Review

A Silkworm Infection Model to Evaluate Antifungal Drugs for Cryptococcosis

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ABSTRACT

The development of effective drugs against fungal diseases involves performing infection experiments in animals to evaluate candidate therapeutic compounds. Cryptococcus neoformans is a pathogenic fungus that causes deep mycosis, resulting in respiratory illness and meningitis. Here we describe a silkworm system established to evaluate the safety and efficacy of therapeutic drugs against infection by Cryptococcus neoformans and the advantages of this system over other animal models. The silkworm assay system has two major advantages: 1) silkworms are less expensive to rear and their use is less problematic than that of mammals in terms of animal welfare, and 2) in vivo screenings for identifying candidate drugs can be easily performed using a large number of silkworms. The pharmacokinetics of compounds are consistent between silkworms and mammals. Moreover, the ED₅₀ values of antibiotics are concordant between mammalian and silkworm infection models. Furthermore, the body size of silkworms makes them easy to handle in experimental procedures compared with other invertebrate infectious experimental systems, and accurate amounts of pathogens and chemicals can be injected fairly easily. These advantages of silkworms as a host animal make them useful for screening candidate drugs for cryptococcosis.

Key words: antifungal drugs, Cryptococcus neoformans, invertebrate animals, silkworm

Introduction

Cryptococcus neoformans is a major human fungal pathogen worldwide¹−³. The organism is frequently isolated in immunocompromised patients and is one of the most common causes of death in AIDS patients, especially in sub-Saharan Africa⁴. Basic research using animal models is essential to elucidate the pathology of C. neoformans infection and to establish treatment and prevention methods against this infection. Various mammalian C. neoformans infection models have been proposed⁵−⁸. The use of mammalian animals to screen for therapeutic drugs, however, is problematic not only in terms of the large numbers of animals required and the cost of rearing, but also due to ethical considerations. To overcome these problems, infection models for C. neoformans using invertebrates such as Drosophila melanogaster, Caenorhabditis elegans, Galleria mellonella, and Bombyx mori have been proposed⁹−ⁱ⁰. The use of invertebrate animals for drug screening experiments is highly advantageous compared with mammals for the following reasons: (1) lower associated costs; (2) smaller space requirements; (3) fewer ethical problems associated with their death; and (4) smaller amounts of the sample can be used because of the small size of the animals (Table 1). In this
Table 1. Comparison of *in vivo* infection models of *Cryptococcus neoformans*

| Model animals | Cost for rearing | Space for rearing | Application to the ethics committee | Escape ability to require biosafety | Time required to die after injection | Temperature after infection | Quantitative injection of samples by a syringe | Reported route of administration | Individual weight | References |
|---------------|------------------|-------------------|------------------------------------|-----------------------------------|-----------------------------------|-----------------------------|---------------------------------|--------------------------------|------------------|-----------|
| Silkworm      | Low              | Small             | Not necessary                      | Low                               | 2-3 days                         | 37°C                        | Easy                           | Intra-hemolymph, intra-gut injections | 1-2 g            | 10)       |
| Fruit fly     | Low              | Small             | Not necessary                      | High                              | 3-4 days                         | 25°C                        | Difficult                      | Intra-hemolymph injection, oral administration | 0.5-2 mg         | 8)        |
| Nematode      | Low              | Small             | Not necessary                      | Low                               | 2-25 days                        | 25°C                        | Difficult                      | Oral administration             | 1 µg             | 7)        |
| Larvae        | Low              | Small             | Not necessary                      | Low                               | 4-20 days                        | 30°C, 37°C                   | Easy                           | Intra-hemolymph injection         | 250 mg           | 9)        |
| Mice          | High             | Large             | Necessary                          | High                              | 6-40 days                        | 37°C (body temperature)      | Easy                           | Intratracheal, intravenous, intraperitoneal injection | 15-40 g          | 6, 30, 43 |

Table was taken with permission from Ishii *et al.* and partly modified.
paper, we describe the usefulness of invertebrate infection models for *C. neoformans*, especially focusing on silkworms.

**Establishment of a silkworm *C. neoformans* infection model**

Silkworms are domesticated animals with well-established rearing methods due to the long history of sericulture. The silkworm body has a moderate size that is easy to handle in experimental procedures, such as for injecting an accurate amount of bacterial sample or drug solution (Fig. 1). We have proposed silkworm models for studying various diseases, such as infectious diseases, diabetes, and chemical-induced tissue injury. We have demonstrated that silkworm disease models are useful for screening candidate therapeutic drugs. In particular, various silkworm infection models are effective for searching for virulence factors of pathogens and therapeutic agents against infectious diseases. Establishing a silkworm infection model for *C. neoformans* will contribute to the development of therapeutically effective antifungal drugs for cryptococcosis in humans. Silkworms can survive for at least 3 days at 37°C, the typical mammalian body temperature. Silkworms died when the *C. neoformans* H99 strain was injected into the hemolymph followed by incubation at 37°C (Fig. 2). On the other hand, injecting silkworms with the heat-killed *C. neoformans* H99 strain did not kill them (Fig. 2). These findings suggest that cells of the *C. neoformans* H99 strain must be viable to kill the silkworms. Pathogenicity is evaluated quantitatively by determining the LD₅₀ value, which is the amount of a pathogen necessary to kill 50% of the silkworm population. A low LD₅₀ value indicates high pathogenicity of the pathogen. The LD₅₀ values of serotype A strains (H99, KN99α, and KN99α), which have high pathogenicity in mammals, were lower in silkworms than those of serotype D strains (KN3501α, KN3501α, and B4500; Table 2). Therefore, one can distinguish less pathogenic strains of *C. neoformans* from highly pathogenic strains based on their effects in silkworms. Furthermore, strains deficient in the *gpa1*, *pka1*, and *cna1* genes, which are reported to be necessary for the pathogenicity of *C. neoformans* against mammals, also exhibited higher LD₅₀ values in the silkworm model than the parent strain. Silkworm infection models of other fungi such as *Candida albicans*, *Candida glabrata*, and *Candida tropicalis* have also been established. The virulence genes of the fungi were identified
using silkworm fungal infection models\textsuperscript{15, 20}. Together, these findings indicate that silkworm infection models are useful for clarifying the pathogenicity of fungi in mammals.

Quantitative evaluation of antifungal drugs using silkworms

The ED\textsubscript{50} values of antibiotics against bacterial infection in silkworm infection models were consistent with those in mammals\textsuperscript{21}. In addition, the LD\textsubscript{50} values of toxic substances were similar between mammals and silkworms\textsuperscript{22}. Therefore, the therapeutic efficacy and toxicity of various compounds can be quantitatively evaluated using silkworms. The value obtained by dividing the ED\textsubscript{50} value by the minimum inhibitory concentration (ED\textsubscript{50} / MIC) is an indicator of the in vivo pharmacodynamics\textsuperscript{23}. The ED\textsubscript{50} / MIC values of antibiotics that are clinically effective in human patients were lower than 10\textsuperscript{23}. Therefore, the ED\textsubscript{50} / MIC values obtained from silkworm infection models appear to be useful indexes for evaluating the pharmacodynamics of antibiotics.

Amphotericin B, flucytosine, fluconazole, and ketoconazole exhibited therapeutic effects upon injection into the silkworm hemolymph (intra-hemolymph injection) in a silkworm infection model for \textit{C. neoformans} (Table 3). The ED\textsubscript{50} / MIC values of amphotericin B, flucytosine, and fluconazole were less than 10. On the other hand, the ED\textsubscript{50} / MIC value of ketoconazole, an external medicine used in humans, was 190 in the silkworm system (Table 3). These results suggest that the silkworm infection model for \textit{C. neoformans} is useful for evaluating the pharmacokinetics of antifungal drugs. Moreover, the difference between ED\textsubscript{50} and LD\textsubscript{50} values determined using silkworms allows us to predict the effective safe dose of antifungal drugs.

Intra-midgut injection, which corresponds to oral administration in humans, can be performed in silkworms. Amphotericin B, which is not absorbed from the intestinal tract in mammals and has no therapeutic effect by oral administration, showed no therapeutic effects following intra-midgut injection into silkworms (Table 4). This result indicates that amphotericin B is not absorbed from the intestinal tract in silkworms, and suggests that the efficacies of orally adminis-

### Table 2. LD\textsubscript{50} of \textit{Cryptococcus neoformans} strains for silkworm

| Strains     | LD\textsubscript{50} (× 10\textsuperscript{6} CFU per larva) | P-value |
|-------------|---------------------------------------------------------------|---------|
| H99         | 6 ± 3                                                          |         |
| KN99a       | 7 ± 1                                                          |         |
| KN99α       | 8 ± 1                                                          |         |
| KN3501a     | > 56                                                          | < 0.0001(vs H99) |
| KN3501α     | > 57                                                          | < 0.0001(vs H99) |
| B4500       | > 72                                                          | < 0.0001(vs H99) |

Table was taken with permission from Matsumoto et al.\textsuperscript{10}.

### Table 3. ED\textsubscript{50}, MIC, ED\textsubscript{50} per MIC, LD\textsubscript{50} and ED\textsubscript{50} per LD\textsubscript{50} of antifungal drugs in silkworm infection model for \textit{Cryptococcus neoformans}

| Antifungal agents | ED\textsubscript{50} (μg g\textsuperscript{-1} of larva) | MIC (μg ml\textsuperscript{-1}) | ED\textsubscript{50} per MIC ratio | LD\textsubscript{50} (mg g\textsuperscript{-1} of larva) | ED\textsubscript{50} per LD\textsubscript{50} ratio |
|-------------------|----------------------------------------------------------|---------------------------------|-----------------------------------|--------------------------------------------------------|---------------------------------|
| Amphotericin B    | 14 ± 10                                                   | 4 ± 2                           | 3.5                               | > 250                                                   | < 0.056                         |
| Flucytosine       | 6 ± 1                                                     | 21 ± 7                          | 0.3                               | 145                                                     | 0.041                           |
| Fluconazole       | 2 ± 1                                                     | 7 ± 6                           | 0.3                               | > 250                                                   | < 0.008                         |
| Ketoconazole      | 19 ± 2                                                    | 0.1 ± 0.1                       | 190                               | > 250                                                   | < 0.076                         |
| Micafungin        | > 125                                                     | > 100                           | > 125                             |                                                        |                                 |

Table was taken with permission from Matsumoto et al.\textsuperscript{10}.
tered antifungal drugs can be predicted using the silkworm infection model for \textit{C. neoformans}.

**Other invertebrate models of \textit{C. neoformans} infection**

\textit{G. mellonella} is a large moth that belongs to Lepidoptera like silkworms. \textit{G. mellonella} has been proposed as an infection model of fungi, including \textit{C. neoformans}\textsuperscript{24−27}. \textit{G. mellonella} can be used to perform infection experiments at 37°C, and therapeutic effects of antifungal drugs were evaluated using the \textit{G. mellonella} infection model for \textit{C. neoformans}\textsuperscript{9, 28}. Because of its large body size, \textit{G. mellonella} can be easily injected with a large volume of sample solution into its hemolymph, similar to silkworms\textsuperscript{29}. Moreover, novel virulence factors of \textit{C. neoformans} have been screened using the \textit{G. mellonella} model\textsuperscript{30}.

\textit{D. melanogaster}, a fruit fly, is widely used as an experimental animal\textsuperscript{31}. An advantage of \textit{D. melanogaster} is that it can be manipulated using various genetic approaches\textsuperscript{32}. The host immune system related to \textit{C. neoformans} infection has been elucidated using mutant libraries of \textit{D. melanogaster}. For example, mutants of Imd and Toll pathways, which act on signal pathways for innate immunity, were analyzed in the \textit{C. neoformans} infection model\textsuperscript{8}. A Toll pathway mutant was susceptible to \textit{C. neoformans} infection, whereas an Imd pathway mutant was not, indicating that the Toll pathway plays a role in innate immunity against \textit{C. neoformans}\textsuperscript{8}. On the other hand, there are some disadvantages to using \textit{D. melanogaster} for evaluation of drug efficacy. The body size of adult flies, which are generally used in infection experiments, is too small (2 - 3 mm) to determine accurate LD\textsubscript{50} and ED\textsubscript{50} values in injection experiments. \textit{C. elegans} is also used as an invertebrate animal model to perform genetic studies of infectious diseases\textsuperscript{33}. Genes related to immune responses against \textit{C. neoformans} infection were identified using \textit{C. elegans} mutants\textsuperscript{34−36}. The capsule, which is necessary for the pathogenicity of \textit{C. neoformans} against mammals, is also needed for the pathogenicity in \textit{C. elegans}\textsuperscript{37}. \textit{C. elegans} has been used to identify virulence factors of \textit{C. neoformans}\textsuperscript{39−36}. Moreover, a \textit{C. neoformans} infection model for \textit{C. elegans} was used to evaluate the therapeutic effects of antifungal reagents\textsuperscript{37, 38}. Like \textit{D. melanogaster}, the body size of \textit{C. elegans} is too small to inject accurate volumes of solutions of pathogens and reagents.

Silkworms have several advantages as animal infection models over \textit{D. melanogaster} and \textit{C. elegans}: (1) silkworms have a larger body size and move more slowly, making it easier to inject accurate volumes of pathogen suspensions and drug solutions; (2) infection experiments at 37°C, the human body temperature, are possible; and (3) samples can be injected through two routes: hemolymph and intestinal tract (Table 1). On the other hand, the availability of genetic tools for manipulation is a major advantage for \textit{D. melanogaster} and \textit{C. elegans} over silkworms. Recently, genetic manipulation techniques, such as the establishment of transgenic animals, have been developed for silkworms\textsuperscript{39−41}. Utilizing these genetic techniques in silkworms will help shed light on host immunity against \textit{C. neoformans} infection.

### Table 4. Intra-midgut administration of amphotericin B does not have therapeutic effects in a silkworm model

| Antifungal agents | i.h. (μg of antifungal agent g\textsuperscript{-1} of larva) of drug administrated by the following route |
|-------------------|---------------------------------------------------|
| Amphotericin B    | 14 ± 10 > 250                                     |
| Flucytosine       | 6 ± 1 9 ± 7                                       |
| Fluconazole       | 2 ± 1 9 ± 3                                       |
| Ketoconazole      | 19 ± 2 14 ± 10                                    |
| Micafungin        | > 125                                             |

Table was taken with permission from Matsumoto \textit{et al.}\textsuperscript{10}. 


Conclusion

A silkworm infection model for *C. neoformans* is useful for evaluating the therapeutic effects of antifungal drugs. Using silkworms for these experiments partially addresses the high cost and animal welfare issues associated with the use of mammals, such as mice and rats. These advantages of silkworms permit *in vivo* screening for identifying candidate antifungal drugs against cryptococcosis.

Conflict of interest

None.

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