

Silkworm fungal infection model for identification of virulence genes in pathogenic fungus and screening of novel antifungal drugs

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Summary

The silkworm infection model has the potential to replace conventional animal models for evaluation of the efficacy and toxicity of investigational antifungal agents. Silkworms are relatively inexpensive, can be simply grown in large numbers and can be easily infected with pathogenic fungi, including mutant strains. Antifungal agents can then be injected into the silkworm either *via* the hemolymph to mimic intravenous administration or directly into the gut for oral administration, and their antifungal effect can be evaluated. Common features regarding the mechanisms of pharmacokinetics between the silkworm and mammals result in consistent therapeutic effectiveness of antifungal agents. ASP2397, a promising new antifungal agent, was discovered using the silkworm model. The conclusion is that silkworms can be a more ethical and less expensive alternative to standard animal models, particularly for the identification and testing of new antifungal agents.

Keywords: Silkworm, fungal infection model, antifungal agents, virulence factors, drug development

1. Introduction

Pathogenic fungi can cause serious deep mycosis, such as pneumonia, in humans. Patients with weakened immune functions, such as those suffering from leukemia or acquired immune deficiency syndrome (AIDS), or those under treatment with immunosuppressive therapy, are predisposed to fungal infections. There are four classes of therapeutic agents for deep mycosis: polyenes, azoles, echinocandins, and fluoropyrimidines. There are limitations in the practical application of these agents in the clinic due to their known adverse effects and antifungal activity. Therefore, the development of novel antifungal agents for deep mycosis is desired.

Animal models mimicking infectious diseases in human are used to understand virulence of pathogenic microorganisms and to evaluate therapeutic effects of drug candidates. Mice and rats have been used as fungal

infection models (1). Sacrificing a large number of mammals for infection experiments incurs not only a high cost but can also raise ethical issues with respect to animal welfare. To solve these problems, we are proposing use of the silkworm as an animal model of infectious diseases (2-5). The silkworm is an insect whose breeding method has been well developed during a long history of sericulture. The cost needed to breed silkworms is relatively low, and the use of insects like silkworms avoids ethical concerns that arise when vertebrate animals are used for research. Therefore, we can easily conduct experiments using a large number of silkworms. Moreover, studies using silkworms are relatively straightforward because they move very slowly and their size is larger as compared to other invertebrate animal models such as the fruit fly or nematode. By using syringes, one can inject accurate volumes of samples containing pathogens or candidate agents into the body fluid of silkworms. Furthermore, injection of the samples into the hemolymph or the gut can be distinguished in silkworms. The former corresponds to intravenous injection in humans, and the latter to oral administration.

The silkworm infection model of *Staphylococcus aureus* has already been shown to be useful for

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screening mutant strains with low virulence (3,4). The mutant strains of *S. aureus* that had low killing ability against silkworms also showed low virulence against mice. Therefore, the silkworm infection model may be useful for understanding common mechanisms of bacterial virulence between silkworms and mice, and possibly humans.

The silkworm shares common mechanisms of drug metabolism with mammals. Namely, chemicals incorporated in the silkworm body are modified by hydroxylation of the first phase reactions by cytochrome P450s followed by the second phase reactions of conjugation to highly water-soluble substances to be excreted. We demonstrated the process by using 7-ethoxycoumarin, which is generally used as a model compound to study drug metabolism in mammals (6). The ED₅₀ values, *i.e.*, the amount of reagent needed for therapeutic effects on half the population of animals, which indicates the therapeutic effect of antibiotics, in the infection model of silkworms, were consistent with the values in mammals (7). The LD₅₀ values, *i.e.*, the amount of reagent needed for killing half the population of animals, which indicates toxicity of chemicals, were also consistent between silkworms and mammals (8). Therefore, the silkworm infection model can be used to evaluate both the therapeutic effects and toxicity of candidate chemicals under consideration as anti-infective agents. By using the *S. aureus* infection model of silkworms, we recently discovered a novel antibiotic, lysocin E (9) suggesting the usefulness of silkworms in the discovery of novel antibiotics.

The above-mentioned results suggest that the silkworm may be appropriate for screening genes responsible for virulence and novel therapeutic agents against other pathogenic microorganisms. In this review, we describe recent progress in the study of silkworm infection models for pathogenic fungi.

2. Silkworm fungal infection model

Silkworms die within a few days after injection with *Aspergillus fumigatus*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, or *Cryptococcus neoformans* (7,10-13). Heat treatment (121°C, 15 min) of the fungi eliminates the killing ability of *C. albicans* or *C. neoformans* (12,14). Killing of the silkworms by *C. neoformans* infection is greatly influenced by temperatures. At 37°C, *C. neoformans* grows in the body of silkworms resulting in a killing effect, whereas at 27°C, the fungus does not kill silkworms. The capsule thickness and the overall size of the cell of *C. neoformans* increase in the silkworm hemolymph at 37°C, where pathogenicity of *C. neoformans* is exhibited, whereas apparent morphological changes are not observed at 27°C (12). The capsule has been shown to be necessary for pathogenicity of *C. neoformans* against mammals. Taken together, these findings suggest

that fungal infection models of the silkworm are useful to understand the mechanisms of pathogenicity of fungi against mammals, including humans.

2.1. Identification of fungal genes responsible for pathogenicity to silkworms

To understand the molecular mechanism of fungal virulence, genetic approaches may provide useful information. To achieve this, we have attempted to isolate mutant strains that lack pathogenicity against silkworms. Silkworms are of an appropriate size for injection by syringes with needles, such that accurate volumes of sample can be injected into the hemolymph of silkworms (Figure 1). Pathogenicity of mutants can be demonstrated quantitatively by determination of the ED₅₀, *i.e.*, the number of cells needed to kill 50% of the population of silkworms. Using this information, one can identify genes responsible for the virulence of pathogens. So far, more than 10 mutants of *C. albicans*, *C. glabrata*, or *C. neoformans* were found to have low pathogenicity against silkworms (15).

Calcineurin complex CMP1 (also called CNA1), a serine/threonine protein kinase of *C. albicans*, and protein kinases SIT4 and YVH1 have been reported to be required for virulence against mammalian animals (16-19). Injection experiments of mutants whose genes encode these protein kinases were artificially disrupted and showed reduced pathogenicities compared to the wild strain (10). Pathogenicity against the silkworm of a disrupted strain of the PTC1 gene encoding another protein kinase also was shown to be decreased (10). Pathogenicity of the disrupted mutant of the PTC1 gene identified by using the silkworm model has also

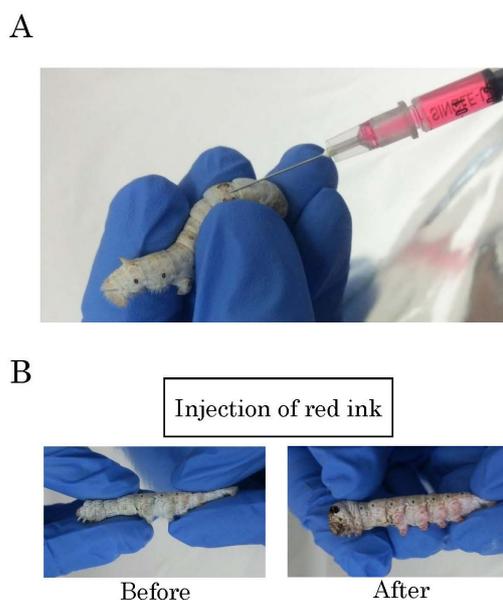


Figure 1. Injection method of liquid into silkworm. (A) Injection of sample into silkworm hemolymph using Syringe. **(B)** Color of silkworm legs (left) change to red (right).

been reported to be decreased in mice (10). The results that genes which are pathogenic against mice are also pathogenic against silkworms suggest that the silkworm model of infection by *C. albicans* is useful for screening virulence factors.

We reported that silkworms ingesting a high glucose diet showed the symptoms corresponding to diabetes in mammals (20,21). We screened *C. glabrata* mutant strains that have low pathogenicity against the diabetic model of silkworms (11). As a result, we found that the *cyb2* gene was needed for pathogenicity against diabetic silkworms (Figure 2). Mutants of the *hap2* and *hap5* genes in which the RNA level of the *cyb2* gene is low also showed low pathogenicity against diabetic silkworms. The *cyb2* gene encodes a protein that has 65% homology with lactate dehydrogenase in *Saccharomyces cerevisiae*, which was found to be an adaptation factor for survival in the intestine. A deficient strain of the *cyb2* gene in infection of the gastrointestinal tract using a diabetic murine model showed decreased adaptation in the mouse cecum (11).

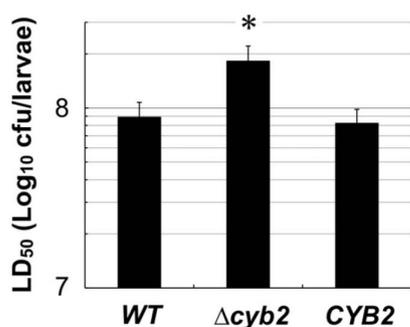


Figure 2. The virulence of *C. glabrata* Δ*cyb2* strain is attenuated in infection model of silkworm. *N* = 10. Asterisks: *p* = 0.05 with Student *t*-test vs. *WT* or *CYB2*. *WT*; wild type strain, Δ*cyb2*: deletant strain, *CYB2*: revertant strain. Ueno K *et al.*, 2011.

These results suggest that the diabetic silkworm/fungal infection model is useful for screening genes needed for virulence of fungi against diabetes patients.

We found that strains deficient in the *can*, *gpa1*, or *pkal* genes, which are required for virulence in *C. neoformans* against mammals, also showed low pathogenicity against silkworms (12). The product of the *can* gene is considered to contribute to the pathogenicity in mammals *via* the calcineurin signaling pathway (22). The product of the *gpa1* gene, an α -subunit of G-protein, was shown to contribute to the capsule formation (23). Pka1 is a protein kinase that functions downstream of Gpa1, and is known to contribute to the capsular formation (24). These results suggest that the fungal infection model of silkworms contributes to understanding the virulence mechanism in *C. neoformans* on a molecular level.

2.2. Evaluation of therapeutic effects of antifungal drugs and screening of novel antifungal drugs using a silkworm fungal infection model

We evaluated the therapeutic effects in silkworms of antifungal agents that currently are used for clinical purposes (7,12). Killing effects of silkworms by *C. albicans* or *C. tropicalis* infection were abolished by the administration of sufficient amount of amphotericin B or fluconazole. The ED₅₀ values were consistent with those in the mouse infection models (Table 1). Injection of amphotericin B, flucytosine, ketoconazole, and fluconazole into the hemolymph showed therapeutic effects against *C. neoformans* infection of the silkworm (Table 2). On the other hand, injection of amphotericin B into the midgut of the silkworm did not show a therapeutic effect, which suggests that it may not be absorbed in the intestinal tract (Table 2). This finding

Table 1. ED₅₀ of antifungal agents in a silkworm-infection model with *C. tropicalis* or *C. albicans*. (Hamamoto H *et al.*, 2004)

Antifungal agents	True fungus	ED ₅₀ in silkworm (μg/g of larva)	MIC (μg/mL)	ED ₅₀ /MIC ratio in	
				Silkworm	Mouse
Amphotericin B	<i>C. tropicalis</i>	1.8	3.2	0.6	0.2
	<i>C. albicans</i>	4.1	1.6	2.6	1.3
Fluconazole	<i>C. tropicalis</i>	1.8	1.6	1.1	7.4
	<i>C. albicans</i>	1.8	0.4	4.5	8.6

Table 2. Therapeutic effects of antifungal agents on silkworm infection by *C. neoformans*. (Matsumoto Y *et al.*, 2012)

Antifungal agents	MIC (μg/mL ⁻¹)	ED ₅₀ (μg of antifungal agent g ⁻¹ of larva) of drug administrated by i.h. or i.m.	
		i.h.	i.m.
Amphotericin B	4 ± 2	14 ± 10	> 250
Flucytosine	21 ± 7	6 ± 1	9 ± 7
Fluconazole	7 ± 6	2 ± 1	9 ± 3
Ketoconazole	0.1 ± 0.1	19 ± 2	14 ± 10
Micafungin	> 100	> 125	N.D.

i.h., intra hemolymph. i.m., intra midgut. N.D., not determined.

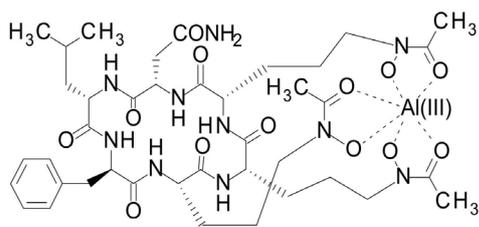


Figure 3. Structure of ASP2397. Nakamura I *et al.*, 2016.

is in keeping with the fact that Amphotericin B is not absorbed in the intestinal tract in mammals, and therefore does not show a therapeutic effect when administered orally. We previously reported common features between silkworms and mammals regarding absorption capacity of various chemicals in the intestine (25). We therefore propose that the silkworm fungal infection model may be useful as an alternative method to assess the intestinal absorption of antifungal drugs.

2.3. Discovery of a novel therapeutic agent against *Aspergillus fumigatus* infection using the silkworm fungal infection model

We recently reported the discovery of a novel therapeutic agent using an *Aspergillus fumigatus* infection model of silkworms (13). *A. fumigatus* killed silkworms 2 days after injection. Amphotericin B and voriconazole showed therapeutic effects in the model. Screening of natural products derived from fungal species allowed us to identify ASP2397 (Figure 3), which showed a therapeutic effect against *A. fumigatus* infection in silkworms. This compound was also therapeutically effective in a mouse infection model of *A. fumigatus*. Initially, *in vitro* antifungal activity was used as an indicator for purification of compounds from a crude extract of a culture supernatant of the fungi that produced the therapeutically effective antifungal. However, a purified fraction which exhibited antifungal activity did not show a therapeutic effect in the silkworm infection model. Therefore, we conducted further purification by monitoring the therapeutic effect in the silkworm model instead of antifungal activity in test tubes. Eventually, ASP2397 was purified and identified as a therapeutically effective compound. These results suggest that therapeutically effective antifungal drugs can be purified by monitoring the therapeutic effect in the silkworm infection model. ASP2397 is expected to demonstrate safety and effectiveness in nonclinical studies and human clinical trials.

3. Conclusion

The silkworm infection model is a promising new approach for the identification of new antifungal agents. This model was used to identify the promising new antifungal agent, ASP2397.

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