Human prokinetic drugs promote gastrointestinal motility in zebrafish

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Key Messages

• We developed a novel zebrafish gastrointestinal (GI) motility model for drug screening and efficacy assessment.
• Zebrafish were fed with Nile red for 16 h, followed by drug treatment for 6 h. Drug effect on zebrafish GI motility was quantitatively assessed using GI tract fluorescent image-based morphometric analysis.
• All 4 human prokinetic drugs (domperidone, metoclopramide, mosapride and magnesium sulfate) increased zebrafish GI motility.
• Our results suggest that larval zebrafish motility model developed in this study is a useful tool for whole-animal in vivo GI transit studies and for assessing prokinetic drugs.

Abstract

Background Gastrointestinal (GI) motility disorders are highly prevalent in populations worldwide and the development of effective and safe drug treatments for GI motility disorders has proven challenging. In this study, taking advantage of the transparency of larval zebrafish, we developed a novel zebrafish GI motility model for drug screening and efficacy assessment.

Methods Zebrafish at 5 days postfertilization were fed 10 μg/L Nile red for 16 h, followed by drug treatment for 6 h. Tested drugs were delivered into the zebrafish by direct soaking. Drug effect on zebrafish GI motility was quantitatively assessed using GI tract fluorescent image-based morphometric analysis. During all the periods of the experiments, the zebrafish were not fed any food. Key Results All four human prokinetic drugs (domperidone, metoclopramide, mosapride, and magnesium sulfate) increased zebrafish GI motility, whereas two drugs that inhibit human GI movement (atropine and anisodamine) and two negative control drugs (glucose and vitamin C) did not show statistically significant effect on zebrafish GI motility. Conclusions & Inferences These results suggest that larval zebrafish motility model developed here is a useful tool for whole-animal in vivo GI transit studies and for assessing prokinetic drugs.

Keywords gastrointestinal motility, Nile red, peristalsis, prokinetic drugs, zebrafish.

Abbreviations: d.p.f, days postfertilization; DMSO, dimethyl sulfoxide; GI, gastrointestinal; MNLC, maximum non-lethal concentration; ROI, region of interest.

INTRODUCTION

In normal digestion, food is transited through the gastrointestinal (GI) tract by rhythmic contractions called peristalsis. Abnormal GI contractions could lead to spasms or paralysis. Such disorders may be primary or secondary and may manifest in a variety of ways, including abdominal distention, recurrent obstruction, severe abdominal colicky pain, severe constipation, gastroesophageal reflux disease, intractable and recur-
dent vomiting, intestinal pseudo-obstruction, irritable bowel syndrome (IBS), fecal incontinence, and functional dyspepsia.1,2 Gastrointestinal motility disorders are highly prevalent in populations worldwide and the development of effective and safe drug treatments for GI motility disorders has proven challenging.3

The propulsion of luminal content is due to integrated activities of the GI smooth muscle cells, the enteric nervous system and extrinsic innervations, pace-making interstitial cells of Cajal, the intestinal mucosa, and endocrine secretions.4,5 Due to its complexity, discovery and development of GI motility drugs face unique challenges. In vitro cell culture lacks organ structure and cannot accurately imitate gut motility. The isolated segments of rat, guinea-pig, and mouse intestines have been extensively used to assess intestinal motility function by measuring electrical signals with or without video imaging for construction of spatio-temporal maps of changes in diameter. However, these ex vivo intestinal experiments are not carried out in an intact animal and thus cannot fully describe intestinal motility under a physiological condition.5,7 Mammalian GI motility models are often time-consuming, labor-intensive, and expensive.8,9 Development of new animal models and rapid assay methods for GI motility research and drug screening are urgently needed.

Zebrafish Danio rerio is emerging as a predictive vertebrate animal model for in vivo assessment of drug efficacy, toxicity, and safety.10–14 An important advantage of the zebrafish animal model is that the morphological and molecular basis of tissues and organs is either identical or similar to other vertebrates, including humans.15,16 The sequence and presumed function of many genes that are important for vertebrates are conserved in the zebrafish.17 The zebrafish GI tract also displays an anatomy and cellular architecture that is similar to the human GI tract, with concentric layers of inner epithelia, connective tissue, circular muscle, and outer longitudinal muscle layers.5,18 Zebrafish has developed a functional GI tract exhibiting spontaneous rhythmic muscular contractions by 5 days postfertilization [d.p.f.], which is also the approximate time that spontaneous feeding begins.19 In the last decade, a few scientists have successfully used zebrafish as a model organism to investigate GI physiology.19, 20 In contrast to other vertebrate models, zebrafish has several advantages, such as external development and transparent embryos and larvae, making it possible to visually observe internal organs including GI movement in vivo in a non-invasive way. In this study, we have developed and validated a novel zebrafish GI motility model that is convenient and predictive for rapid in vivo screening and efficacy assessment of GI motility drugs.

MATERIALS AND METHODS

Zebrafish handling

Adult AB strain zebrafish were housed in a light- and temperature-controlled aquaculture facility with a standard 14 : 10 h light/dark photoperiod and fed live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for nature mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28 °C in fish water [0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 mS/cm, and hardness 53.7–71.6 mg/L CaCO3]. The embryos were washed and staged at 6 and 24 h postfertilization.21 The zebrafish facility at Hunter Biotechnology Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA LAC) International.

Fluorescent dye and drugs

Fluorescent dye Nile red was bought from Sigma-Aldrich (St. Louis, MO, USA) and used as a tracer to visualize the zebrafish GI transit. Human GI motility modulators domperidone, metoclopramide, mosapride, magnesium sulfate, atropine, anisodamine, and negative control drugs glucose and vitamin C were selected for the development and validation of the zebrafish GI motility model. All the tested drugs were purchased from Sigma-Aldrich. Stock solutions were prepared in either 100% dimethyl sulfoxide (DMSO) or ultrapure water and serial dilutions were made before each experiment. Zebrafish treated with 1.0% DMSO or fish water were used as vehicle controls. Untreated zebrafish were used to confirm that the vehicle solvent did not have an adverse effect on the zebrafish. Ammonia concentration was measured at the end of experiments and no ammonia accumulation was detected in fish water. Dissolved oxygen concentration in fish water was kept >80% during the experiments.

Determination of zebrafish gut filling time

Zebrafish exhibit a functional GI tract with spontaneous and rhythmic muscular contractions and exogenous feeding starting by 5 d.p.f.22–24 Consequently, we chose 5 d.p.f. zebrafish as an optimal stage for the GI motility model development. The zebrafish were placed in a 6-well microplate at a density of 30 zebrafish per well with 3 mL of fish water and Nile red was added to the wells at a final concentration of 10 μg/L. Zebrafish were incubated at 28 °C in the dark, anesthetized, and imaged under a fluorescent stereomicroscope (Nikon AZ 100; Tokyo, Japan) at 1, 2, 3, 4, 6, 10, 16, and 20 h after adding Nile red. Fluorescent dye intensity in zebrafish GI tract, which is representative of the amount of GI tract contents, was quantified and compared among the zebrafish groups fed Nile red at different time points to determine zebrafish gut filling time. Zebrafish gut filling time was defined as the time period at which the zebrafish GI tract was most filled with Nile red.

Optimization of zebrafish drug treatment period

After determining the zebrafish gut filling time, we used a well-characterized and extensively used prokinetic drug domperidone to optimize the drug treatment period for assessing efficacy of GI motility modulators in the model. Zebrafish at 5 d.p.f. were loaded with 10 μg/L Nile red for 16 h, then the dye was removed and zebrafish were washed three times. The Nile red-fed zebrafish...
were further treated with 30 mg/L domperidone for various time periods: 1, 2, 4, 6, 9, 24, and 30 h, respectively, and the optimal zebrafish drug treatment period was selected.

**Determination of maximum non-lethal concentration (MNLC)**

To determine the MNLC of a drug, 6 d.p.f zebrafish were treated with a testing drug for 6 h and mortality was recorded at the end of treatment. Dead zebrafish was defined as the absence of heartbeat under a dissecting stereomicroscope (Nikon AZ 100). In the initial tests, five concentrations (0.1, 1, 10, 100, and 500 mg/L) were used for each drug. If a MNLC could not be found from the initial tests, additional concentrations within the range of 0.01–2000 mg/L were tested. Mortality curves were generated using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) and the MNLC was determined with logistic regression.

**Assessment of drug effect on zebrafish GI motility**

Six known human GI motility modulators including drugs that increase GI motility (domperidone, metoclopramide, mosapride, and magnesium sulfate) and those that decrease GI motility (atropine and anisodamine), and two negative control drugs (glucose and vitamin C) were selected for the validation of the zebrafish GI motility model. The zebrafish were fed Nile red for 16 h, followed by drug treatment for 6 h at three concentrations (1/10 MNLC, 1/3 MNLC, MNLC). At the end of the treatment, 20 zebrafish from each group were randomly selected for GI tract image acquisition. The gut of a 6 d.p.f zebrafish larva is situated predominantly posterior to the liver and anterior to the cloacal pore. The zebrafish were anesthetized by 0.2% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich), immobilized in 3% methyl cellulose, and placed in the dorsolateral position with anterior left. Images of gut region of interest (ROI) were acquired at 80× magnification under the same exposure time and fluorescence gain using a fluorescent stereomicroscope installed with a DS-F1c color cooled digital camera (Nikon). Quantitative image analysis of GI tract total fluorescence was performed using NIS Elements D advanced software (Nikon) and data were expressed as mean ± SEM. Total fluorescent signal ($S$) = area of ROI × average fluorescent intensity in ROI. Drug effect on zebrafish GI motility was quantified based on the total fluorescent signal in zebrafish GI tract.\textsuperscript{11} Effect of a test drug or compound on GI motility was calculated using the formula below:

\[
\text{Drug effect on GI emptying} (\%) = \left(1 - \frac{S_{\text{vehicle}}}{S_{\text{compound}}} \right) \times 100\%
\]

A positive percentage indicates that the tested drug promotes GI motility and a negative percentage suggests that the tested drug suppresses GI motility.

**Statistics**

One-way ANOVA followed by the Dunnett’s test was used to compare differences among groups. All statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA) and $p < 0.05$ was considered statistically significant. For quantitative analysis, all data were presented as mean ± SEM and results were statistically compared between drug-treated and vehicle-treated zebrafish groups.

**Quality control standard**

Successful experiments must meet all the milestones below: (i) zebrafish natural death in untreated and vehicle-treated groups was ≤10%; (ii) there was no statistical difference ($p > 0.05$) in assessed endpoint or signal between untreated and vehicle-treated groups; (iii) intraplate and interplate coefficient of variation (CV) was ≤25%; and (iv) positive control drug worked ($p < 0.05$ as compared with model group and mean difference >1SD of model group).

**RESULTS**

To develop a zebrafish motility model for drug screening and efficacy assessment, zebrafish gut filling time was first determined. The zebrafish at 5 d.p.f were fed Nile red in a 6-well microplate and images were acquired under a fluorescent stereomicroscope at various time points from 1 to 20 h of Nile red feeding [Fig. 1] and intensity of the fluorescent tracer through the GI tract of individual zebrafish was quantified.

**Figure 1** Zebrafish gut filling time determination. (A) Representing images from zebrafish GI tract filled with fluorescent dye Nile red after loaded with the dye for various times: 0, 1, 16, and 20 h. SB, swim bladder; GI, gastrointestinal; scale: 500 μm. (B) Time-course of zebrafish GI tract fluorescent intensity.
Zebrafish gut filling time was defined as a time period at which zebrafish GI tract was filled with Nile red at maximum. As reported earlier by other investigators, we confirmed that Nile red was a non-absorbable fluorescent tracer. This dye could be easily observed as it traveled along the zebrafish GI tract and could be in sufficient amounts to gradually fill the zebrafish GI lumen. At the feeding concentration used in this study (10 μg/L) and the feeding time periods up to 20 h, Nile red had no observable effects or toxicity to zebrafish. We found that the fluorescent intensity from Nile red in the zebrafish GI tract increased with time and that the fluorescent intensity reached its maximum at 16 and 20 h after fed Nile red, indicating that Nile red intake of zebrafish GI tract was saturated at these time points. Nile red feeding for 16 h was, therefore, chosen as the zebrafish gut filling time.

After obtaining the zebrafish gut filling time, next we used a known human prokinetic drug domperidone to optimize the drug treatment period for in vivo screening and assessment of the GI tract motility modulators in the zebrafish. The zebrafish were incubated with 10 μg/L Nile red for 16 h [zebrafish gut filling time]. After removing the Nile red and washing three times, zebrafish were further treated with domperidone for various time periods from 1 to 30 h [Fig. 2A]. At the end of the treatment, zebrafish images were taken under a fluorescent stereomicroscope and the fluorescent intensity in zebrafish GI tract was quantitatively analyzed. In the untreated control group, the zebrafish GI emptying rate was from 22% [1 h] to 35% [30 h], and zebrafish GI tract fluorescent image profiles between 6 and 30 h after Nile red feeding were closely similar [Fig. 2B]. Gastrointestinal emptying rates in domperidone-treated zebrafish were from 36% [1 h treatment] to 69% [30 h treatment]. Compared with the untreated control group, domperidone significantly facilitated GI emptying of Nile red of zebrafish GI tract in a time-dependent fashion, starting at 6 h [p < 0.001], and no significant difference between 6 and 30 h [p > 0.05]. Because domperidone treatment for 6 h resulted in a statistically increased GI emptying of Nile red that was similar to longer treatment times as indicated in Fig. 2A and also taken into account experimental convenience, 6 h was selected as the optimized drug treatment period. Thus, we optimized a zebrafish GI motility model for drug screening and efficacy assessment using 5 d.p.f zebrafish fed 10 μg/L Nile red for 16 h, followed by drug treatment for 6 h.

To validate the zebrafish GI motility assay developed above, four known human prokinetic drugs [domperidone, metoclopramide, mosapride, and magnesium sulfate], two known human GI motility suppressors [atropine and anisodamine], and two negative control drugs (glucose and vitamin C) were selected and tested in the model. Three concentrations at 1/10 MNLC, 1/3 MNLC, and MNLC were assessed for each drug (Table 1). Maximum non-lethal concentration was 30 mg/L for domperidone, 1000 mg/L for metoclopramide, 10 mg/L for mosapride, 2000 mg/L for magnesium sulfate, 100 mg/L for atropine, 100 mg/L for anisodamine, 100 mg/L for Vitamin C, and 3% for glucose, respectively.

As expected, human prokinetic drugs domperidone, metoclopramide, mosapride, and magnesium sulfate significantly promoted zebrafish GI emptying by 7.4–39.4%, –4.1 to 18.1%, 8.7–81.9%, and 13.3–74.1%, respectively. Statistically significant positive effect on zebrafish GI motility was observed at MNLC for domperidone [p < 0.01], metoclopramide [p < 0.05], and magnesium sulfate [p < 0.001], and at 1/3MNLC [p < 0.001] and MNLC [p < 0.001] for mosapride (Table 2, Fig. 2). Atropine and anisodamine suppressed zebrafish GI motility and reduced GI emptying (indicated as negative percentages), but no statistical differences were found when compared with the vehicle control [p > 0.05]. Negative control drugs vitamin C and glucose had no statistically significant effect on zebrafish GI motility [p > 0.05; Fig. 3].

**DISCUSSION**

Nile red [9-diethylamino-5H-benzo[α]phenoxazine-5-one] is a fluorescent dye and initially used to label intracellular lipid droplets in live cultured peritoneal...
macrophages and smooth muscle cells. Recently, Nile red has been widely used in fat metabolism disease models as a non-toxic and vital staining dye. When zebrafish are exposed to Nile red, yolk sac, a lipid rich tissue, can be observed under a fluorescent microscope. Zebrafish yolk sac is absorbed mostly by 5–6 d.p.f, and at this developmental stage, Nile red-filled zebrafish GI tract could be visualized clearly. In the preliminary studies, we found that Nile red is a non-absorbable tracer that can be excreted through the zebrafish GI tract and that Nile red could be used as a vital dye to assess zebrafish GI motility and potentially for GI motility drug screening.

Zebrafish develop a functional GI tract and start spontaneous feeding by 5 d.p.f. In this study, we determined that larval zebrafish gut filling time with Nile red was ~16 h and that the effect of prokinetic drugs on zebrafish GI motility was assessable after 6 h of drug treatment. Thus, we developed a zebrafish GI motility model for drug screening and efficacy assessment using 5 d.p.f zebrafish loaded with 10 μg/L Nile red for 16 h, followed by drug treatment for 6 h. Drug effects on zebrafish GI motility were quantified using the GI tract fluorescent image-based morphometric assay. In the model validation studies, all four human prokinetic drugs (domperidone, metoclopramide, mosapride, and magnesium sulfate) increased zebrafish GI motility, whereas two human GI motility suppressing drugs (atropine and anisodamine) did not show significant effect on zebrafish GI movement, suggesting that the zebrafish motility model we developed in this

Table 1 Drug concentrations used for assessing GI motility in zebrafish larvae

<table>
<thead>
<tr>
<th>Drugs</th>
<th>1/10MNLC</th>
<th>1/3MNLC</th>
<th>MNLC</th>
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<tbody>
<tr>
<td>Domperidone*</td>
<td>3</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>100</td>
<td>300</td>
<td>1000</td>
</tr>
<tr>
<td>Mosapride*</td>
<td>1</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>200</td>
<td>600</td>
<td>2000</td>
</tr>
<tr>
<td>Atropine</td>
<td>10</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>Anisodamine*</td>
<td>0.3%</td>
<td>1%</td>
<td>3%</td>
</tr>
<tr>
<td>Glucose†</td>
<td>10</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

*The drugs were prepared in DMSO, others were in ddH2O. †Mass percentage.

Table 2 Quantitative analysis of drug effect on gastrointestinal emptying

<table>
<thead>
<tr>
<th>Drugs</th>
<th>1/10MNLC</th>
<th>1/3MNLC</th>
<th>MNLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domperidone</td>
<td>7.4</td>
<td>15.0</td>
<td>39.4**</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>-4.1</td>
<td>-6.3</td>
<td>18.1*</td>
</tr>
<tr>
<td>Mosapride</td>
<td>8.7</td>
<td>31.0***</td>
<td>81.9***</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>13.3</td>
<td>5.7</td>
<td>74.1***</td>
</tr>
<tr>
<td>Atropine</td>
<td>2.3</td>
<td>14.2</td>
<td>-21.0</td>
</tr>
<tr>
<td>Anisodamine*</td>
<td>13.6</td>
<td>3.0</td>
<td>-3.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.9</td>
<td>14.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>-1.8</td>
<td>12.1</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

*Compared with vehicle control: *p < 0.05, **p < 0.01, ***p < 0.001. A positive percentage indicates that the tested drug promotes GI motility and a negative percentage suggests that the tested drug suppresses GI motility.

Figure 3 Zebrafish GI fluorescent intensity after exposure to the tested drugs for 6 h. GI fluorescent signal was significantly reduced in zebrafish treated with all four promoting peristalsis drugs domperidone, metoclopramide, mosapride, and magnesium sulfate (*p < 0.05, **p < 0.01, ***p < 0.001). However, two drugs that inhibit human GI movement (atropine and anisodamine) and two negative control drugs (glucose and vitamin C) did not show significant effect on zebrafish GI motility. All values were expressed as the mean ± SEM.
study is suitable for screening and assessing prokinetic drugs.

Our original intention for this study was to develop an in vivo zebrafish motility model that could be used for assessing both prokinetic drugs and GI transit inhibitors. Surprisingly, the zebrafish motility model optimized in this report worked well for human prokinetic drugs but not for human GI tract movement suppressors. We realized that it might be difficult to assess both pro- and anti-motility drugs in the same zebrafish model mainly because the optimized Nile red loading time for kinetic drug assessment was in fact not suitable for assessing drugs that inhibit GI tract movement. As reported in this study, we found that the effect of a prokinetic drug could be assessed in zebrafish whose GI tract was filled with Nile red at maximum. Perhaps the effect of GI motility suppressors could be better assessed in zebrafish using alternative approaches like alternative tracers or incomplete loading. We are now close to optimizing another zebrafish model that could be used for assessing GI motility inhibitors and will present this new model in our future report.

In this study, all the experiments were performed in 6-well microplates that were wrapped with aluminum foil for the dye [Nile red] to be protected from light. Zebrafish lived in the dark during experiments. Unexpectedly, we found that zebrafish GI fluorescent intensity was only slightly reduced in untreated control zebrafish from 6 to 30 h after removing Nile red, indicating that zebrafish GI motility is at a very low frequency in the dark environment. This phenomenon was also reported by other investigators and may be related to circadian rhythms. It will be interesting to investigate the physiological factors that regulate this delay. Many studies have suggested that the GI tract displays biologic rhythms in basal gastric acid output, epithelial cell proliferation, motility, and appetite regulation. The zebrafish GI tract moves during the day, frequently following awakening or a meal, but rarely moves during the night. When treated with glucose, we demonstrated that the zebrafish GI fluorescent intensity decreased, but with no significant difference.

Zebrafish as a model organism offers opportunities for rapid in vivo drug discovery and development. The process of GI transit is complex, requiring the coordination of many different tissues and external influences. Larval zebrafish GI transit model developed in this study was a live and physiology-associated whole-animal assay. This model is a useful tool for whole-animal in vivo GI transit studies and for screening GI motility promoting drugs. The use of zebrafish as an alternative animal model for prokinetic drug screening and assessment could save time, decrease costs, and reduce drug failure at later stages of drug development. Extended studies are needed to further validate this model using more human drugs and negative control compounds, and to develop this model into a higher throughput screening or even an automatic assay system.

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CONFLICTS OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTION

C-QL designed the research; JZ and S-YG performed the research; JZ and YZ analyzed the data; JZ and C-QL wrote the paper.

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