Original Article

Evaluation of therapeutic effects and pharmacokinetics of antibacterial chromogenic agents in a silkworm model of *Staphylococcus aureus* infection

Tomoko Fujiyuki, Katsutoshi Imamura, Hiroshi Hamamoto, Kazuhisa Sekimizu*

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan.

ABSTRACT: The therapeutic effect of dye compounds with antibacterial activity was evaluated in a silkworm model of *Staphylococcus aureus* infection. Among 13 chromogenic agents that show antibacterial activity against *S. aureus* (MIC = 0.02 to 19 μg/mL), rifampicin had a therapeutic effect. The ED_{50} value in the silkworm model was consistent with that in a murine model. Other 12 dyes did not increase survival of the infected silkworms. We examined the reason for the lack of therapeutic efficacy. Amidol, pyronin G, and safranin were toxic to silkworms, which explained the lack of therapeutic effects. Fuchsin basic and methyl green disappeared quickly from the hemolymph after injection, suggesting that they are not stable in the hemolymph. Although coomassie brilliant blue R250/G250, cresyl blue, and nigrosin showed no toxic effects or instability in the hemolymph, they also did not have a therapeutic effect. The *in vitro* antibacterial actions of these dyes were inhibited by silkworm plasma or bovine serum albumin and filtration experiments demonstrated that cresyl blue bound to plasma proteins in the silkworm, suggesting that plasma protein binding inhibited the therapeutic efficacy of these four dyes. These findings indicate that drug screening using the silkworm infection model is useful for evaluating toxicity and pharmacokinetics of potential antibiotics.

Keywords: Silkworm, plasma protein binding, antibacterial, dye

1. Introduction

In the course of developing drugs to treat infectious diseases, chemical compounds with antibacterial activity *in vitro* are tested for their therapeutic efficacy *in vivo* in animal infection models. A serious problem is that most of compounds that exhibit antibacterial activity *in vitro* do not have therapeutic effects in animal infection models due to toxicity and pharmacokinetic issues. Thus, for efficient drug discovery, protocols must be established to exclude agents without therapeutic effects at earlier stages of drug development. Evaluation of the therapeutic effects of potential antibiotics has been performed using mammalian models, but conventional methods using a large number of mammals are problematic due to high costs and ethical concerns. Therefore, the development of a non-vertebrate infection model to test drug efficacy in the early stages of development is highly desirable.

We have established insect models of human pathogenic bacterial and viral infection using the silkworm, *Bombyx mori* (*1-3*), and proposed the utilization of the silkworm model for drug discovery (*4*). Our previous studies indicated that lethal doses of chemicals in silkworm were consistent with those in mammals, when normalized by body weights (*5*). Silkworms and mammals share conserved mechanisms for the pharmacokinetics of chemicals: absorption, distribution, metabolism, and excretion (ADME) (*5-7*). Therefore, the silkworm infection model is potentially useful for the evaluation of toxicity and ADME of candidate compounds in therapeutic drug screening for infectious diseases.

In mammals, the binding of drugs to plasma proteins like albumin is an important factor in the distribution of drugs in the animal body. In invertebrates, however, information regarding interactions of drugs with plasma proteins is scarce. Therefore, it is not known whether the silkworm model can be used to evaluate the inhibition of therapeutic effects induced by plasma protein binding of drug candidate agents.

To evaluate factors that affect pharmacokinetics, including plasma protein binding, on the therapeutic effects of antibacterial agents in the silkworm infection model, we considered dyestuffs as suitable model agents. Some of dyes have antibacterial activity (*8,9*).

**Address correspondence to:*
Dr. Kazuhisa Sekimizu, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
e-mail: sekimizu@mol.f.u-tokyo.ac.jp

In addition, dye concentration in samples can be easily quantified by measuring absorbance. Here we describe the therapeutic effects and pharmacologic properties of dyes in the silkworm model of *S. aureus* infection.

2. Materials and Methods

2.1. *Animals*

Bombyx mori eggs (Fu•Yo × Tsukuba•Ne) were purchased from Ehima Sansyu (Ehime, Japan), and raised to the fifth instar larval stage by feeding with artificial food Silkmate 2S (Nosan Corporation, Yokohama, Japan). All of the animals used in this study were fifth instar larvae.

2.2. *Chromogenic compounds*

Rifampicin, methyl green, amidol (Wako Pure Chemical Industries, Osaka, Japan), Coomassie brilliant blue (CBB) R250, CBB G250, nigrosin (Nakalai Tesque, Kyoto, Japan), malachite green (Tokushu Chemicals, Tokyo, Japan), crystal violet (Sigma, St. Louis, MO, USA), cresyl blue, pyronin G (Tokyo Chemical Industry, Tokyo, Japan), toluidine blue O (Chroma-gesellschaft Schmid, Stuttgart, Germany), fuchsin basic, and safranin (Kanto Chemical, Tokyo, Japan) were dissolved in phosphate buffered saline (PBS, 10 mM sodium phosphate, 137 mM sodium chloride, 3 mM potassium chloride, pH 7.4) or dimethyl sulfoxide. Commercially available CBB R250 is a mixture of chromogenic compounds in different forms (*10-12*). Therefore, we purified CBB R250 from the purchased reagent using thin layer chromatography (TLC). The sample (30 mg) in methanol was applied to a preparative TLC plate (silica gel 60 F254, Merck KGaA, Darmstadt, Germany) and developed in 1-butanol/acetic acid/water (15:3:7). The main band with blue color was scraped off, eluted with methanol, and filtered through a 0.45-μm filter. The methanol was removed by evaporation and the sample was analyzed using liquid chromatography-mass spectrometry. The molecular mass of the principal compound in the fraction was estimated to be 804, which is previously reported molecular mass of CBB R250 (*11*).

2.3. *Assay of antibacterial activity of dyes*

A clinical strain of *S. aureus*, MSSA1 (*13*), was cultured in Luria-Bertani 10 (LB 10) medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl per liter) at 37°C for 24 h. The full growth culture was diluted to 1/1,000 in Mueller-Hinton (MH) medium and then a 100-μL aliquot was added to each well of 96-well plate. The dye solution was serially diluted 2-fold and 100 μL of each dilution was added to the bacterial solution, and cultured at 37°C for 18 to 24 h. Bacterial growth was visually determined. The MIC (minimum inhibitory concentration) was defined as the lowest concentration of the dye that inhibited the bacterial growth. To evaluate the effect of silkworm plasma or bovine serum albumin (BSA) on the antibacterial activity of the dyes, the silkworm plasma (final concentration, 25%) or BSA (fraction V, Nakalai Tesque; final concentration, 25 mg/mL) was added to MH medium, and the MICs of the dyes were determined. The silkworm plasma was prepared as follows: the legs of fifth instar larvae were cut and the hemolymph was collected. 2-Mercaptoethanol (2-ME; final concentration, 0.1%) was added to the samples to inhibit melanization and the hemocytes were removed by centrifugation for 5 min at 8,000 rpm (R10A2, Hitachi Koki, Tokyo, Japan).

2.4. *Calculation of the theoretically minimal effective dose of antibacterial dye required for the treatment of infected silkworms*

The fifth instar larva (2 g) used for therapeutic assay has a hemolymph with volume of approximately 500 μL (*5*). Therefore, injection of 50 μL dye solution into the silkworm hemolymph results in a 1:10 dilution. The putative minimal effective dose of dye minimally required to achieve therapeutic effect on the infected silkworm, tEDmini (μg/g•larva), was theoretically defined based on the following formula using the MIC of the dye.

$$\text{tEDmini (μg/g•larva)} = \frac{\text{MIC (μg/mL)} \times 0.5\text{ (mL)} \times 10}{2\text{ (g•larva)}}$$

2.5. *Determination of the dye LD_{50} in silkworms*

The larvae were reared for 1 day at 27°C with feeding. The larvae (n = 3-5) were injected with 2-fold serial dilutions of the dye solution (50 μL) into the hemolymph and reared at 27°C. The number of surviving larvae was counted 2 days later. The LD_{50} was determined by the survival curve as the dose that killed half of the larvae (LD_{50}).

2.6. *Evaluation of therapeutic effects of dyes*

Full growth *S. aureus* culture was diluted 10-fold with 0.9% NaCl. A 50 μL aliquot was injected into the hemolymph of the silkworm. Dye solution (50 μL) diluted with PBS was further injected into the hemolymph (*6*). The silkworms were reared at 27°C and the number of surviving silkworms was counted 2 days later. Vancomycin (200 μg/mL) was administered as a positive control. To determine the number of viable bacteria in the hemolymph, the hemolymph was collected 1 day after the injection of CBB R250 and diluted in 0.9% NaCl before spreading on mannitol-

salt agar plates. The plates were incubated at 37°C overnight and the colonies were counted.

2.7. *Determination of the concentration of chromogenic compound in the hemolymph of the injected silkworm*

Rifampicin solution (3.5 mg/mL in 30% dimethyl sulfoxide, 0.9% NaCl, 50 μL) was injected into the hemolymph of the silkworm. The animals were reared at 27°C and the hemolymph was collected after 20 sec, 1, 5, 10, 15, 30, 60 min, 1.5, 2, 4, 8 h. 2-ME (1 μL) was added to approximately 100 μL of hemolymph and an equal volume of acetone was added to the hemolymph, followed by vigorous shaking. The sample was then centrifuged at 20,000 × g for 5 min and the OD_{475} of the supernatant was measured. Hemolymph of a silkworm injected with the buffer (30% dimethyl sulfoxide, 0.9% NaCl) was used as the background.

A solution (50 μL, in PBS) of fuchsin basic (5 mg/mL), methyl green (15 mg/mL), CBB R250 (3.7 mg/mL), CBB G250 (5 mg/mL), cresyl blue (8 mg/mL), or nigrosin (9 mg/mL) was injected into the hemolymph of the silkworm. The animals were reared at 27°C and the hemolymph was collected after 2 days and the sample was prepared as described above. The OD value, at absorption maximum of each dye, of the supernatant was measured. Hemolymph of a silkworm injected with PBS was used as the background.

2.8. *Assay for cresyl blue binding to the silkworm plasma proteins*

A solution of cresyl blue (2 μL, 8 mg/mL) was mixed with the silkworm plasma (200 μL) diluted with phosphate buffer (10 mM sodium phosphate, 150 mM sodium chloride, 5 mM potassium chloride, 1 mM calcium chloride, pH 7.2). The sample was applied to YM-3 filter for ultra filtration (3 kDa cut-off, Millipore, Billerica, MA, USA) and centrifuged at 12,000 × g for 30 min at 4°C. The OD_{635} of the filtered solution was measured and the amount of the dye that passed through the membrane was calculated. Protein concentration in the filtered solution was determined by Lowry's method after trichloroacetic acids precipitation. The findings revealed that most of the proteins did not pass through the filter.

3. Results

First, we measured the antibacterial activity *in vitro* of 44 randomly selected chromogenic compounds *in vitro* and chose the 13 dyes for which the MIC was less than 20 μg/mL (Table 1). We then tested whether the dyes were therapeutically effective in silkworms infected with *S. aureus*. Rifampicin had a therapeutic effect and the ED_{50} value was 0.08 μg/g•larva. This is consistent with the value in a murine model (0.062 μg/g) (*14*). None of others had a therapeutic effect, even at various doses. In addition to evaluating the therapeutic effect by counting the number of surviving silkworms, we examined whether CBB R250 suppresses the growth of *S. aureus* in the silkworm hemolymph. Our findings indicated that CBB R250 administration did not decrease the number of viable bacteria in the hemolymph (Figure 1), whereas vancomycin suppressed bacterial growth in the hemolymph. To understand the reason for the lack of therapeutic effects of these dyes in the silkworm infection model, we studied their toxicity, stability in the hemolymph, and plasma protein binding properties as follows.

For antibacterial agents to show therapeutic effects, their concentrations in the hemolymph after injection should be higher than their MIC values and, at the same time, they should not be toxic to the host animals. As the MIC value enables us to calculate the minimal required dose for the therapeutic effect in infected silkworms (see "Material and Methods"), we defined the value as the theoretical minimal effective dose required

Table 1. Antibacterial activity, toxicity, and therapeutic effect of dyes in the silkworm model of *S. aureus* infection

Dye	MIC (μg/mL)	tEDmini (μg/g•larva)	LD_{50} (μg/g•larva)	ED_{50} (μg/g•larva)	ED_{50}/MIC
Rifampicin	0.02	0.05	ND	0.08	4
Amidol	19	48	54	> 9	> 0.5
Pyronin G	2	5	11	> 13	> 7
Safranin	10	25	34	> 23	> 2
Fuchsin basic	6	15	100-500	> 230	> 38
Methyl Green	13	33	530	> 160	> 12
CBB R250	4	10	> 660	> 83	> 17
CBB G250	7	18	> 800	> 72	> 10
Cresyl Blue	4	10	530	> 150	> 39
Nigrosin	5	13	490	> 170	> 33
Crystal Violet	0.2	0.5	15	> 9	> 45
Malachite Green	0.3	0.8	15	> 10	> 33
Toluidine Blue O	14	35	1,000	> 700	> 50
Vancomycin	1	2	ND	0.3*	0.3*

*; the values are cited from Hamamoto *et al.*, 2004. tEDmini; theoretical minimal effective dose required for therapeutic effect in the infected silkworm. ND; not determined.

for the therapeutic effect (tEDmini), and compared it with the LD_{50} value of the dye. The tEDmini values of amidol, pyronin G, and safranin were 48, 5, and 25 μg/g•larva, respectively, which were all greater than 1/3 of the LD_{50} values (Table 1). These results suggest that these three dyes were not therapeutically effective in the silkworm infection model because they were toxic to the silkworms.

The other nine dyes with antibacterial activity showed smaller tEDmini values lower than 1/10 of the LD_{50} (Table 1). These dyes still did not have therapeutic effects in the infected silkworms. We previously proposed that an antibacterial agent whose ED_{50}/MIC values were lower than 10 had proper pharmacokinetics characteristics (*6*). While the ED_{50}/MIC value of rifampicin was 4, the values of the nine dyes were over 10 (Table 1), suggesting that these dyes have pharmacokinetic problem in the silkworm and thus are not therapeutically effective. To test this notion, we examined the stability of these compounds in the silkworm hemolymph. The concentration of the chromogenic compound in the hemolymph can be easily measured by absorbance at the maximum absorption wavelength. This method revealed that the half-life of rifampicin at α phase was 3 min, indicating rapidly distributed (Figure 2). The half-life at β phase was 3 h, suggesting that rifampicin is present in the hemolymph over the MIC during 2 days after injection. The period of the half-life at β phase was consistent with that of human (*15*). As for other dyes, the concentration of fuchsin basic and methyl green in the hemolymph was below the MIC at 2 days after injection (Table 2), suggesting that these dyes are rapidly eliminated from the hemolymph. We concluded that these chromogenic compounds do not have therapeutic effects due to their rapid distribution into the tissues, rapid metabolism, and/or rapid excretion.

The concentrations of CBB R250, CBB G250, cresyl blue, and nigrosin in the hemolymph were higher than the MIC values even 2 days after injection, but they still did not show the therapeutic effects (Tables 1 and 2), suggesting that a factor other than distribution, metabolism, and excretion of the dyes inhibited the therapeutic effects.

Therapeutic effects of drugs are sometimes prevented by plasma protein binding, which is included in the issues of distribution of ADME. It is well known that CBB R250, CBB G250, and nigrosin bind to proteins (*16-18*). We examined whether the antibacterial activity of CBB R250, CBB G250, cresyl blue, and nigrosin were inhibited in the presence of the silkworm plasma. Adding the silkworm plasma fraction increased the MICs of these four dyes (Table 3). The protein concentration in the hemolymph of fifth instar larva ranges from approximately 10 to 100 mg/mL (*19,20*). Adding 25 mg/mL BSA also increased the MICs of the dyes, whereas adding the silkworm

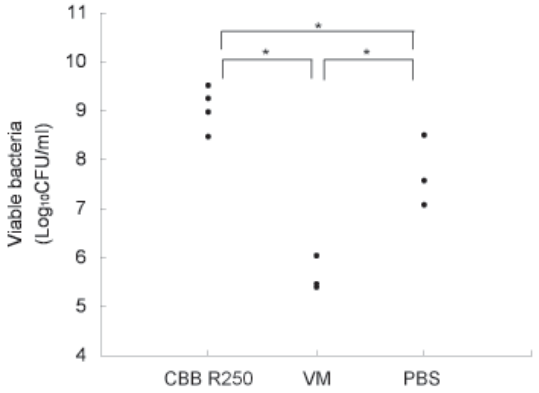

Figure 1. Evaluation of the therapeutic effect of CBB R250 based on the number of viable bacteria in the hemolymph after injection into the silkworm. A 10-fold diluted solution (50 μL) of full growth *S. aureus* culture was injected into the hemolymph of fifth instar larva, and then an equal volume of CBB R250, PBS, or vancomycin was injected into the hemolymph. The hemolymph was collected 1 day later and the number of colonies of viable bacteria was counted. Each dot indicates the number of viable bacteria in the hemolymph from one individual larva. * Student's *t*-test, $p < 0.05$.

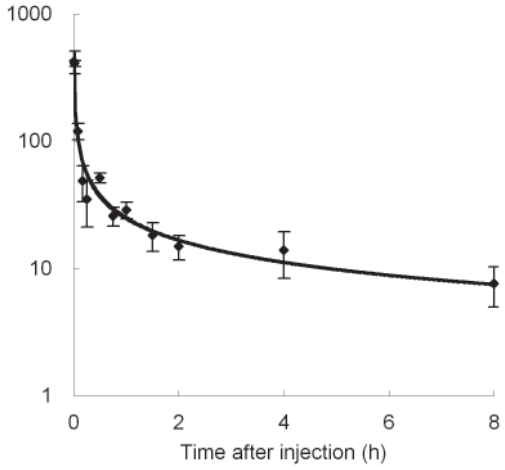

Figure 2. Pharmacokinetics of rifampicin in the silkworm. Rifampicin solution (50 μL, 3.5 mg/mL) was injected into the silkworm hemolymph and then the hemolymph was collected at different time after injection. The rifampicin concentration in the hemolymph was calculated by OD_{475} value. Data are presented as mean ± S.E.M. ($n = 3$).

Table 2. Dye concentration in the silkworm hemolymph after injection

Dye	MIC (μg/mL)	Concentration in hemolymph after 2 days (μg/mL)
Fuchsin basic	6	0 ± 0 ($n = 5$)
Methyl green	13	1 ± 1 ($n = 5$)
CBB R250	4	14 ± 5 ($n = 7$)
CBB G250	7	7 ± 3 ($n = 5$)
Cresyl blue	4	12 ± 4 ($n = 4$)
Nigrosin	5	9 ± 1 ($n = 4$)

Data shown are the MICs and the concentrations in the hemolymph of dyes (μg/mL). The concentration was measured 2 days after dye injection (250 μg/larva for fuchsin basic, 750 μg/larva for methyl green, 185 μg/larva for CBB R250, 250 μg/larva for CBB G250, 400 μg/larva for cresyl blue, 450 μg/larva for nigrosin) into the hemolymph. Values are presented as mean ± S.E.M. N; number of the tested silkworms.

plasma or BSA did not affect the MIC of vancomycin. Cresyl blue is a basic dye that binds to various biologic substances such as nucleic acids, although information regarding its protein binding properties is limited to only a few species of proteins (*21*,*22*). To examine whether cresyl blue binds to silkworm plasma proteins, we performed a filtration assay (see "Materials and Methods"). Adding the silkworm plasma decreased the amount of the filtrated dye (Figure 3). When mixed with the maximum amount (99%) of plasma, only 4% of cresyl blue penetrated through the filter, indicating that 96% of the dye wad bound to the plasma proteins. These results suggest that the antibacterial activities of CBB R250, CBB G250, cresyl blue, and nigrosin are inhibited in the hemolymph by plasma protein binding.

4. Discussion

In the present study, we examined the therapeutic effects of 13 different chromogenic compounds with *in vitro* antibacterial activity against *S. aureus*, and demonstrated that rifampicin had a therapeutic effect and the ED_{50} value and the elimination half-life were consistent with those in mammals. The other 12 dyes were not therapeutically effective in the silkworm infection model due to toxicity or ADME problems. Some of the dyes (CBB R250, CBB G250, cresyl blue, and nigrosin) had no therapeutic effects, while apparent concentrations in the hemolymph were maintained higher than their MICs. These dyes lost their antibacterial activity in the presence of silkworm plasma or BSA, which suggested that these dyes bind to silkworm plasma proteins and the concentrations of the free compounds are too small in the hemolymph to produce therapeutic effects in the silkworm infection model. We previously reported that the pharmacokinetics of various compounds are similar between silkworms and mammals (*5*-*7*). Here we reported that the influence of plasma protein binding on drug distribution, which is a known phenomenon in mammals, is also observed in silkworms. To our knowledge, this is the first report that the therapeutic effectiveness of drugs can be inhibited by plasma protein binding in invertebrates as well as in vertebrates.

Table 3. Effect of the presence of the silkworm plasma (25%) and BSA (25 mg/ml) on the antibacterial activity of dyes

Dye	None	Plasma	BSA
CBB R250	7 ± 2 (n = 3)	> 263 (n = 3)	> 1,050 (n = 3)
CBB G250	7 ± 0 (n = 3)	600 ± 184 (n = 3)	> 900 (n = 3)
Cresyl Blue	5 ± 1 (n = 4)	> 16 (n = 4)	> 131 (n = 4)
Nigrosin	3 ± 0 (n = 3)	> 163 (n = 4)	> 325 (n = 3)
Vancomycin	1 ± 0 (n = 2)	1 ± 0 (n = 2)	1 ± 0 (n = 2)

The MICs of the dyes are shown (μg/mL). A CBB R250 reagent was obtained from the supplier and used without purification in this experiment. Values are presented as mean ± S.E.M.

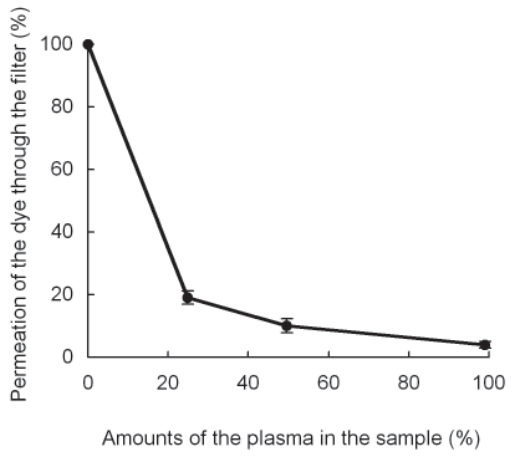

Figure 3. Binding of cresyl blue to the silkworm plasma proteins. Silkworm plasma was mixed with buffer in various ratios and then cresyl blue was added. The sample was applied to ultra-filtration. The OD_{635} of the filtrated solution was determined and the permeation of the dye was calculated. Data are presented as mean ± S.E.M. (n = 3).

Further, we showed that the silkworm model can be used to evaluate the toxicity of candidate agents of drugs for infectious diseases. We previously reported that the LD_{50} values of cytotoxic agents in silkworm are consistent with those in mammals (*5*). Therefore, therapeutically effective agents in the silkworm infection model are expected to show therapeutic effectiveness without toxicity in mammals. The silkworm infection model is advantageous for simultaneous evaluation of the toxicity and therapeutic effects of drug candidates.

Drug candidates whose concentrations in free form in the blood are reduced by plasma protein binding often do not show therapeutic effects. Efficient exclusion of such candidates in the early phase of drug development is necessary for productive drug discovery. To advance drug development, candidate compounds must have the low toxicity and good property of ADME properties. We propose the use of a silkworm model for these aims. Silkworm models of diseases do not cost compared with mammalian models and are associated with fewer ethical issues and biohazard risks. Furthermore, silkworm larvae are large enough to handle for the injection of reagent solutions and for the collection of hemolymph for analysis. Silkworm models of infectious diseases have been established using bacteria, virus, and fungi (*1*,*3*,*6*). Although other invertebrate infection models have also been proposed (*23*,*24*), the silkworm is highly advantageous for studies of pharmacokinetics and this model can likely be extended to various human diseases. Actually a silkworm model of human sepiapterin reductase deficiency was recently reported (*25*). Utilization of

the silkworm model of various diseases will be helpful to exclude candidate therapeutic agents that are not effective at an early stage of drug development.

Acknowledgements

We thank Kiyomi Kyougoku, Yumiko Matsuzawa, and Aya Yoshino for their technical assistance. This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences of NIBIO and grants from Genome Pharmaceuticals Co. Ltd. T. Fujiyuki is the recipient of a Grant-in-Aid from Japan Society for the Promotion of Science for young scientists (21-40175).

References

1. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb Pathog. 2002; 32:183-190.
2. Kaito C, Sekimizu K. A silkworm model of pathogenic bacterial infection. Drug Discov Ther. 2007; 1:89-93.
3. Orihara Y, Hamamoto H, Kasuga H, Shimada T, Kawaguchi Y, Sekimizu K. A silkworm baculovirus model for assessing the therapeutic effects of antiviral compounds: Characterization and application to the isolation of antivirals from traditional medicines. J Gen Virol. 2008; 89:188-194.
4. Kurokawa K, Hamamoto H, Matsuo M, Nishida S, Yamane N, Lee BL, Murakami K, Maki H, Sekimizu K. Evaluation of target specificity of antibacterial agents using *Staphylococcus aureus ddlA* mutants and D-cycloserine in a silkworm infection model. Antimicrob Agents Chemother. 2009; 53:4025-4027.
5. Hamamoto H, Tonoike A, Narushima K, Horie R, Sekimizu K. Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. Comp Biochem Physiol C Toxicol Pharmacol. 2009; 149:334-339.
6. Hamamoto H, Kurokawa K, Kaito C, Kamura K, Manitra Razanajatovo I, Kusuhara H, Santa T, Sekimizu K. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrob Agents Chemother. 2004; 48:774-779.
7. Hamamoto H, Kamura K, Razanajatovo IM, Murakami K, Santa T, Sekimizu K. Effects of molecular mass and hydrophobicity on transport rates through non-specific pathways of the silkworm larva midgut. Int J Antimicrob Agents. 2005; 26:38-42.
8. Bentley R. Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence beta-lactams). J Ind Microbiol Biotechnol. 2009; 36:775-786.
9. Sörgel F. The return of Ehrlich's 'Therapia magna sterilisans' and other Ehrlich concepts? Series of papers honoring Paul Ehrlich on the occasion of his 150th birthday. Chemotherapy. 2004; 50:6-10.
10. Wilson CM. Studies and critique of amido black 10B, coomassie blue R, and fast green FCF as stains for proteins after polyacrylamide gel electrophoresis. Anal Biochem. 1979; 96:263-278.
11. Kundu SK, Robey WG, Nabors P, Lopez MR, Buko A. Purification of commercial coomassie brilliant blue R-250 and characterization of the chromogenic fractions. Anal Biochem. 1996; 235:134-140.
12. Rosenthal HL, Berger RA, Tyler AN, Moore BW. Isolation of a component from commercial coomassie brilliant blue R-250 that stains rubrophilin and other proteins red on polyacrylamide gels. Biochim Biophys Acta. 1988; 965:106-113.
13. Akimitsu N, Hamamoto H, Inoue R, Shoji M, Akamine A, Takemori K, Hamasaki N, Sekimizu K. Increase in resistance of methicillin-resistant *Staphylococcus aureus* to beta-lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals. Antimicrob Agents Chemother. 1999; 43:3042-3043.
14. Rothstein DM, Farquhar RS, Sirokman K, Sondergaard KL, Hazlett C, Doye AA, Gwathmey JK, Mullin S, van Duzer J, Murphy CK. Efficacy of novel rifamycin derivatives against rifamycin-sensitive and -resistant *Staphylococcus aureus* isolates in murine models of infection. Antimicrob Agents Chemother. 2006; 50:3658-3664.
15. Nitti V, Virgilio R, Partricolo MR, Iuliano A. Pharmacokinetic study of intravenous rifampicin. Chemotherapy. 1977; 23:1-6.
16. Congdon RW, Muth GW, Splittgerber AG. The binding interaction of coomassie blue with proteins. Anal Biochem. 1993; 213:407-413.
17. Fazekas de St Groth S, Webster RG, Datyner A. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochim Biophys Acta. 1963; 71:377-391.
18. Ortega M. Use of nigrosine for staining proteins after electrophoresis on filter paper. Nature. 1957; 179:1086-1087.
19. Fujiwara Y, Yamashita O. Developmental changes and hormonal regulation of mRNA of *Bombyx mori* larval serum protein (BmLSP). J Seric Sci Jpn. 1998; 67:393-401.
20. Etebari K, Matindoost L, Mirhoseini SZ, Turnbull MW. The effect of BmNPV infection on protein metabolism in silkworm (*Bombyx mori*) larva. Invertebrate Survival Journal. 2007; 4:13-17.
21. Kelley EG, Miller EG Jr. Reactions of dyes with cell substances; II. The differential staining of nucleoprotein and mucin by thionine and similar dyes. J Biol Chem. 1935; 110:119-140.
22. Lillie RD, Pizzolato P. Acid alcoholic brilliant cresyl blue stain for gastric and other mucins. Histochimie. 1969; 17:138-144.
23. Mylonakis E, Casadevall A, Ausubel FM. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog. 2007; 3:e101.
24. Scully LR, Bidochka MJ. Developing insect models for the study of current and emerging human pathogens. FEMS Microbiol Lett. 2006; 263:1-9.
25. Meng Y, Katsuma S, Daimon T, Banno Y, Uchino K, Sezutsu H, Tamura T, Mita K, Shimada T. The silkworm mutant *lemon* (*lemon lethal*) is a potential insect model for human sepiapterin reductase deficiency. J Biol Chem. 2009; 284:11698-11705.

(Received June 8, 2010; Accepted August 8, 2010)