A silkworm–baculovirus model for assessing the therapeutic effects of antiviral compounds: characterization and application to the isolation of antivirals from traditional medicines

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Ganciclovir, foscarnet, vidarabine and ribavirin, which are used to treat viral infections in humans, inhibited the proliferation of a baculovirus (Bombyx mori nucleopolyhedrovirus) in BmN4 cells, a cultured silkworm cell line. These antiviral agents inhibited the proliferation of baculovirus in silkworm body fluid and had therapeutic effects. Using the silkworm infection model, the antiviral activity of Kampo medicines was screened and it was found that cinnamon bark, a component of the traditional Japanese medicine Mao-to, had a therapeutic effect. Based on the therapeutic activity, the antiviral substance was purified. Nuclear magnetic resonance analysis of the purified fraction revealed that the antiviral activity was due to cinnzeylanine, which has previously been isolated from Cinnamomum zeylanicum. Cinnzeylanine inhibits the proliferation of herpes simplex virus type 1 in Vero cells. These results suggest that the silkworm–baculovirus infection model is useful for screening antiviral agents that are effective for treating humans infected with DNA viruses.

INTRODUCTION

To develop antiviral agents, compounds that show inhibitory effects on the proliferation of the target virus in cultured mammalian cells are screened in libraries of synthetic compounds and chemicals obtained from natural resources. The therapeutic effects of candidate substances are then examined by using mammalian models. Most compounds selected from in vitro-cultured cell systems have no therapeutic effects, because of their pharmacodynamics in host animals. Therefore, a large number of mammalian animals must be killed to collect data in the preclinical stages, which is costly and leads to ethical issues (EU, 1986; Orlans et al., 1998). To overcome these problems, the use of invertebrate animals for evaluating the therapeutic effects of antiviral agents in the early stages of screening has been proposed.

We reported previously that silkworms are a useful animal model of infection from bacteria pathogenic to humans (Hamamoto et al., 2004; Kaito et al., 2002). Silkworms can be cultivated by using artificial food at any time of the year. Furthermore, a large number of silkworms can be produced at low cost. We demonstrated that antibiotics used clinically to treat infected humans are effective in silkworms, and that the ED50 values of the antibiotics used in the silkworm infection model are consistent with those obtained in mammalian animal models (Hamamoto et al., 2004, 2005; Hamamoto & Sekimizu, 2005; Kaito et al., 2002, 2005). Silkworms have enzymes, P450s and conjugating enzymes (Hamamoto et al., 2005; Li et al., 2005; Luque et al., 2002) that are involved in metabolizing
antibiotics. Silkworms are large enough for antiviral agents to be injected into their midgut and haemolymph by using syringes.

Baculoviruses infect silkworms (Kool et al., 1995; Rohrmann, 1994; Szewczyk et al., 2006). Among the baculoviruses, *Bombyx mori* nucleopolyhedrovirus (BmNPV) is used as a vector for the overproduction of recombinant proteins. Proliferation of *Autographa californica* nucleopolyhedrovirus (AcNPV) in cultured cells is inhibited by ganciclovir, an antiviral agent used clinically to treat infected humans (Ansari & Emery, 1999; Safronetz et al., 2003). The amino acid sequence of BmNPV DNA polymerase has a high similarity (96%) to that of AcNPV. BmNPV DNA polymerase also has a high similarity, especially with regard to functional domains, to the herpesvirus and cytomegalovirus DNA polymerases. Therefore, it is expected that ganciclovir would inhibit the DNA polymerases of all of these viruses.

In this paper, we describe methods for evaluating, in the silkworm infection model, the therapeutic effects of antiviral agents used clinically to treat human patients. We also demonstrate the purification of antiviral agents in Kampo medicines by using the silkworm infection model.

**METHODS**

**Animals and reagents.** Silkworm eggs (*Hu-Yo × Tsukuba-Ne*) were purchased from Ehime Sansyu and raised until the fourth-instar larval stage, using artificial food (Silkmate 2S; Nosan Corporation). Silkworm larvae were reared as described previously (Hamamoto et al., 2004; Hamamoto & Sekimizu, 2005). Reagent-grade powders of ganciclovir and vidarabine were kindly provided by Tanabe Seiyaku Co. Ltd. and Mochida Pharmaceutical Co. Ltd., respectively. Ribavirin and fosfocarnet were purchased from Duchefa and Alfa Aesar, respectively. Ribavirin and foscarnet were purchased from Duchefa and Alfa Aesar, respectively. Ribavirin was dissolved in 0.9% NaCl, and foscarnet was purchased from Duchefa and Alfa Aesar, respectively.

**Evaluation of therapeutic effects of antiviral agents used clinically to treat infected humans and of plant extracts from Kampo medicines in silkworms infected with baculovirus.**

Artificial diets were fed to fifth-instar silkworm larvae on the first day for 1 day, and baculovirus (BmNPV FP #128; Katsuma et al., 1999) solution containing $1.6 \times 10^4$ virions in 0.05 ml was injected into the haemolymph by using a disposable syringe (Terumo) with a 27 G needle. Silkworms were raised until the fourth-instar larval stage, using artificial food (Silkmate 2S; Nosan Corporation). Silkworm larvae were reared as described previously (Hamamoto et al., 2004; Hamamoto & Sekimizu, 2005). Reagent-grade powders of ganciclovir and vidarabine were kindly provided by Tanabe Seiyaku Co. Ltd. and Mochida Pharmaceutical Co. Ltd., respectively. Ribavirin and fosfocarnet were purchased from Duchefa and Alfa Aesar, respectively. Unless otherwise stated, all other reagents were reagent-grade commercial products.

Vidarabine and ganciclovir were dissolved in 0.9% NaCl containing 20% DMSO. Foscarnet and ribavirin were dissolved in 0.9% NaCl. Each sample (0.05 ml) was injected into the haemolymph of silkworms. Three days after injection, the silkworm haemolymph was harvested and the number of surviving silkworms injected with test samples was counted and the ED50 values were calculated.

Vidarabine and ganciclovir were dissolved in 0.9% NaCl containing 20% DMSO. Foscarnet and ribavirin were dissolved in 0.9% NaCl. Each sample (0.05 ml) was injected into the haemolymph of silkworms. Three days after injection, the silkworm haemolymph was harvested and the number of surviving silkworms injected with test samples was counted and the ED50 values were calculated.

**Table 1. ED50 and IC50 values of antiviral drugs**

ED50 values were calculated from the results shown in Fig. 1 as the amount of drug required for 50% survival of silkworms infected with BmNPV. IC50 values were determined by inhibition of BmNPV proliferation in BmN4 cells. Mean values from two independent experiments are shown.

<table>
<thead>
<tr>
<th>Antiviral drug</th>
<th>ED50* [μg (g larva)^{-1}]</th>
<th>IC50 † [μg ml^{-1}]</th>
<th>ED50:IC50 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir</td>
<td>31</td>
<td>32</td>
<td>1.0</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>84</td>
<td>105</td>
<td>0.8</td>
</tr>
<tr>
<td>Vidarabine</td>
<td>290</td>
<td>63</td>
<td>4.6</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>10</td>
<td>11</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Measured in silkworms.
†Measured in BmN4 cultured cells.
detector, were pooled and concentrated, followed by measurement of the therapeutic effects in the silkworm infection model. Active fractions, which were eluted at 15.8 min, were analysed by $^1$H nuclear magnetic resonance ($^1$H-NMR) and $^{13}$C-NMR at 500 and 125 MHz, respectively.

**Assay for anti-herpes simplex virus type 1 (HSV-1) activity of cinnzeylanine.** Anti-HSV-1 activity was measured by plaque-reduction assay (Tanaka et al., 2004). Vero cells (Tanaka et al., 2003) were grown to the confluent stage in 24-well titre plates, in Dulbecco’s modified Eagle medium (Nacalai) supplemented with heat-treated fetal bovine serum (1%). Medium was changed to 0.25 ml 199 medium (Sigma) supplemented with 1% heat-treated fetal bovine serum. HSV-1 F strain (100 p.f.u.) (Ejercito et al., 1968; Kawaguchi et al., 2003) was adsorbed to the cells for 1 h, followed by incubation with 200 ml 199 medium containing 50 mM HEPES/KOH (pH 7.5), test sample and antibody against HSV-1 for 45 h. Cells were fixed with methanol and stained with crystal violet, followed by counting of plaques under a microscope.

**Calculation of ED$_{50}$ and IC$_{50}$ values.** ED$_{50}$ values were calculated by probit analysis. IC$_{50}$ values were determined graphically.

**RESULTS**

**Therapeutic effects of antiviral agents against the killing effect of baculovirus in silkworms**

Ribavirin, ganciclovir, foscarnet and vidarabine are used clinically to treat virus-infected human patients. These antiviral agents inhibited the proliferation of baculovirus in BmN4 cells, which are derived from silkworms. IC$_{50}$ values were determined as the concentration of these reagents needed to produce 50% inhibition of plaque formation (Table 1). We determined whether these antiviral compounds have therapeutic effects in silkworms infected with baculovirus. When 1.6 x 10$^4$ p.f.u. baculovirus was injected into the haemolymph of silkworms, all of the animals died within 5 days (Fig. 1a). Each of the antiviral agents produced a dose-dependent increase in the number of surviving silkworms (Fig. 1b). We calculated the ED$_{50}$ values, i.e. the amount of the agents required to produce a silkworm survival rate of 50% (Table 1). The ED$_{50}$ values obtained were consistent with the amount of the antiviral agents used for clinical administration in humans. Silkworms were still alive after injection of >500 µg of these drugs, indicating that the toxicity of these drugs in silkworms was negligible.

Next, we examined whether these antiviral agents inhibited the proliferation of baculovirus in the body fluid of silkworms. We harvested haemolymph from silkworms and determined the number of viruses by using a plaque-forming assay, and found that the increase in the number of viruses in the haemolymph was inhibited by the antiviral agents (Fig. 1c). These results indicate that the therapeutic effects of these antiviral agents can be explained by the inhibition of viral proliferation in the silkworm body. The results also suggest that silkworms infected with...
baculovirus are a useful model for evaluating the therapeutic effects of antiviral agents.

**Purification of antiviral agent in Kampo medicine based on the therapeutic effects determined by using the silkworm infection model**

We speculated that this silkworm–baculovirus infection model would be useful for evaluating new antiviral drugs. Thus, we screened for antiviral agents in natural sources by using this model. We examined the therapeutic effects of Mao-to, Kakkon-to and Shosaiko-to. Mao-to showed positive results (Fig. 2a).

Mao-to is composed of four plant-derived medicines: ephedra herb, apricot kernel, licorice root and cinnamon bark. We examined the therapeutic effects of the hot-water extracts of each crude drug in the silkworm–baculovirus infection model, and found that cinnamon bark had therapeutic activity. We purified the antiviral agent in cinnamon bark further by using the therapeutic assay. The antiviral component was extracted efficiently with chloroform. The chloroform extract was purified further with chromatography using Silicagel C200, Sephadex LH20 and reversed-phase HPLC with υBondasphere (Table 2). The final purification using HPLC with υBondasphere produced a single peak (Fig. 2b), indicating that the fraction was highly homogeneous. The fraction inhibited the proliferation of BmNPV in BmN cultured cells (Fig. 2e). The ED₅₀ value of the fraction was 1 μg per larva, which was 190-fold lower than that of the chloroform extract. The ED₅₀ value was 3% of that of ganciclovir [31 μg (g
larva)−1] in the silkworm–baculovirus infection model (Fig. 2c).

The purified fraction was analysed by 1H- and 13C-NMR, and the structure of the compound was determined (Fig. 2d). This compound was identified as cinnzeylanine, which was detected previously in the bark of Cinnamomum zeylanicum (Isogai et al., 1977) and Cinnamomum cassia (Yagi et al., 1980). The antiviral activity of cinnzeylanine has not been reported previously. Our results are, to our knowledge, the first to demonstrate that cinnzeylanine has a therapeutic effect against baculovirus infection in silkworms.

**Cinnzeylanine inhibition of HSV-1 proliferation in Vero cells**

We next examined whether cinnzeylanine inhibited the proliferation of HSV-1, an infectious virus in humans. The effects of various concentrations of cinnzeylanine on the proliferation of HSV-1 in Vero cells, a cell line derived from monkey, were tested by using a plaque-reduction assay (Fig. 3). Cinnzeylanine reduced the number of HSV-1 plaques. The cinnzeylanine concentration that decreased the plaque number by 50% (IC50) was 230 μg ml−1. Vero cells remained attached to the culture dish even in the presence of 320 μg cinnzeylanine ml−1, which abolished HSV-1-induced plaque formation completely.

**DISCUSSION**

**Evaluation of therapeutic effects of antiviral agents in the silkworm–baculovirus infection model**

The present study demonstrated that antiviral agents that are used clinically for treating humans are effective in the silkworm–baculovirus infection model. These antiviral agents, which target DNA polymerase, inhibited the proliferation of baculovirus in cultured cells (Table 1). We consider that the mechanism of action of these antiviral agents is inhibition of the increase in the number of viral particles in infected cells (Fig. 1c). Inhibition of the proliferation of baculovirus by ganciclovir, vidarabine and foscarnet is thought to be due to the inhibition of viral DNA replication. The mechanism of ribavirin for inhibiting baculovirus proliferation is unknown, although ribavirin reportedly acts as a mutagen (Chevaliez et al., 2007; Crotty et al., 2000; Graci et al., 2007) and as an inhibitor of IMP dehydrogenase (Lowe et al., 1977; Parker, 2005). Ribavirin might inhibit the proliferation of baculovirus by these mechanisms.

We suggest that the silkworm–baculovirus infection model is useful for evaluating the therapeutic effects of antiviral agents against DNA viruses. To evaluate the therapeutic effects of compounds against RNA virus infection, including severe acute respiratory syndrome coronavirus and human influenza viruses, further studies of a corresponding RNA virus that infects silkworms are needed. Cytoplasmic polyhedron virus, which is classified as a member of the family Reoviridae and contains RNA-dependent RNA polymerase (Hagiwara & Matsumoto, 2000), might be a candidate model virus for reovirus and severe acute respiratory syndrome coronavirus.

Baculovirus does not have a gene encoding a nucleotide kinase that phosphorylates ganciclovir. Therefore, ganciclovir might be phosphorylated by a silkworm cellular kinase, resulting in the inhibition of viral DNA replication, although it is possible that a protein kinase induced by

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (units)</th>
<th>Recovery (%)</th>
<th>Weight (mg)</th>
<th>ED50 μg (g larva)−1</th>
<th>ED50 μg (g larva)−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>I CHCl3 extract</td>
<td>32 000</td>
<td>100</td>
<td>12 000</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>II C200 column chromatography</td>
<td>11 000</td>
<td>35</td>
<td>660</td>
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<tr>
<td>III LH20 column chromatography</td>
<td>3 400</td>
<td>12</td>
<td>34</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IV μBondasphere column chromatography</td>
<td>1 000</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3.** Inhibitory effect of cinnzeylanine on HSV-1 proliferation in Vero cells.

**Table 2.** Summary of the purification of the component of cinnamon bark with antiviral activity

Therapeutic activity was determined by using the silkworm infection model. One unit indicates the amount required for the survival of 50% of silkworms infected with BmNPV. ED50 values were calculated based on the assumption that the mass of the silkworm body was 2 g.
baculovirus infection phosphorylates ganciclovir. This would explain why a higher concentration of ganciclovir was needed to inhibit baculovirus proliferation in BmN cells than to inhibit HSV-1 proliferation in Vero cells. Godeau et al. (1992) reported that baculovirus expressing the thymidine kinase of HSV-1 was highly sensitive to ganciclovir, which supports this hypothesis.

Historically, screening of antiviral substances has involved inhibition of virus proliferation in cultured cells induced by candidate compounds. A problem with this method is that most of the candidate compounds that have antiviral activity in vitro are not effective for virus proliferation in host animals, due to their pharmacodynamic characteristics in the host animals. The pharmacodynamics of compounds in animal bodies are governed by adsorption, distribution, metabolism and excretion. To determine the pharmacodynamics of each compound, experiments with model animals are essential. We propose the use of the silkworm infection model prior to the use of mammalian infection models for general screening of therapeutic compounds. We demonstrated previously that (i) antibiotics that are effective in human patients are also effective in silkworms infected with bacteria or fungi pathogenic to humans, and (ii) ED50 values, which provide a quantitative basis for assessing the therapeutic effect of antibiotics, are consistent between silkworms and mammals (Hamamoto et al., 2004, 2005; Hamamoto & Sekimizu, 2005). These findings suggest that we can predict the pharmacodynamic characteristics of antibiotics in mammals by using the silkworm infection model. We propose that the pharmacodynamic characteristics of antiviral agents in the infection models of silkworms and mammals will be consistent. The ratio between the IC50 (the effective concentration of the compound needed for inhibiting virus proliferation in cultured cells by 50%) and the ED50 (the evaluation of the amount of the compound needed to produce a therapeutic effect) will be useful for determining the pharmacodynamics of the compound. A small value would indicate better pharmacodynamics. We used several antiviral agents in this study that are clinically effective in humans infected with pathogenic viruses. The ED50:IC50 value for all of the tested agents typically used for clinical purposes was <.5. We propose that candidate compounds that have an ED50:IC50 value of <.5 should be considered as promising candidate antiviral agents for clinical purposes.

**Antiviral effect of cinnzeylanine**

Mao-to, Kakkon-to and Shosaiko-to have a long history as treatments for patients with influenza in Kampo medicine, but none of the compounds that are effective against viral infection have been identified. In this study, Mao-to had a therapeutic effect in the baculovirus-infected silkworm, and we purified an antiviral substance from a hot-water extract of cinnamon bark, one of the four components of Mao-to. Our assay measured the therapeutic effects in silkworms infected with baculovirus. The purified substance was cinnzeylanine, whose structure was identified previously. The antiviral activity of cinnzeylanine had not been determined previously. A remarkable feature of cinnzeylanine is that this compound shows therapeutic effects following administration into the midgut. Our previous study demonstrated that mammalian intestines and silkworm midgut have common permeability characteristics for chemical compounds (Hamamoto et al., 2005). Therefore, we can expect that oral administration of cinnzeylanine is effective in humans. We also showed that cinnzeylanine inhibited the proliferation of HSV-1 in Vero cells. Taking these findings together, we propose that cinnzeylanine is a good candidate antiviral agent against HSV infection. To use cinnzeylanine in humans, chemical modifications of this compound are needed to increase the antiviral effect. The silkworm infection model will also be useful for optimizing chemically modified candidates.

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