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Evaluation of drug toxicity profiles based on the phenotypes of ascidian *Ciona intestinalis*

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ABSTRACT

In vivo toxicity evaluation using model organisms is an important step for the development of new drugs. Here, we report that *Ciona intestinalis*, a chordate invertebrate, is beneficial to drug toxicity evaluation for the following reasons: rapid embryonic and larval development, resemblance to vertebrates, ease of management, low cost, transparent body, and low risk of ethical issues. The dynamic phenotypic change of *Ciona* larvae during metamorphosis prompted us to examine the effect of cytotoxic drugs on its development by quantifying six toxicity endpoints: degenerated tail size, ampulla length, rotation of body axis, stomach size, heart rate, and body size. As a result, mitochondrial respiratory inhibitors, tubulin polymerization/depolymerization inhibitors, or DNA/RNA synthesis inhibitors showed distinct toxicity profiles against these six endpoints, but drugs with the same targets showed a similar toxicity profile in *Ciona*. Our results suggest *Ciona* is an effective animal model for profiling drug toxicity and exploring the mechanisms of drugs with unknown targets.

1. Introduction

Toxicity evaluation is an important step for development of new drugs. For toxicity evaluation, *in vivo* assays using mammalian animals including rats, mouse, monkeys, or rabbits have been employed to predict toxicity effects on humans. Although these results offer a better prediction than the results of *in vitro* assay using cultured cells, they are costly and time-consuming. To circumvent these problems, for example, zebrafish (*Danio rerio*) is increasingly employed as an animal model for *in vivo* toxicity evaluation, taking advantage of its low cost, ease of management, rapid development, and a transparent body convenient for observation [1–3]. Indeed, toxicity evaluation using zebrafish can provide insights into toxicity information including cardiotoxicity, hepatotoxicity, and neurotoxicity, which resemble human toxicity profiles [3–5]. However, using zebrafish or other vertebrate animals for toxicity evaluations has ethical and legal limitations due to the exposure of these animals to pain or distress. Furthermore, toxicity profiles elucidated by zebrafish alone, or by equivalent "affordable" invertebrate animals such as the fruit fly, nematode worm, or sea urchin are usually not sufficient to confirm drug toxicity, suggesting the need for a more effective animal model for drug toxicity evaluation.

Ciona intestinalis is a chordate invertebrate, the closest living relatives of vertebrates. *Ciona* tadpole larvae consist of small numbers of cells and contain a notochord and a nerve cord as seen in vertebrates [6], and have similar organs as humans including a heart, stomach, intestine, esophagus, testis, and ovary. In 2002, the whole genome of *Ciona* was sequenced [7], and *Ciona* is now in the midst of mainstream developmental biology [8]. Embryonic and larval development of *Ciona* is as rapid as that of the zebrafish. Adult organs begin to form as early as 2 dpf (days post fertilization) after metamorphosis [9,10], and a series of dynamic morphogenetic movements occur, characterized by the degeneration of the tail, removal of larval tunic, and rotation of the body axis. Transparent body, low husbandry costs, and large numbers of individuals are also common advantages shared with the zebrafish. Moreover, since *Ciona* does not have a pain-related channel [11], and are not vertebrates, they are less restricted than the zebrafish by ethical and legal issues.

Previously, *Ciona* have been treated with more than 351 types of drugs, suggesting *Ciona* is sensitive enough to cause drug-induced effects [12]. In the present study, we investigated whether *Ciona* is useful as a new animal model for drug toxicity. In particular, we focused on the phenotypes in *Ciona* larvae treated with cytotoxic

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drugs. By using the toxicity endpoints affected by cytotoxic drugs, we aimed to use *Ciona* not only for assessing the presence of drug toxicity, but also for characterizing drug toxicity.

2. Material and methods

2.1. Biological materials and drug treatment

Ciona were cultivated in Maizuru, or Misaki (National Bio-Resource Project) of Japan. Adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained by dissecting the gonadal and sperm ducts, respectively, and cross-fertilization was performed in plastic petri dishes. After fertilization, embryos were cultured at 18 °C until 47 hpf (hours post fertilization). Of note, at 24 hpf, deformed larvae, or tadpole-shaped larvae not attached to the bottom of the dish were eliminated from the dish. After collecting the larvae from the dish, larvae were evenly distributed to a 12-well plate, with each well containing about a hundred larvae suspended in 1 ml of sea water, followed by an addition of 10 μl of drug at 48 hpf.

2.2. Drugs

We chose the following drugs commonly known to induce cytotoxic effects in various species through known modes of action: rotenone (Sigma, St. Louis, USA), antimycin A (BioVision, Mountain View, USA), oligomycin A (Cayman Chemical, Michigan, USA), MPP^+ (1-methyl-4-phenylpyridinium) (Sigma, St. Louis, USA) known as mitochondria respiratory inhibitor, paclitaxel (Wako Pure Chemical, Osaka, Japan), vinblastine (Wako Pure Chemical) known as tubulin polymerization/depolymerization inhibitor, cisplatin (Wako Pure Chemical), and doxorubicin (Sigma) known as DNA/RNA synthesis inhibitor.

2.3. Quantification

Pictures of larvae were taken using a microscopy camera (WRAYCAM-SR300, WRAYMER, Osaka, Japan) attached to a stereoscopic microscope (OLYMPUS, SZX16, Tokyo, Japan), with a zoom ratio of 8:1. Pictures were used for quantifying the size of the degenerated tail, stomach, and body, and the length of ampulla using ImageJ software. The angle of the body axis was measured by protractor. Heart rate was measured for 30 s.

2.4. Hierarchical cluster analysis

First, each dataset for toxicity endpoints was normalized by z-score using all the concentrations of eight drugs. Next, these normalized datasets for toxicity endpoints were analyzed by cluster analysis to examine the relevance among toxicity endpoints. Then, normalized data were aligned with the relevance of toxicity endpoints in each drug using further cluster analysis to examine the relevance among the toxicity profiles of drugs. Cluster analysis of toxicity endpoints and toxicity profiles of drugs was done by complete linkage based on correlation distance, using R version 3.1.1 (http://www.R-project.org).

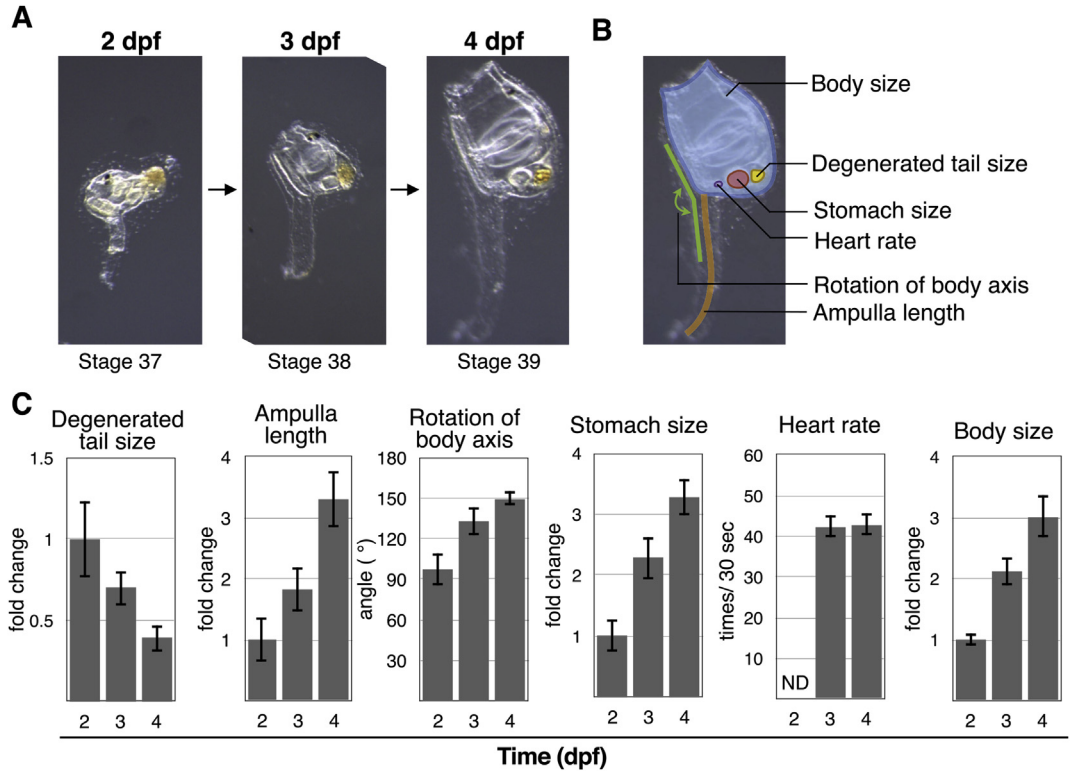

Fig. 1. Six developmental indicators were used to evaluate *Ciona* development. (A) Phenotypes of *Ciona* larvae at 2, 3, and 4 dpf. (B) Six developmental indexes of 4 dpf *Ciona* larvae in A, including degenerated tail size (yellow), ampulla length (orange), rotation of body axis (green), stomach size (red), heart rate (purple), and body size (blue). (C) Quantification of developmental indexes of *Ciona* larvae from 2 hpf to 4 dpf. Degenerated tail size, ampulla length, and body size were calculated as fold change relative to the larvae at 2 hpf. Rotation of body axis was measured using protractor, and heart rate was measured for 30 s. Data were obtained from at least two independent experiments and averaged (n = 10). ND indicates not detected. Error bars are s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Setting of developmental indicators in Ciona

From 2 dpf to 4 dpf, adult organs of *Ciona* begin forming dynamically and larval organs such as the tail muscle and notochord are degenerated in the process of metamorphosis (Fig. 1A). According to the developmental stage of *Ciona* after larval stage (FABA2 [13]) tail absorption and ampulla elongation initiate from Stage 32 (27 hpf) and Stage 35 (30 hpf), respectively. Subsequently, body axis rotation occurs during Stages 36–40 (30–57 hpf), the size of the stomach increases at Stage 38 (3 dpf), and heart beat initiates around 3 dpf, accompanying the remarkable increase in body size of the *Ciona*. Here, we quantified these important developmental events at 2–4 dpf: degenerated tail size, ampulla length, rotation of the body axis, stomach size, heart rate, and body size. As shown in Fig. 1B and C, quantifying these six events showed remarkable differences between 2 dpf and 4 dpf, confirming their usefulness as developmental indicators in *Ciona*.

3.2. Effect of rotenone and vinblastine on developmental indicators

Next, we assessed the developmental toxicity of cytotoxic drugs in larval *Ciona* using the above six developmental indicators. When larval *Ciona* at 2 dpf were treated with rotenone known as a mitochondrial respiratory inhibitor, the absorption of the degenerated tail, elongation of ampulla, heart rate, and body growth were significantly suppressed dose-dependently at 4 dpf (Fig. 2A and C). We next examined the effect of paclitaxel, a tubulin depolymerization inhibitor, on the developmental indicators (Fig. 2B and D). Paclitaxel also inhibited the elongation of ampulla, heart rate, and body growth as observed in the treatment with rotenone. However, unlike rotenone, paclitaxel did not inhibit absorption of the degenerated tail and, furthermore, did inhibit the rotation of body axis. These results suggest the developmental indicators we chose can be used as "toxicity endpoints" for assessing the presence of drug toxicity. Furthermore, the inhibition patterns of toxicity endpoints are not the same among drugs, suggesting that these inhibition patterns are representing drug toxicity profiles.

3.3. Comparison of toxicity profiles among targets

The above results promoted us to examine whether differences in toxicity profiles among drugs depend on their targets. We added antimycin A, oligomycin A, and MPP^+ as mitochondrial respiratory inhibitors, vinblastine as a tubulin polymerization inhibitor, and cisplatin and doxorubicin as DNA/RNA synthesis inhibitors to quantify toxicity endpoints. We carried out two independent

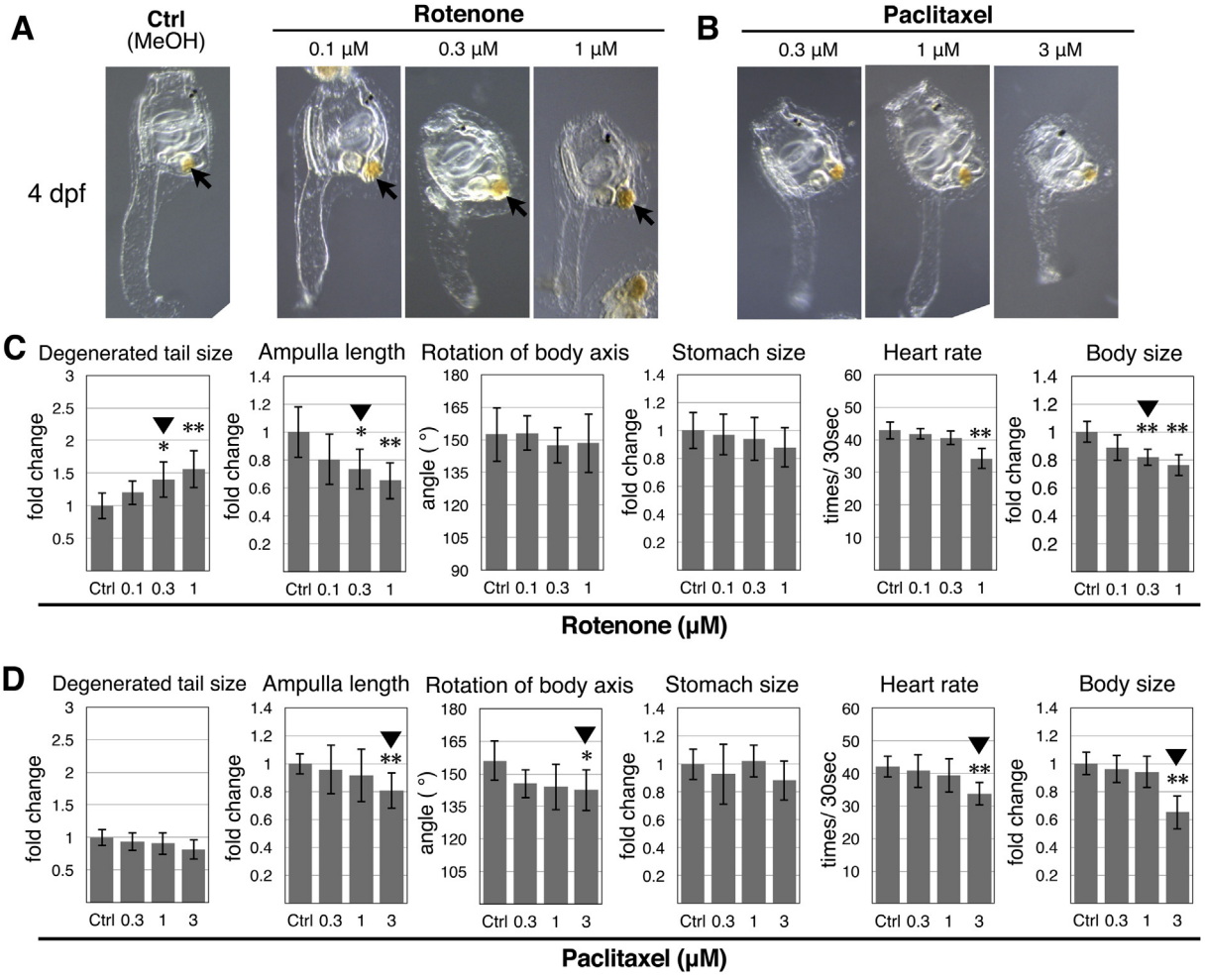

Fig. 2. Rotenone and paclitaxel showed different toxicity profiles. *Ciona* larvae at 2 dpf were treated with rotenone or paclitaxel, and observed at 4 dpf by a stereoscopic microscope. Degenerated tail size, ampulla length, stomach size, body size were calculated as fold change relative to control. Data were obtained from at least two independent experiments, and averaged (n = 10). Black triangle indicates significant inhibition at MTC. Error bars are s.e.m. $^*p < 0.01$, $^{**}p < 0.001$ compared with control (ctrl), two-tailed *t*-tests. (A, B) Phenotypes of the larvae treated with rotenone (A) or paclitaxel (B). Arrows indicate degenerated tail. (C, D) Quantification of developmental indexes of the larvae treated with rotenone (C) or paclitaxel (D).

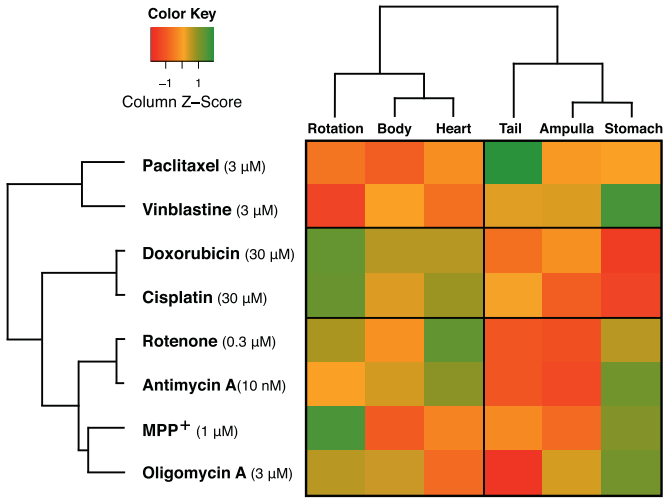

Fig. 3. Cluster analysis of the toxicity profile of cytotoxic drugs. Toxicity profiles of drugs (Doxorubicin, cisplatin, paclitaxel, vinblastine, rotenone, antimycin A, MPP^+, oligomycin A) were clustered using the datasets composed by six normalized toxicity endpoints for each drug. Cluster analysis was performed based on the complete-linkage method using correlation distance. Rows and columns indicate drugs and toxicity endpoints, respectively. The heat map shows a gradient color scale from red, indicating relatively strong inhibition, to green, indicating relatively weak inhibition among toxicity endpoint. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments and obtained highly reproducible data. Each toxicity endpoint was normalized by z-score using all concentrations of eight drugs. We chose minimum toxic concentration (MTC) in which either toxicity endpoint was significantly affected ($p < 0.01$) (Fig. 2C and D, Suppl. Fig. 1), and performed a hierarchical cluster analysis of toxicity profiles of each drug at MTC. The results are displayed in the form of a heat map in Fig. 3. The heat map employs a gradient color scale from green, indicating a relatively weak effect, to magenta, indicating a relatively strong effect, interpolated over yellow, indicating a relatively moderate effect among six toxicity endpoints in each drug.

We expected the drugs showing a similar mode of action to be clustered in the same tree. Indeed, tubulin polymerization/depolymerization inhibitors (paclitaxel and vinblastine), DNA/RNA synthesis inhibitors (doxorubicin and cisplatin), and mitochondrial respiratory inhibitors (rotenone, antimycin A, MPP^+, and oligomycin A) were all clustered in the same positions.

Moreover, the characteristic features of toxicity endpoints affected by the drugs in *Ciona* were classified into two clusters. One cluster contained rotation of body axis, body growth, and heart rate; they were relatively sensitive to tubulin polymerization/depolymerization inhibitors, but insensitive to DNA/RNA synthesis inhibitors. Another cluster contained absorption of degenerated tail, ampulla elongation, and stomach formation; these were sensitive to DNA/RNA synthesis inhibitors, but relatively insensitive to tubulin polymerization/depolymerization inhibitors. In addition, tail absorption and ampulla elongation, but not stomach formation, were strongly suppressed by the mitochondrial respiratory inhibitors.

These results suggest the developmental drug toxicity profiles of six endpoints we set depended on the mechanisms of action behind the drugs, allowing us to predict the mode of action by the new drugs, or offer a way to classify them based on functional similarities.

4. Discussion

Current reports regarding toxicity evaluation using *Ciona* embryogenesis [14–16] assess the presence of toxicity based on mortality rate or abnormal developmental rate. Here, we demonstrated an evaluation system for drug toxicity that quantifies the developmental phenotypic changes of *Ciona* using six toxicity endpoints. These endpoints enabled us to assess not only the presence of toxicity but also the toxicity profiles represented by the inhibitory patterns of six toxicity endpoints. Unfortunately, these toxicity profiles cannot be applied directly to the toxicity profiles of adult humans since most of the organs used in toxicity endpoints except for the stomach and the heart do not exist in humans, and toxicity evaluation was performed on developing *Ciona* larvae, not adults. However, considering the sensitivity of detecting drug toxicity, employing six toxicity endpoints during embryonic and larval development is advantageous due to substantial changes in toxicity endpoints with drug treatment over a wide range, as seen from 2 dpf to 4 dpf. Furthermore, since previous studies show embryonic and larval fish are more sensitive to cytotoxic drugs than adults [17–19], *Ciona* larvae relative to fish might be more sensitive to toxicity drugs than adults.

Using this toxicity evaluation system, we detected toxicity in *Ciona* at MTC. As shown in Table 1, MTCs in *Ciona* roughly corresponded to concentrations of each drug similar to toxic effects experienced by zebrafish. Paclitaxel shows no toxic effect on zebrafish larvae near MTC, but can inhibit cleavage of fertilized eggs in sea urchin at 10 μM [20]. Nevertheless, MTCs were validated in other species, and toxicity profiles at MTCs may be caused by sufficient inhibition of the targets. At MTCs, we obtained a variety of toxicity profiles that were dependent on the drugs, but also similar toxicity profiles in drugs with similar modes of action. However, it is not clear how these toxicity profiles are subjected to the mode of action; therefore, future studies are needed to describe more precisely the inhibitory mechanisms of toxicity endpoints.

In the present study, we demonstrated the possibility of using *Ciona* as a new animal model to evaluate drug toxicity. Toxicity assay using *Ciona* is also a promising step toward high-throughput screening: adult *Ciona* produce a higher egg yield than zebrafish,

Table 1
Comparative toxicity of drugs employed in this study vs. previous studies using other species.

Drugs	Present study	Literature			
	MTC	Concentration	Species	Description	Reference
Paclitaxel	3 μM	10 μM	Sea urchin	Inhibition of cleavage of fertilized eggs.	[20]
Vinblastine	3 μM	3 μM	Zebrafish	Lethal effect on larvae (LC50 = 3.05 μM).	[21]
Doxorubicin	30 μM	>43 μM	Zebrafish	Lethal effect on embryos or larvae.	[18]
Cisplatin	30 μM	50 μM	Zebrafish	Induction of hair cell death.	[22]
Rotenone	0.3 μM	0.3 μM	Zebrafish	Lethal effect on embryos (LC50 = 300 nM).	[23]
Antimycin A	10 nM	2.9 nM	Zebrafish	Lethal effect on embryos (LC50 = 2.9 nM).	[23]
MPP^+	1 μM	1 μM	Zebrafish	Induction of loss of dopaminergic cells.	[24]
Oligomycin A	3 μM	1.23 μM	Zebrafish	Lethal effect on embryos (LC50 = 1.23 μM).	[23]

LC50 is the concentration that is lethal for 50% of embryos.

allowing assays using thousands of synchronized embryos with large numbers of drugs. Future studies of toxicity evaluation using *Ciona* can also facilitate the development of new drugs, saving time and costs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.119

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.119.

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