Acute oral toxicity test of chemical compounds in silkworms

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1. Introduction

Acute toxicity of chemical compounds can be evaluated by calculating LD$_{50}$ values, i.e., the dose that will kill 50% of animals in a test group. From the viewpoint of animal welfare, sacrificing a large number of mammals in an experiment raises ethical issues. At the end of 2002, the Organization for Economic Cooperation and Development (OECD) deleted TG 401, which is the method for evaluating the acute oral toxicity of compounds by calculating LD$_{50}$ values in mammals (1). Given the issue of animal welfare, the fixed dose procedure (FDP; OECD 420), the acute toxic class method (ATC; OECD 423), and the up and down procedure (UDP; OECD 425) have been developed as alternatives to animal testing in TG 401. However, mammals must be sacrificed even when using these alternate methods. Cytotoxic tests of mammalian cells could predict the acute toxicity of compounds in mammal by measuring IC$_{50}$ values (2). However, predicting the toxicity of compounds in mammals with those tests would be difficult since the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body are not taken into account. Techniques to predict the acute toxicity of compounds in mammals without sacrificing those animals must be devised to overcome these problems.

Silkworms have been bred over the long history of sericulture, and subjecting silkworms to a toxicity test poses fewer ethical problems. Moreover, techniques to rear a large number of genetically uniform individuals have been established. The current authors also recently reported that metabolic pathways in silkworms are similar to those in mammals and that the toxicity of some compounds to silkworms accords with their toxicity to mammals once weight is taken into account (3). Moreover, silkworms are large enough to inject a precise amount of a sample solution into either the hemolymph or the midgut (4). Hemolymph is akin to blood in humans and injection into the midgut is akin to oral administration in humans. In light of these facts, an acute oral toxicity test in silkworms is a useful way to evaluate the toxicity of compounds in mammals.

This study performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines as standard substances for a cytotoxicity test, and median lethal dose (LD$_{50}$) were recalculated for each compound. Acute oral LD$_{50}$ values in mammals are listed in OECD guidelines and acute oral LD$_{50}$ values in silkworms were determined in this study. R$^2$ for the correlation between LD$_{50}$ values in mammals and LD$_{50}$ values in silkworms was 0.66. In addition, the acute oral toxicity test in silkworms was performed by two different facilities, and test results from the facilities were highly reproducible. These findings suggest that an acute oral toxicity test in silkworms is a useful way to evaluate the toxicity of compounds in mammals.

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were calculated for each compound. LD$_{50}$ values in silkworms were calculated in this study and LD$_{50}$ values in mammals are listed in OECD guidelines. LD$_{50}$ values in silkworms and LD$_{50}$ values in mammals were subjected to linear regression analysis. The acute oral toxicity test in silkworms was conducted two different facilities, and this study evaluated the between-laboratory reproducibility of their results.

2. Materials and Methods

2.1. Acute oral toxicity tests in silkworms

The toxicity of 59 compounds was evaluated in silkworms using an acute oral toxicity test. These compounds (Table 1) are listed as standard substances in OECD Guidelines. *Bombyx mori* eggs (Hu Yo × Tsukuba Ne or Kinshu × Showa) were purchased from Ehime Sansyu (Ehime, Japan) or Kougensha (Nagano, Japan) and raised as previously described (6,7). A two-fold dilution series containing each compound (50 μL) was injected into the midgut of fifth instar silkworm larvae with a disposable plastic syringe (Terumo, Tokyo, Japan). Silkworms were fed with Silkmate 2S (Nosan Co., Yokohama, Japan) every day and reared at 27°C. After 2 or 7 days, survival rates were measured and LD$_{50}$ values were calculated based on the survival curve ($n = 7$).

Acute oral LD$_{50}$ values in silkworms were determined at 2 days and acute oral LD$_{50}$ values in mammals are listed in OECD guidelines. Linear regression analysis of the common logarithm of LD$_{50}$ values in silkworms and LD$_{50}$ values in mammals was performed to determine the correlation between these 2 values.

2.2. Reproducibility of an acute oral toxicity test in silkworms conducted by two different facilities

Two different facilities (Laboratory 1: Genome Pharmaceuticals Institute Co., Ltd., Tokyo, Japan, Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory, Kanagawa, Japan) performed an acute oral toxicity test in silkworms, and this study evaluated the between-laboratory reproducibility of their results.

3. Results

3.1. Comparison of acute oral toxicity test results in silkworms and in mammals

LD$_{50}$ values in mammals and IC$_{50}$ values in 3T3 cells and NHK cells are listed in OECD guidelines. Linear regression analysis of the LD$_{50}$ values was performed, and the regression line and the determination coefficient were defined as below.

\[
\log (\text{LD}_{50} \text{ in mammals}) = 0.499 \times \log (\text{IC}_{50} \text{ 3T3 cells}) + 1.65 \quad (R^2 = 0.33)
\]

\[
\log (\text{LD}_{50} \text{ in mammals}) = 0.445 \times \log (\text{IC}_{50} \text{ NHK cells}) + 1.81 \quad (R^2 = 0.37)
\]

LD$_{50}$ values for 59 compounds were calculated based on an acute oral toxicity test in silkworms (Table 1). LD$_{50}$ values in silkworms were calculated in this study and LD$_{50}$ values in mammals are listed in OECD guidelines. Linear regression analysis of the 2 values was performed, and the regression line and the determination coefficient were defined as below.

\[
\log (\text{LD}_{50} \text{ in mammals}) = 0.860 \times \log (\text{LD}_{50} \text{ in silkworms}) + 0.168 \quad (R^2 = 0.66)
\]

Some of the 59 compounds produced LD$_{50}$ values in silkworms that differed from LD$_{50}$ values in mammals listed in OECD guidelines (Table 1). The LD$_{50}$ values for physostigmine, nicotine, lindane, carbamazepine, propylparaben, and sodium hypochlorite were 10 or more times lower in silkworms than in mammals. The LD$_{50}$ values for cycloheximide, sodium selenate, and phenylthiourea were 10 or more times higher in silkworms than in mammals.

3.2. Reproducibility of an acute oral toxicity test in silkworms performed by two different facilities

LD$_{50}$ values in silkworms that are calculated by different facilities must be highly reproducible if an acute oral toxicity test in silkworms is to serve as an alternative to acute oral toxicity testing in mammals. Each facility calculated the LD$_{50}$ values in silkworms, and the Pearson’s product-moment correlation coefficient of the correlation between those 2 values was 0.88

4. Discussion

Cytotoxicity tests of compounds in mammalian cells or cell lines have been proposed as a way to predict the acute oral toxicity of compounds in mammals (2). IC$_{50}$ values in 3T3 cells or NHK cells and LD$_{50}$ values for 59 compounds in mammals are listed in OECD guidelines (5). Linear regression analysis of those IC$_{50}$ values yielded an $R^2$ of 0.33 and linear regression analysis of LD$_{50}$ values in mammals yielded an $R^2$ of 0.37. LD$_{50}$ values that were calculated in the acute oral toxicity test in silkworms and LD$_{50}$ values listed in OECD guidelines for mammals were subjected to linear regression analysis, which yielded an $R^2$ of 0.66 (Figure 1). These results suggest that, in contrast to a toxicity test in mammalian cells, an acute oral toxicity...
Table 1. Acute oral LD$_{50}$ values in silkworms calculated in this study

| No. | Name                        | Ref. acute oral LD$_{50}$ (mg/Kg) | Laboratory 1 | Laboratory 2 |
|-----|-----------------------------|-----------------------------------|--------------|--------------|
|     |                             | LD$_{50}$ (Day 2) (mg/Kg)         | LD$_{50}$ (Day 7) (mg/Kg) | LD$_{50}$ (Day 2) (mg/Kg) | LD$_{50}$ (Day 7) (mg/Kg) |
| 1   | 1,1,1-Trichloroethane       | 12,078                            | 6,200        | 5,200        | N.D.          | 1,700        |
| 2   | 2-Propanol                  | 5,105                             | 22,000       | 22,000       | 14,000        | 11,000       |
| 3   | 5-Aminosalicylic acid       | 3,429                             | 460          | 160          | N.D.          | N.D.         |
| 4   | Acetaminophen               | 2,163                             | 1,700        | 870          | 2,300         | 1,800        |
| 5   | Acetonitrile                | 3,598                             | 43,000       | 36,000       | 25,000        | 20,000       |
| 6   | Acetyl salicylic acid       | 1,506                             | 1,400        | 1,400        | 1,900         | 1,500        |
| 7   | Arsenic III trioxide        | 25                                | 100          | 100          | 16            | 16           |
| 8   | Atropine sulfate            | 819                               | 3,400        | 1,100        | N.D.          | 1,300        |
| 9   | Boric acid                  | 3,426                             | 3,100        | 1,600        | 1,300         | 830          |
| 10  | Busulfan                    | 12                                | 42           | 42           | N.D.          | N.D.         |
| 11  | Cadmium II chloride         | 135                               | 57           | 51           | 140           | 87           |
| 12  | Caffeine                    | 310                               | 560          | 290          | 800           | 300          |
| 13  | Carbazepine                 | 2,805                             | 280          | 120          | N.D.          | 210          |
| 14  | Carbon tetrachloride        | 3,783                             | 3,400        | 2,400        | 1,200         | 1,200        |
| 15  | Chloral hydrate             | 638                               | 1,700        | 990          | 1,600         | 1,580        |
| 16  | Citric acid                 | 5,929                             | 10,000       | 9,500        | 6,500         | 6,500        |
| 17  | Colchicine                  | 15                                | 23           | 1.8          | N.D.          | 5.4          |
| 18  | Cupric sulfate H$_2$O       | 474                               | 650          | 460          | 940           | 610          |
| 19  | Cycloheximide               | 2                                 | 1,875        | 1,875        | 2,300         | 2,100        |
| 20  | Dibutyl phthalate           | 8,892                             | 45,000       | 30,000       | N.D.          | 18,000       |
| 21  | Diclofloxine                | 59                                | 310          | 15           | 0.42          | 0.42         |
| 22  | Diethyl phthalate           | 9,311                             | 3,800        | 3,500        | 4,400         | 4,000        |
| 23  | Digoxin                     | 28                                | 96           | 6.1          | 24            | 17           |
| 24  | Dimethylformamide           | 5,309                             | 20,000       | 18,000       | 25,000        | 16,000       |
| 25  | Ethanol                     | 11,324                            | 56,000       | 34,000       | 28,000        | 25,000       |
| 26  | Ethylene glycol             | 7,161                             | 33,000       | 41,000       | 46,000        | 46,000       |
| 27  | Gibberellic acid            | 6,040                             | 7,100        | 4.4          | N.D.          | N.D.         |
| 28  | Glycerol                    | 19,770                            | 35,000       | 35,000       | 54,000        | 54,000       |
| 29  | Haloperidol                 | 330                               | 1,100        | 69           | N.D.          | N.D.         |
| 30  | Hexachlorophene             | 82                                | 20           | 12           | 67            | 59           |
| 31  | Lactic acid                 | 3,639                             | 36,000       | 30,000       | 5,100         | 4,500        |
| 32  | Lithium I carbonate         | 590                               | 220          | 5.4          | N.D.          | N.D.         |
| 33  | Mercury II chloride         | 40                                | 120          | 50           | 58            | 40           |
| 34  | Methanol                    | 8,710                             | 6,900        | 3,900        | 8,300         | 8,000        |
| 35  | Nicotine                    | 70                                | 15           | 3.3          | 3.6           | 3.7          |
| 36  | Phenol                      | 548                               | 860          | 790          | 1,000         | 940          |
| 37  | Phenylthiourea              | 3                                 | 320          | 170          | 240           | 240          |
| 38  | Potassium Cyanide           | 7                                 | 25           | 25           | 74            | 82           |
| 39  | Potassium I chloride        | 2,799                             | 6,800        | 5,800        | 10,000        | 7,900        |
| 40  | Procainamide                | 1,950                             | 7,000        | 4,100        | N.D.          | 5,900        |
| 41  | Propranolol HCL             | 466                               | 570          | 540          | 540           | 490          |
| 42  | Propylparaben               | 6,332                             | 670          | 370          | 640           | 580          |
| 43  | Sodium chloride             | 4,046                             | 6,500        | 5,000        | 6,800         | 4,900        |
| 44  | Sodium Dichromate Dihydrate | 51                                | 170          | 170          | 610           | 530          |
| 45  | Sodium hypochlorite         | 10,328                            | 1,500        | 980          | 830           | 650          |
| 46  | Sodium I fluoride           | 127                               | 110          | 70           | 110           | 96           |
| 47  | Sodium oxalate              | 633                               | 1,100        | 1,100        | 1,300         | 1,300        |
| 48  | Sodium selenate             | 3                                 | 50           | 39           | 150           | 110          |
| 49  | Thallium I sulfate          | 25                                | 92           | 10           | 1,100         | 1,100        |
| 50  | Trichloroacetic acid        | 5,229                             | 5,200        | 3,500        | 1,500         | 1,200        |
| 51  | Valproic acid               | 995                               | 3,500        | 3,500        | 7,900         | 6,100        |
| 52  | Verapamil HCL               | 111                               | 490          | 370          | 1,200         | 1,100        |
| 53  | Xylene                      | 4,667                             | 2,200        | 2,200        | 2,100         | 1,100        |
| 54  | Amitriptyline HCL           | 348                               | 390          | 210          | 340           | 290          |
| 55  | Chloramphenicol             | 3,491                             | 1,100        | 980          | 350           | 320          |
| 56  | Epinephrine Bitartrate      | 4 (mouse)                        | 14           | 14           | N.D.          | N.D.         |
| 57  | Lindane                     | 100                               | 27           | 12           | 2.3           | 2.3          |
| 58  | Physostigmine               | 5                                 | 0.76         | 0.46         | 0.75          | 0.65         |
| 59  | Styrenoline                 | 6                                 | 44           | 4.1          | N.D.          | N.D.         |

*Ref. acute oral LD$_{50}$* means LD$_{50}$ values for adult laboratory rats according to the literature (5) unless otherwise specified.
test in silkworms yields results that are more closely in line with the results of an acute oral toxicity test in mammals. A previous study by the current authors reported that some compounds are metabolized in silkworms in the same manner as in mice (3). An in vitro cytotoxicity test cannot reflect the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body, and this leads to disparities in the oral acute toxicity and cytotoxicity of some compounds. These problems might be overcome through the use of silkworms and more consistent results with mammalian test could be obtained rather than cytotoxicity test.

In this study, the LD$_{50}$ values for some compounds were 10 or more times lower in silkworms than in mammals or 10 or more times higher in silkworms than in mammals. Compounds with LD$_{50}$ values that were 10 or more times lower in silkworms were physostigmine, nicotine, lindane, carbamazepine, propyl paraben, and sodium hypochlorite. Compounds with LD$_{50}$ values that were 10 or more times higher in silkworms were cycloheximide, sodium selenite, and phenyl thiourea. Physostigmine is a carbamate insecticide and dichlorvos has been used as an organic phosphorus-based insecticide; physostigmine and dichlorvos are known to inhibit acetylcholinesterase (8,9). Nicotine, an acetylcholine receptor agonist, has been used as an insecticide (10). Lindane is an insecticide that is known to be a GABA receptor antagonist (11). Therefore, silkworms may be more sensitive to these compounds than mammals. Cycloheximide was one compound with an LD$_{50}$ value that was 10 or more times higher in silkworms than in mammals. Cycloheximide may be hydrolyzed in the midgut of silkworms because of the alkaline environment there (pH 9-11).

This study evaluated the reproducibility of an acute oral toxicity test in silkworms by having two different facilities perform the test. Each facility calculated LD$_{50}$ values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88. Laboratory 1: Genome Pharmaceutical Institute Co., Ltd. Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory.
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