history, including moulting and reproduction. While many molecular details underlying hormone signalling remain unknown, the study on hormonal pleiotropy offers intriguing insights into phenotypic integration and the mechanisms underlying the regulation of processes. Several lines of evidence imply that MIH secreted from the X-organs and a few other centres is likely to be the modulator of ecdysteroid synthesis in addition to MIH-mediated control of vitellogenesis. Various approaches have been used to enhance the growth of crustaceans, such as the use of antibiotics, a high cholesterol diet etc. Nevertheless, interference with MIH binding to the Y-organ will certainly play a significant role in increasing the size of crustaceans. An understanding of the molecular basis for MIH–Y-organ interaction and inhibition of gene expression accountable for ecdysteroid synthesis has been greatly enhanced by the recent research. A combination of crystallographic structure analysis and structure assisted drug discovery can be employed to develop specific competitive inhibitors of MIH. The design and molecular docking can used to determine the binding mechanism as well as studies on novel competitive inhibitors against MIH that interact favourably to the binding site. In contrast to all previous strategies, to use either antibiotics leading to the development of drug resistant bacterial strains or eyestalk ablation which also increases the mortality rate, competitive inhibitors of MIH could be much more potent for accelerating growth and development in crustaceans. As discussed above, MIH could be involved in the regulation of diverse physiological functions together with moulting and reproduction. The structure of receptor guanylyl cyclase and its binding sites could provide more insights in the regulation of these functions which will certainly help to further develop and improve specific inhibitors that can enhance growth in crustaceans.

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Conflict of Interest

The authors declare no conflict of interest.

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