MINI-REVIEW

Drosomycin, an essential component of antifungal defence in *Drosophila*

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Abstract

Drosomycin is an inducible antifungal peptide of 44 residues initially isolated from bacteria-challenged *Drosophila melanogaster*. The systemic expression of drosomycin is regulated by the Toll pathway present in fat body, whereas inducible local expression in the respiratory tract is controlled by the Immune Deficiency (IMD) pathway. Drosomycin belongs to the cysteine-stabilized α-helical and β-sheet (CSαβ) superfamily and is composed of an α-helix and a three-stranded β-sheet stabilized by four disulphide bridges. Drosomycin exhibits a narrow antimicrobial spectrum and is only active against some filamentous fungi. However, recent work using recombinant drosomycin expressed in *Escherichia coli* revealed its antiparasitic and anti-yeast activities. Two evolutionary epitopes (α- and γ-patch) and the m-loop have been proposed as putative functional regions of drosomycin for interaction with fungi and parasites, respectively. Similarity in sequence, structure and biological activity suggests that drosomycin and some defensin molecules from plants and fungi could originate from a common ancestor.

Keywords: innate immunity, antifungal peptide, CSαβ motif, plant defensin, Toll signal pathway.

Introduction

Antimicrobial peptides (AMPs), the first line of host defence of multicellular organisms, play a crucial role in eliminating infection from various bacteria, fungi, viruses and protozoa

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(Zasloff, 2002; Bulet *et al.*, 2004). The majority of AMPs are small and cationic molecules generally with 12–50 amino acids in length. Most have a positive net charge at physiological pH because of the presence of a high content of arginines and lysines. They usually adopt an amphipathic structure in which the positively charged and hydrophilic domains are separated from the hydrophobic domains (Hancock & Diamond, 2000; Hancock, 2001). Such a structure associated with their cationic character is suited to interacting with negatively charged membranes of microbes, resulting in permeabilization and cell death (Bulet *et al.*, 2004). Beyond the plasma membrane, a series of extracellular and/or intracellular targets have also been identified to be involved in the metabolic inhibition mechanism of AMPs (Brogden, 2005). Despite diverse primary sequences, these peptides can be grouped into three major classes based on their secondary structure features (Bulet *et al.*, 1999; Zasloff, 2002; Bulet *et al.*, 2004). The most common class contains peptides with an α-helical conformation (Tossi *et al.*, 2000; Giangaspero *et al.*, 2001). The second class comprises peptides with intramolecular disulphide bonds, which display β-sheet or α-helical/β-sheet mixed structures (Dimarcq *et al.*, 1998). The third class includes peptides with overrepresentation of certain residues such as proline, glycine, histidine, arginine and tryptophan (Bulet *et al.*, 1999; Zasloff, 2002; Bulet *et al.*, 2004). In addition, some larger proteins and protein fragments have emerged as novel class of AMPs, such as lactoferrins and their derived peptides, and complement-derived peptides and kinocidins (Yount *et al.*, 2006).

Although widely distributed in animals, plants and fungi, AMPs are especially rich in insects (Bulet *et al.*, 1999). In *Drosophila melanogaster*, some 20 inducible AMPs have been identified, which are grouped into seven types, diptericin, drosocin, cecropin, attacin, defensin, metchnikowin and drosomycin. Each type exhibits a definite spectrum of antimicrobial activity (Lemaitre & Hoffmann, 2007). Of them, drosomycin, the first inducible antifungal peptide from insects, was initially isolated by Fehlbaum *et al.* from 2000 bacteria-challenged adult *D. melanogaster* (Fehlbaum

et al., 1994). However, this highly bacteria-induced peptide does not have any antibacterial activity. Instead, it has strong inhibitory effects on some phytopathogenic filamentous fungi, which are also targeted by many plant defensins (Terras *et al.*, 1993; Fehlbaum *et al.*, 1994). This is not unexpected given the significant sequence similarity between drosomycin and plant defensins (Fehlbaum *et al.*, 1994; Landon *et al.*, 1997). Phylogenetically, drosomycin is restrictedly distributed in *D. triauraria* (*montium* subgroup), *D. ananassae* (*ananassae* subgroup) and five species of the *melanogaster* subgroup. In addition, three drosomycins from the coleopteran species were found in the expressed sequence tag (EST) database (Sackton *et al.*, 2007; Tian *et al.*, 2008). In *D. melanogaster*, besides drosomycin, there are six additional paralogues (named drosomycin-1 to -6), all located within a 56 kb region of the left arm of chromosome 3 and comprising a multigene family (Khush & Lemaitre, 2000; Jiggins & Kim, 2005), in which only drosomycin and drosomycin-2 have been confirmed to have antimicrobial activity (Fehlbaum *et al.*, 1994; Tian *et al.*, 2008).

As a key antifungal component, drosomycin and its paralogues not only provide an ideal molecular model to elucidate the detailed immune response of *Drosophila* against fungi, but also exhibit potent therapeutic potential for developing anti-infective drugs. This review presents the history (Fig. 1) and current knowledge in the field of drosomycin, emphasizing structural and biological features, the functional surface and mode of action as well as evolution.

Sequence and structural features

Drosomycin is a cationic antifungal peptide of 44 residues with four intramolecular disulphide bridges. The amino acid sequence of drosomycin was determined by Edman degradation combined with reduction and alkylation (Fehlbaum *et al.*, 1994) (Fig. 2A). Using the recombinant peptide expressed in the yeast system, Michaut *et al.* assigned the disulphide array of drosomycin (Michaut *et al.*, 1996). Subsequently, its Nuclear Magnetic Resonance (NMR) structure was determined, which shows a typical CSαβ folding composed of one α-helix and a twisted three-stranded β-sheet (Landon *et al.*, 1997) (Fig. 2B,C). The first short β-strand is connected to the C-terminus of the molecule through the disulphide bridge Cys1–Cys8, and the following N-terminal loop is linked to the second β-strand by the disulphide bridge Cys2–Cys5. The third β-strand includes an invariant motif (CXC) that links to the α-helix with another invariant motif (CXXXC) by two disulphide bridges (Cys3–Cys6, Cys4–Cys7). This compact structure confers drosomycin remarkable stability to heating, protease-mediated degradation as well as to pH alterations (Landon *et al.*, 1997; Bulet *et al.*, 1999).
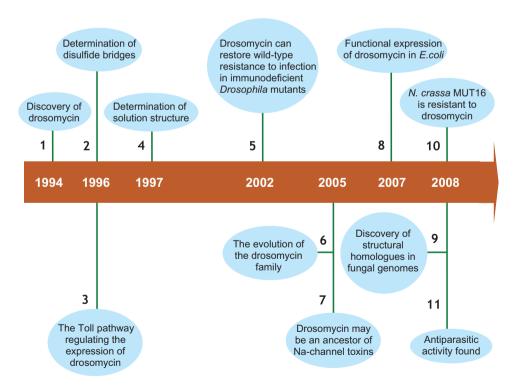

Figure 1. The history of drosomycin. Data sources: 1: Fehlbaum *et al.* (1994); 2: Michaut *et al.* (1996); 3: Lemaitre *et al.* (1996); 4: Landon *et al.* (1997); 5: Tzou *et al.* (2002); 6: Jiggins & Kim (2005); 7: Zhu *et al.* (2005); 8: Yuan *et al.* (2007); 9: Zhu (2008); 10: Gao & Zhu (2008); 11: Tian *et al.* (2008).
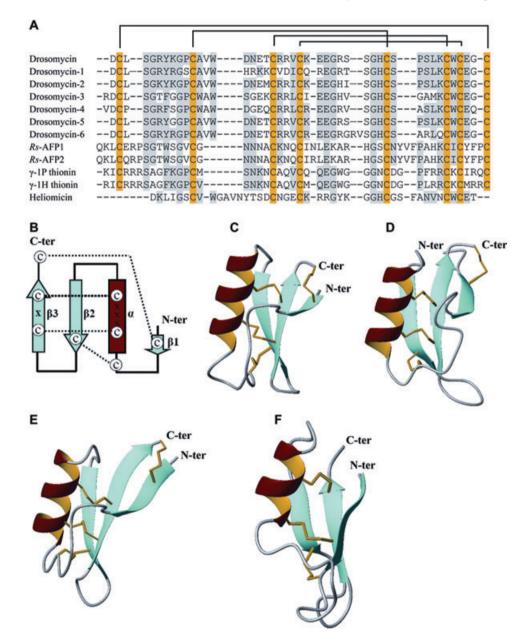
Figure 2. Sequences and structures of drosomycin and related peptides. (A) Multiple sequence alignment of drosomycins from *Drosophila melanogaster* (Jiggins & Kim, 2005), plant defensins *Rs*-AFP1 and *Rs*-AFP2 from the radish *Raphanus sativus*, the γ-1P and 1H thionins from wheat and barley (Fehlbaum *et al.*, 1994; Landon *et al.*, 1997), as well as heliomicin, an insect antifungal defensin from *Heliothis virescens* (Lamberty *et al.*, 1999). Cysteines are highlighted in yellow. Conservation replacements between drosomycins and other defensins are shadowed in grey. (B) The topology of drosomycin. The cylinder and arrow represent α-helix and β-sheet, respectively, and dotted lines indicate positions of disulphide bridges. (C–F) Three-dimensional structures of drosomycin (pdb entry 1MYN) (C), *Rs*-AFP1 (1AYJ) (D), γ-1H thionin (1GPS) (E), and Heliomicin (1I2U) (F).

Sequence and structural comparison reveals that drosomycin has striking similarity to antifungal cysteine-rich plant defensins such as *Rs*-AFP1, *Rs*-AFP2, γ-1P and γ-1H thionins. These plant defensins and drosomycins share a conserved sequence pattern that includes eight cysteines and eight other residues: Ser4, Gly9, Asn16, Glu26, Gly31, Pro35, an aromatic amino acid in position 7 and a basic residue in position 38 (numbered according to drosomycin), most of which are probably involved in protein structure stabilization or in protein folding (Landon *et al.*, 1997; Bulet *et al.*, 1999; Landon *et al.*, 2000) (Fig. 2). Interestingly, heliomicin also shares sequence and structural similarity to drosomycin (Lamberty *et al.*, 1999) (Fig. 2F). A remarkable difference between them is that heliomicin lacks the fourth

Table 1. IC$_{50}$ of drosomycin and drosomycin-2 against fungi and the yeast. Data are derived from the references Fehlbaum *et al.* (1994) and Tian *et al.* (2008)

Microorganism	IC$_{50}$ (μM)	
	Drosomycin	Drosomycin-2
Geotrichum candidum	1.50	4.50
Neurospora crassa	0.42	0.75
Saccharomyces cerevisiae	12.00	5.50
Alternaria brassicola	0.9	–
Alternaria longipes	1.4	–
Ascochyta pisi	3.2	–
Botrytis cinerea	1.2	–
Fusarium culmorum	1.0	–
Fusarium oxysporum	4.2	–
Nectria haematococca	1.8	–

IC$_{50}$, Inhibitory Concentration 50%.

disulphide bridge linking the N- and C-termini compared with drosomycin. We suspect that heliomicin may also be a homologue of drosomycin.

Biological activity

Although bacterial challenge can lead to its up-expression, drosomycin is a strict antifungal peptide with strong potency against filamentous fungi, including *Neurospora crassa*, *Geotrichum candidum*, *Fusarium culmorum* etc. (Fehlbaum *et al.*, 1994; Yuan *et al.*, 2007; Gao & Zhu, 2008; Tian *et al.*, 2008). For most of the fungi tested, the Inhibitory Concentration 50% (IC$_{50}$) values are within the micromolar ranges, suggesting drosomycin's high potency in restricting fungal growth (Table 1). At high concentration, drosomycin inhibits spore germination and no hyphae were observed, whereas at lower concentration it delays growth of hyphae and subsequently leads to abnormal morphology of the fungi (Fehlbaum *et al.*, 1994).

The *in vivo* function of drosomycin in *Drosophila* innate immunity has been evaluated by using mutant flies without drosomycin expression that exhibit a lower survival rate than wild-type flies when challenged with fungi. By contrast, constitutive expression of drosomycin in *imd; spätzle* double mutants can restore a wild-type level of survival against both *N. crassa* and *F. oxysporum*. Enhanced resistance to *F. oxysporum* and *Aspergillus fumigatus* was observed in immunodeficient transgenic *D. melanogaster* carrying two copies of drosomycin genes but not in the lines carrying one copy (Tzou *et al.*, 2002). These experiments highlight the importance of drosomycin in antifungal immune response (Lemaitre *et al.*, 1996; Tzou *et al.*, 2002). Additional evidence comes from the infection assay, in which *Drosophila* larvae show higher survival rates under the wild-type *N. crassa* than *N. crassa* MUT16 (Gao & Zhu, 2008).

Drosomycin-2, a paralogue of drosomycin, is also an antifungal peptide lacking antibacterial activity. This recom-

binant peptide can cause partial lysis of hyphae of *N. crassa* and *G. candidum* and decreases the cell number of *Saccharomyces cerevisiae*. Drosomycin and drosomycin-2 both inhibit the growth of the fungi *N. crassa*, *G. candidum* and the yeast *S. cerevisiae*, but they display differential potency on Drosomycin is threefold more effective with *G. candidum*, whereas drosomycin-2 is twofold more effective in inhibiting the growth of *S. cerevisiae* (Tian *et al.*, 2008).

Recently, Tian *et al.* reported for the first time antiparasitic activity of drosomycin and drosomycin-2 that can inhibit the development of the parasite *Plasmodium berghei* ookinetes with differential potency. The marked antiparasitic activity makes drosomycins good candidates for use in the development of transgenic insects to control related parasite diseases. In addition, the successful expression producing large amounts of recombinant drosomycins makes it possible to develop these peptides as new antiparasitic drugs (Yuan *et al.*, 2007; Gao & Zhu, 2008; Tian *et al.*, 2008).

Regarding the antifungal activity of other drosomycin paralogues, Yang *et al.* reported functional divergence of the six isoforms of *D. melanogaster* Drs-IC, Drs-ID, Drs-IE, Drs-IF, Drs-IG and Drs-II, corresponding to drosomycin-1–6. It is worth mentioning that Tian *et al.* observed the activity of drosomycin-2 against *N. crassa*, whereas Yang *et al.* found that drosomycin-2 has no effect on *N. crassa*. (Yang *et al.*, 2006; Tian *et al.*, 2008). Configurational change in the folding process is a possible reason for the loss of biological activity.

Functional surface

Knowledge regarding the functional surface of drosomycin is little so far because of the lack of an efficient expression system to produce adequate amounts of peptide and its mutants for functional analysis. Given that drosomycin and *Rs*-AFP2, a plant defensin from radish seed, share a similar sequence, structure and antifungal spectrum (Landon *et al.*, 2000), it is reasonable to infer functional sites of drosomycin by a comparison with those of *Rs*-AFP2, which have been obtained by mutational analysis (De Samblanx *et al.*, 1997). In this way, Landon *et al.* proposed that the active site of drosomycin is formed by the hydrophobic cluster Leu3, Pro10, Pro35, Leu37 and Trp40, where the basic residue Lys38 is embedded (Landon *et al.*, 2000) (Fig. 3A). More recently, on the basis of the discovery of the differential antifungal and antiparasitic potency of drosomycin and drosomycin-2, Tian *et al.* carried out an evolutionary tracing analysis with the sequences of the drosomycin family. They identified two evolutionary epitopes (named α-patch and γ-patch) and the m-loop, possibly representing the functional region of drosomycin (Fig. 3B,C). The α-patch is primarily concentrated on the helical region and composed of Tyr7, Thr18, Arg21, Val22, Glu25, whereas the γ-patch region consists of Ser36 and Lys38. Residue Ser29
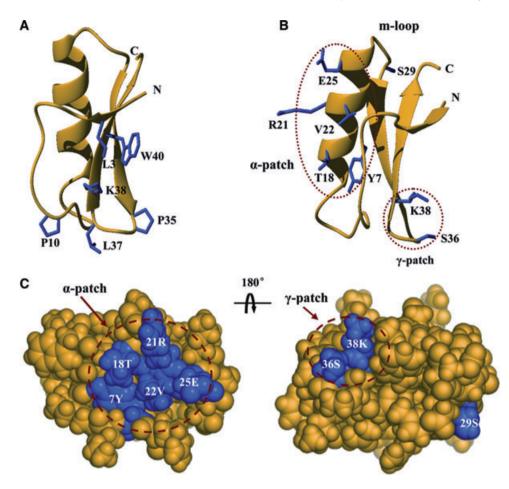
Figure 3. The putative functional sites of drosomycin. (A) Functional sites delineated by comparison with *Rs*-AFP2. (B) Evolutionary tracing residues forming the α- and γ-patch. (C) Mapping of the evolutionary epitopes on the sphere structure of drosomycin.

is alone situated on the m-loop. The α-patch appears to be a functional region involved in interaction with fungi. The γ-patch is located at the γ-core, a crucial functional surface of some CSαβ-type defensins (Yount & Yeaman, 2004). The m-loop is recognized as a putative antiparasitic region containing one Arg and two Gly residues that are located at two termini of the loop. The Gly residues may confer flexible conformation in this region and promote the interaction of drosomycin with the parasite (Tian *et al.*, 2008). It is also worth mentioning that, although the putative functional sites revealed by evolutionary tracing are different in some regions from those proposed by Landon *et al.*, the importance of the γ-core is convergently highlighted by these two different methods (Landon *et al.*, 2000). Further mutational analysis based on the above work will undoubtedly be needed to identify key residues that are involved in these two distinct functions of drosomycin.

Mode of action

Drosomycin can cause hyphae lysis of susceptible fungi such as *B. cinerea* and *N. crassa*, in which cytoplasmic material extrudes along the hyphae (Fehlbaum *et al.*, 1994; Gao & Zhu, 2008). Similarly, drosomycin-2 also causes partial lysis of hyphae of *N. crassa* and *G. candidum* (Tian *et al.*, 2008). However, the detailed binding molecule on the fungal membrane for drosomycin remains to be identified. In this respect, several drosomycin-related proteins provide some clues. It has been found that *Rs*-AFP2 and heliomicin both interact with fungal glucosylceramides (Thevissen *et al.*, 2004), whereas DmAMP1, a plant defensin from the seed of dahlias, binds to fungal mannosyldiinositolphosphoryl-ceramide, an acid complex sphingolipid (Aerts *et al.*, 2008). Interestingly, as mentioned above, drosomycin lacks activity against a specific mutant strain of *N. crassa* (MUT16) obtained by chemical mutagenesis. This is also a common feature observed in several plant defensins (Ferket *et al.*, 2003). In comparison with *N. crassa*, the MUT16 strain displays clear differences in the sphingolipid profile, which has been considered as a factor associated with such resistance towards plant defensins (Ferket *et al.*, 2003). Given that drosomycin and plant defensin are both effective against *N. crassa* and inactive against *N. crassa* MUT16, Gao & Zhu proposed

that these two structurally related molecules may possess a similar mode of action, in which the role of sphingolipid is highlighted (Gao & Zhu, 2008). Supported by these facts, this mutant seems to be a useful model for investigating the mode of action of drosomycin with *N. crassa*. According to the two-step mode of action of plant defensin *Rs*-AFP2 (Thevissen *et al.*, 2003), Tian *et al.* also elucidated a similar mode for drosomycin interacting with fungi, where the α-patch and γ-patch might be involved in binding and subsequent membrane permeability, respectively (Tian *et al.*, 2008).

The expression and regulatory pathways of drosomycin

In *Drosophila*, drosomycin can be expressed systemically in the fat body and locally in a variety of epithelial tissues (Fehlbaum *et al.*, 1994; Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). During the systemic response, drosomycin is rapidly synthesized by the fat body, a functional homologue of the mammalian liver, and secreted into the haemolymph (Fehlbaum *et al.*, 1994), which is controlled by the Toll signal pathway (Lemaitre *et al.*, 1996), a key immune cascade initially recognized in the establishment of the dorsoventral axis during embryo development (Belvin & Anderson, 1996). In general, the Toll pathway is responsible for regulation of antimicrobial peptide genes involved in clearing the infection of Gram-positive bacteria and fungi. In this process, the spätzle protein, a ligand of the Toll receptor, has been characterized as a crucial initiator for the activation of this pathway (Michel *et al.*, 2001). For essential extracellular and the intracellular components of the Toll pathway, see references (Belvin & Anderson, 1996; Hultmark, 2003; Lemaitre, 2004; Naitza & Ligoxygakis, 2004; Lemaitre & Hoffmann, 2007).

Apart from inducible systemic expression in the fat body, the use of a drosomycin-green fluorescent protein reporter gene revealed that a variety of epithelial tissues (eg those of the respiratory, digestive and reproductive tracts) can also express drosomycin, and this expression is independent of the Toll pathway (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). There are two distinct types of local immunity of drosomycin: inducible local expression and constitutive local expression. Inducible local immunity is activated by natural local infection but not by bacterial injection into the haemocoel, which often results in the initiation of systemic immunity. This inducible local expression of drosomycin can be observed in nearly all the epithelial tissues and expression in the tracheae is via the IMD pathway rather than the Toll pathway, whereas constitutive local expression, which is restricted to the salivary glands and the female reproductive organs, is independent of the Toll and IMD pathways (Ferrandon *et al.*, 1998). The *homeobox* gene product Caudal functions as the transcription modulator respon-

sible for constitutive local expression in salivary glands; in addition, the *moleskin* gene, which mediates Caudal nuclear localization is also necessary (Han *et al.*, 2004; Ryu *et al.*, 2004). Interestingly, the Toll and IMD pathways have been found to have a synergistic effect in controlling the expression of drosomycin via the formation of Nuclear Factor-Kappa B (NF-κB) factor heterodimers (Bangham *et al.*, 2006).

Recently, the constitutive expression pattern of seven drosomycin genes in the four developmental stages (eggs, larva, pupa and adult) has also been reported (Tian *et al.*, 2008). It was found that all seven drosomycin genes are shut off in the egg, and drosomycin-1 and -6 can not be detected in any of the four stages. Drosomycin, drosomycin-2, -3, -4 and -5 are expressed in the larva and adult, whereas in the pupa, only drosomycin and drosomycin-2 are detected.

Evolution

As mentioned previously, drosomycin and plant defensins have marked sequence and structural similarity and both lack activity against *N. crassa* MUT16. This mechanical similarity provides new evidence for a homologous relationship between them. Furthermore, the finding of drosomycin-like peptides in some fungal genoms through a bioinformatic approach allows the construction of a phylogenetic tree that highlights the monophyletic origin of drosomycin and drosomycin-like peptides from insects, plants and fungi (Zhu, 2008). However, it can not be excluded that drosomycin in insects could be a consequence of horizontal gene transfer because of its restricted phylogenetic distribution. Isolation and characterization of drosomycin-related genes from non-insect species are needed to reach a decisive conclusion (Gao & Zhu, 2008).

Some immune-related genes involved in direct interaction with pathogens may coevolve with microorganisms, as identified by accelerated substitutions of some antimicrobial peptide genes in vertebrates and termites, which are presumably driven by positive selection (Tennessen, 2005). Although Jiggins & Kim did not detect a positive selection signal in drosomycin by using maximum likelihood methods, Zhu *et al.* identified three positively selected sites, in which one is located in the α-patch and the other one in the m-loop, two putative functional regions of drosomycin predicted by evolutionary tracing (Jiggins & Kim, 2005; Zhu *et al.*, 2005; Tian *et al.*, 2008). The opinion of adaptive evolution of drosomycin is further strengthened by the observation that drosomycin and drosomycin-2 have different potency against different microbes (Tian *et al.*, 2008).

Interestingly, a recent structure-based evolutionary analysis of the CSαβ superfamily revealed a close relationship between drosomycin and scorpion Na-channel toxins (Zhu *et al.*, 2004, Zhu *et al.*, 2005). In particular, BmKITc, a

scorpion depressant toxin, shares about 50% sequence similarity with drosomycin in the region corresponding to the CSαβ motif. Compared with drosomycin, BmKITc extends its C-terminus by 14 residues and has an N-turn insertion, suggesting that drosomycin might be an ancestor of Na-channel toxins (Zhu *et al.*, 2005). By grafting these two structural motifs onto the drosomycin scaffold, Zhu *et al.* generated an engineering toxin-like molecule exhibiting selective potency against tetrodotoxin-resistant (TTX-R) Na$^+$ currents of rat dorsal root ganglion (DRG) cells. This provides novel experimental evidence for their evolutionary link. Recent work presented here also highlights the key importance of drosomycin in innate immunity response of *Drosophila* against diverse pathogens and offers clues for exploring antifungal and antiparasitic drugs by using it as template or scaffold in drug design.

Acknowledgements

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