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Developmental and Full-Life Cycle Exposures to Guanylurea and Guanylurea–Metformin Mixtures Results in Adverse Effects on Japanese Medaka (*Oryzias latipes*)

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Abstract: Metformin is currently thought to be the highest drug by weight released into the aquatic environment, as a direct result of its widespread use in the treatment of a number of human health disorders. The removal of metformin from wastewaters is directly related to the formation of guanylurea (metformin's only known persistent degradation product), which is generally present at higher concentrations in surface waters than the parent compound. With metformin use rising steadily, it is important to characterize the effects of guanylurea on nontarget aquatic organisms. We recently demonstrated the effects of developmental exposure to environmentally relevant concentrations of metformin on the growth of early life stage (ELS) medaka as well as effects on the body weight of adult male fish following full-life cycle exposures. In the present study, we describe similar effects of guanylurea exposure on these endpoints and life stages. Guanylurea led to effects on growth in a 28-d ELS assessment that were similar to those of metformin; however, these effects occurred at concentrations in the ng/L range compared with the µg/L range for metformin. A possible sex-dependent association with body weight changes was also observed in adults following a 165-d full-life cycle exposure to guanylurea alone or in a mixture with metformin. To our knowledge, the present is the first study to report the toxicity of guanylurea to nontarget aquatic organisms. *Environ Toxicol Chem* 2019;38:1023–1028. © 2019 SETAC

Keywords: Developmental toxicity; Guanylurea; Metformin; Nontarget organism toxicity; Pharmaceuticals

INTRODUCTION

The occurrence of pharmaceuticals in the aquatic environment has become a progressively important issue because of the increased use and excretion from anthropogenic pathways (e.g., sewage outfalls, landfill leachate, and runoff from animal feedlots). Concentrations of pharmaceuticals in aquatic environments are typically measured in the ng L^{-1} – µg L^{-1} range, and their detection is increasing in frequency with improving analytical methods (Corcoran et al. 2010; Fatta-Kassinos et al. 2011; Tanwar et al. 2014). Pharmaceutical compounds are generally present in the aquatic environment at concentrations well below the effective dose required for target organisms. However, in many instances the effect concentrations for nontarget aquatic organisms, including fish, have not been fully elucidated. Furthermore, some pharmaceuticals and personal care products that are used in high volume are considered to be pseudopersistent, despite their relatively short half-lives. This is because of their continuous renewal/discharge into aquatic environments, resulting in a high likelihood of lifelong exposure for aquatic biota (Ebele et al. 2017).

Metformin is currently thought to be the highest drug by weight released into the aquatic environment (Blair et al. 2013; Kolpin et al. 2002; Oosterhuis et al. 2013; Scheurer et al. 2012; Trautwein et al. 2014). The environmental prevalence of metformin is attributed to its widespread use in the treatment of a number of human health disorders, including common diseases such as type 2 diabetes, polycystic ovary syndrome, and cancers where insulin resistance is a factor (Morley et al. 2017). The use of metformin is rising steadily, with an estimated 80 million prescriptions written for it in 2015 in the United States alone (up from 59 million in 2011) making it the seventh most commonly prescribed drug in the United States. Metformin is also 1 of only 2 oral glucose-lowering agents to be included in the last several iterations of the World Health Organization's (2015) list of essential medications, underscoring its global importance.

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Interestingly, metformin is not biotransformed via metabolic processes in the human body. Consequently, an estimated 70% of the parent compound is excreted unchanged in urine and feces (Gong et al. 2012; Pentikäinen et al. 1979). The removal of metformin in wastewater-treatment plants (WWTPs) has been found to be directly related to the formation of guanylurea, which is the only presently known recalcitrant degradation product of metformin (Trautwein et al. 2014; Table 1). Although the degradation pathway is not yet fully elucidated, a bacterial double dealkylation (whereby both methyl groups are removed at the terminal nitrogen) has been proposed for the conversion of metformin to guanylurea in WWTPs (Markiewicz et al. 2017).

Metformin has been detected in European surface waters at concentrations up to 3 µg/L, with guanylurea concentrations exceeding those of metformin by an order of magnitude or more (Blair et al. 2013; Ghoshdastidar et al. 2015; Oosterhuis et al. 2013; Scheurer et al. 2012). In light of the fact that biotransformation and degradation products are often found at higher concentrations in the aquatic environment (and can exhibit different toxicities from the parent compound) it is critical to consider the action and comparative toxicity of major metabolites when evaluating risk. This is particularly true for pharmaceuticals, which are specifically designed to bind receptors in biota, many of which are highly conserved across taxa and can cause effects in nontarget organisms.

In a previous 28-d study, we demonstrated effects on growth in early life stage (ELS) medaka following exposure to environmentally relevant concentrations of metformin (Ussery et al. 2018). Therefore, one goal of the present study was to characterize the developmental toxicity of environmentally relevant concentrations of guanylurea on the growth of ELS medaka, as well as to examine the persistence of ELS effects on growth into adulthood. Given the co-occurrence of guanylurea and metformin in aquatic environments, the final goal of the present study was to examine the interactive effects of a guanylurea and metformin co-exposure in a full-life cycle exposure setting.

MATERIALS AND METHODS

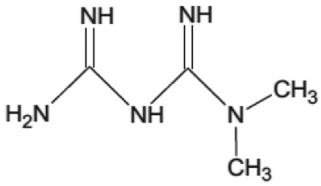
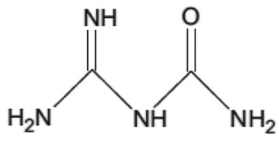
Animal care

The animal care protocol used in the present study is described in more detail in Ussery et al. (2018). Briefly, adult Japanese medaka (FLFII strain) were obtained from the National Institute of Basic Biology (Okazaki, Japan) and housed in 70-L tanks in the Aquatics Facility at the University of Ontario Institute of Technology (Oshawa, Ontario, Canada). All embryos were obtained by gently stripping eggs from each gravid female, then placed in rearing solution (0.1% sodium chloride, 0.0163% magnesium sulfate heptahydrate, 0.004% calcium chloride dihydrate, 0.003% potassium chloride, and 0.0001% methylene blue, prepared in deionized water) and kept in a climate-controlled room (25 °C) for 1 h to assess viability. Viable embryos were housed until hatch in a temperature-controlled room set at 25 °C with a 16:8-h light:dark photoperiod cycle. Chambers for all larval and adult fish were kept at 26 ± 1 °C using a 16:8-h light:dark photoperiod cycle for the entire experiment. Standard water quality parameters (temperature, water hardness, pH, nitrates, and nitrites) were monitored daily to ensure that acceptable levels were maintained.

ELS study

To determine the effects of guanylurea exposure on ELS medaka, 118 h postfertilization embryos were separated by sex by extricating males with fluorescing leucophores via fluorescence microscopy (Leica DM 2000 microscope). Male and female medaka were kept separate for the remainder of the exposure to determine if sex acted as an additional biological variable that influenced toxicity. Embryos were randomly assigned to 1 of 5 nominal concentrations (100, 32, 10, 3.2, and 1.0 ng/L) of waterborne guanylurea (Sigma-Aldrich; Chemical Abstracts Service no. 141-83-3) or a control with laboratory water only. Guanylurea concentrations <0.25 µg/L (method detection limit [MDL]) are reported as nominal concentrations throughout. Although limitations in our analytical methods prevented us from detecting <0.25 µg/L with the requisite degree of certainty, concentrations below our MDL are still well below those commonly detected in surface waters (and are therefore considered environmentally relevant). Any treatment concentrations that were unable to be confirmed analytically will be clearly identified in the present study as nominal concentrations (those not clearly identified as nominal were confirmed using the methods described in the *Determination of treatment concentration* section).

TABLE 1: Chemical structure, molecular formula and weight, and pK_a of metformin and guanylurea

	Metformin	Guanylurea
Chemical structure		
Molecular formula	$C_4H_{11}N_5$	$C_2H_6N_4O$
Molecular weight (g/mol)	129.2	102.1
pK_a	2.8[a] and 12.3	8.0 and 13.5
logK_{OW}	–2.64	—

[a]From Hernández et al. (2015) and Scheurer et al. (2012).

Embryos were maintained in plastic Petri dishes (Fisher Scientific; 60 × 15 mm), each containing 20 mL of the assigned treatment solution, renewed daily. Four replicates per concentration per sex were used, with each replicate containing 20 eggs (80 eggs/treatment/sex). Embryos were monitored daily under a dissecting stereomicroscope (Leica EZ4D, ×20 magnification) using the developmental staging methods described by Iwamatsu (2004) and Wakamatsu et al. (2003). On hatch, larval fish were transferred to 1-L plastic trays corresponding with their exposure concentration and replicate number for the remainder of the 28-d study (with subsampling time points at days 7 and 14). Waterborne exposures were achieved using a flow-through system (Watson-Marlow 200 Series 16-channel peristaltic pump) that delivered stock concentrations from 1-L amber bottles. Larval fish were fed 15 mL of concentrated live premium grade brine shrimp (Brine Shrimp Direct) twice per day for the entirety of the exposure. Trays were vacuumed once per day to remove uneaten brine shrimp and waste.

Life-cycle growth study

The life-cycle study was executed in the same manner as the ELS study; however, 2 modifications were made based on the results of the ELS study: 1) sex was not used as an initial biological variable, and 2) the treatment concentrations were amended. A 1.0 ng/L nominal treatment was chosen because it was the experimental lowest-observed-effect concentration found in the ELS study. A 7.5 µg/L treatment was also included because it more closely reflects the current levels found in the environment. In addition, a mixture (3.2 µg/L metformin + 7.5 µg/L guanylurea; a molar ratio of 1:3.5 metformin [20 nM]:guanylurea [70 nM]) treatment was added to the experiment because this represents concentrations of each compound that are typically found in surface waters. For continuity with the units used for the rest of the exposures, the mixture treatment will be reported using µg/L (instead of nM).

Larvae were monitored through hatch, placed in 1-L plastic trays for 28 d, then moved to 70-L glass aquaria in the corresponding treatments, and housed there for the remainder of the 165-d study. Waterborne guanylurea concentrations were achieved via a flow-through system using the methods described. Three replicate tanks were used per treatment, with 15 fish per replicate. Medaka were fed 15 mL of concentrated live brine shrimp twice per day for the first 28 d of the exposure. The volume of brine was increased by 5 mL every other week, to a maximum brine volume of 80 mL. Tanks were vacuumed after each feeding to remove remaining brine and debris.

Biological endpoints

Fish were euthanized in water containing tricaine methanesulfonate (200 mg/L) buffered with sodium bicarbonate (in equimolar ratios) at the end of each exposure period (28 or 165 d). To assess a potential difference in growth rate, ELS fish were subsampled at days 7, and 14 of the 28-d exposure. Individual fish length was measured using a digital caliper, and wet weight was measured using a Mettler Toledo MX5 microbalance (28 d posthatch [dph]) or a Mettler Toledo balance (AB204-S; 165 dph). Larval fish were immediately flash-frozen in liquid nitrogen and stored at –80 °C for future analysis. For all statistical analyses involving ELS fish, measurements from individual larvae were used to generate a replicate mean, and replicate means were further averaged to generate treatment means. In addition, condition factor was calculated using the medaka established equation: weight (g) × 105/length $(mm)^3$ (Foran et al. 2003).

Determination of treatment concentration

Briefly, water samples were collected from all flow-through systems in each experiment and analyzed at the Water Quality Centre at Trent University (Peterborough, Ontario, Canada). Water samples were collected twice during the ELS study, once on day 7 posthatch ($n = 4$/treatment) and once on day 21 posthatch ($n = 4$/treatment). During the life-cycle study, flow-through systems were sampled on days 55 ($n = 3$/treatment), 110 ($n = 3$/treatment), and 155 ($n = 3$/treatment). Water samples were placed in 60-mL polypropylene copolymer bottles and stored at 4 °C in the dark until transport to Trent University. Concentrations of waterborne treatments were determined using a Shimadzu 10 A liquid chromatograph coupled with an AB Sciex Qtrap 5500 mass spectrometer. The MDL for guanylurea was determined to be 0.25 µg/L. Therefore, it was necessary to use nominal exposure concentrations to describe treatments <0.25 µg/L. In the adult growth study, water samples were collected from all treatment tanks to monitor the concentration of both guanylurea and metformin. Similar to the ELS study, nominal exposure concentrations were used to describe treatments <0.25 µg/L; however, to estimate the concentration present in the 1.0 ng/L treatment tanks, the stock concentration (prepared to a nominal concentration of 1.0 µg/L) was measured. Treatment tanks should be 1000-fold lower than the stock bottle concentration (measured treatment and stock bottle concentrations are available in Supplemental Data, Table S1).

Statistical analysis

All data were analyzed using SigmaPlot (Systat Software) unless otherwise specified. Normality of all data was confirmed using a Shapiro–Wilk test prior to statistical analysis. Effects of sex on size (length and wet wt) of 28-dph fish were assessed using a 2-factor analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Treatment effects on length and weight for larval medaka (pooled sexes) were determined using a 2-factor ANOVA (with treatment concentration and time as factors) followed by Tukey's post hoc test. Treatment effects on adult growth metrics were also determined using a one-factor ANOVA, followed by Tukey's post hoc test. Tests of statistical significance were set at an α value of 0.05. For both the ELS and adult studies, measurements from individual fish were used

to generate a replicate mean, and replicate means were further used to generate treatment means.

RESULTS AND DISCUSSION

ELS study

No significant differences in larval mortality ($df = 5$, $F = 0.43$, $p = 0.831$), hatch success ($df = 5$, $F = 0.401$, $p = 0.846$), or time-to-hatch ($df = 5$, $F = 1.914$, $p = 0.089$) were observed in any of the guanylurea treatments, relative to controls (Supplemental Data, Table S2). Similar to the parent compound metformin (Ussery et al. 2018), sex did not significantly affect the size of 28-dph fish exposed to guanylurea in the present study (length, $df = 5$, $F = 0.266$, $p = 0.931$; wt, $df = 5$, $F = 0.464$, $p = 0.803$); thus, the growth metrics were not separated by sex for the remainder of the ELS analysis.

Guanylurea exposure significantly decreased fish length over the course of the 28-d study (Figure 1). Significant decreases in mean body weight ($df = 17$, $F = 1626$, $p < 0.001$; Figure 1A) and mean fish length ($df = 17$, $F = 316$, $p < 0.001$; Figure 1B) were detected in all guanylurea-exposed fish by 28 dph, regardless of concentration. Because body size is an important factor that determines the likelihood a larval fish will successfully evade predation and survive to sexual maturity, exposure to guanylurea during development may have consequences for the fitness of ELS fish through reduced body size (Crowder et al. 1992).

Full-life cycle study

There were no significant treatment effects of guanylurea alone or in combination with metformin on any developmental endpoints measured in the full-life cycle portion of the present study. These included percent larval mortality ($df = 3$, $F = 1.20$, $p = 0.838$), hatch success ($df = 3$, $F = 0.64$, $p = 0.644$), and time-to-hatch ($df = 3$, $F = 1.66$, $p = 0.356$; Supplemental Data, Table S3). Again, we did not observe any obvious deformities in control or treated fish.

No significant effects on adult (165 dph) medaka body size (length, wet wt, or condition factor) were observed in male or female fish exposed to guanylurea alone or in combination with metformin ($p > 0.05$; Tables 2 and 3). Although not statistically significant, male medaka appeared to be more sensitive to guanylurea exposure in the present study, with the most marked effects observed in males exposed to the guanylurea–metformin mixture. It is likely that the lack of statistical significance in this instance is attributable to the low number of replicate tanks ($n = 3$) included in the present study because the magnitude of change in body weight was fairly substantial in males (male body wt was decreased by 11% in the mixture, 9% in the 7.5 µg/L guanylurea treatment, and 6% in the nominal treatment concentration of 1.0 ng/L). Given the design of the present study (which utilized 3 replicates and a total of 150 fish), the calculated power to detect a 10% difference in size was 0.2. The authors recommend that future studies wishing to detect a 10% difference in size use at least 6 times the number of fish used in the present study, which will detect a 10% difference in size at a power of 0.8. Furthermore, other studies have reported similar sex-dependent sensitivities in male medaka and fathead minnows exposed to metformin (Niemuth and Klaper 2015; Ussery et al. 2018). Additional studies are needed to confirm these results with a higher degree of statistical confidence.

Comparative toxicity

We previously described the effects on growth of ELS medaka following exposure to environmentally relevant concentrations of waterborne metformin (Ussery et al. 2018). In the present study, we demonstrate that metformin's major metabolite, guanylurea,

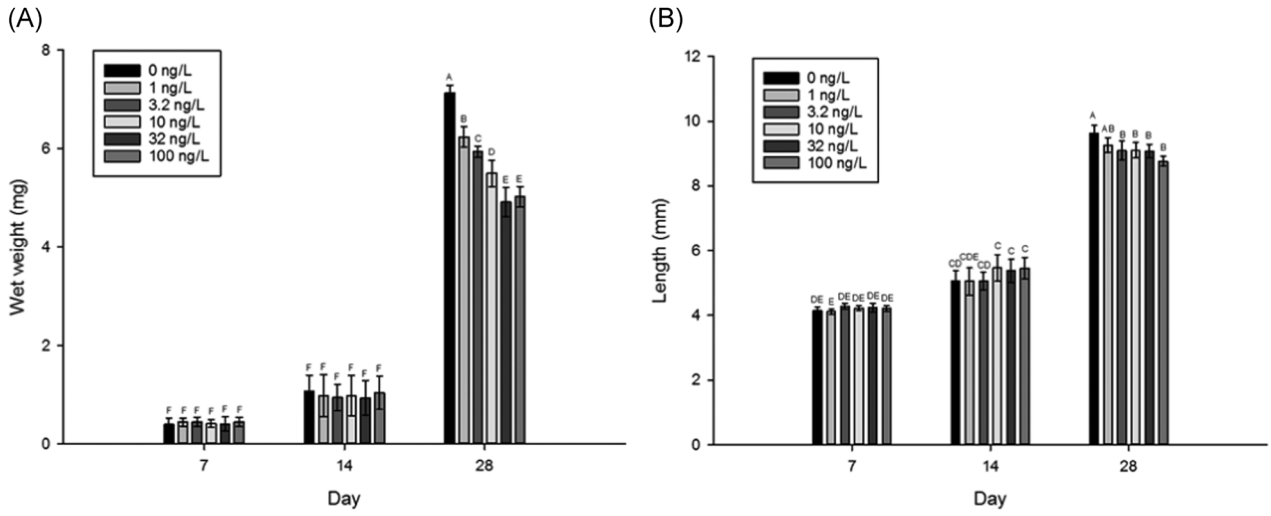


FIGURE 1: Mean wet weight (**A**) and length (**B**) of 28-d-old Japanese medaka (±standard error) by day and guanylurea treatment concentration (day 7, $n = 4$ [with 8 fish/replicate]; day 14, $n = 4$ [with 10 fish/replicate]; day 28, $n = 4$ [with 40 fish/replicate]). Letters indicate significantly different groups (one-factor analysis of variance, followed by Tukey's honestly significant difference, $\alpha = 0.05$). Nominal exposure concentrations are used to represent treatments. Concentrations <0.25 µg/L guanylurea fall below the method limit of detection; thus, nominal exposure concentrations are used to represent treatments.

TABLE 2: Mean weight, length, and condition factor (K) for adult male medaka exposure to guanylurea alone and in combination with metformin for 165 d[a]

Concentration	Mean weight (mg ± SE)	Mean length (mm ± SE)	Mean K (±SE)
Control	412.0 (26.7)	33.5 (0.454)	1.08 (0.029)
Guanylurea (1.0 ng/L)	389.0 (8.71)	33.3 (0.225)	1.04 (0.006)
Guanylurea (7.5 µg/L)	375.0 (6.21)	32.7 (0.270)	1.07 (0.014)
Mixture	368.0 (9.82)	32.5 (0.304)	1.06 (0.021)

[a]Mixture = 3.2 µg/L metformin + 7.5 µg/L guanylurea ($n = 3$). No significant growth differences were seen in male Japanese medaka exposed to any treatment concentration compared to control males (one-factor analysis of variance followed by Tukey's honestly significant difference, $\alpha = 0.05$; weight, $p = 0.204$; length, $p = 0.088$; condition factor, $p = 0.621$). Samples from the stock solution were used to monitor the treatment concentration for the 1–ng/L treatments because 1 ng/L is far below the method detection limit of the machine (= 0.25 µg/L).
SE = standard error.

causes similar effects on the growth of ELS fish. However, these effects occur at exposure concentrations < 0.25 µg/L, whereas effects were seen at metformin exposures of 1 µg/L or greater. Our results suggest that guanylurea exerts effects similar to those of metformin on ELS fish at much lower concentrations. In fact, the nominal guanylurea concentrations in the present study are 3 orders of magnitudes lower than the metformin concentrations required to elicit similar responses in ELS medaka, as seen by the 13% decrease in growth in each of the lowest treatments (i.e., nominal guanylurea treatment of 1.0 ng/L led to growth effects comparable to those in fish exposed to 1.0 µg/L metformin). This finding is of profound significance because guanylurea is commonly present in surface waters at concentrations nearly 3 times those of metformin. Effects on larval size can have effects at higher levels of organization, particularly for sensitive species, because body size is one of the main determinants of survival to sexual maturity for ELS fish in the wild (Crowder et al. 1992).

Despite the effects seen on larval size, no significant effect on adult size was seen; however, we did see similar, nonsignificant effects on the body weight of exposed male fish. These results, combined with the paucity of knowledge surrounding the effects of metformin and guanylurea on nontarget aquatic organisms, highlight the need for further toxicological studies on these compounds. Whole-organism screening studies that provide information about molecular initiating events and affected cellular pathways/physiological processes would be particularly valuable.

TABLE 3: Mean weight, length, and condition factor (K) for adult female medaka exposure to guanylurea alone and in combination with metformin for 165 d[a]

Concentration	Mean weight (mg ± SE)	Mean length (mm ± SE)	Mean K (±SE)
Control	449.0 (5.31)	33.9 (0.398)	1.16 (0.022)
Guanylurea (1.0 ng/L)	472.0 (12.0)	34.6 (0.124)	1.14 (0.021)
Guanylurea (7.5 µg/L)	415.0 (10.8)	33.2 (0.312)	1.14 (0.012)
Mixture	427.0 (30.2)	33.7 (0.412)	1.13 (0.041)

[a]Mixture = 3.2 µg/L metformin + 7.5 µg/L guanylurea ($n = 3$). No significant difference in female medaka growth was seen when compared to control females (weight, $p = 0.213$; length, $p = 0.266$; condition factor, $p = 0.581$). Samples from the stock solution were used to monitor the treatment concentration for the 1 ng/L treatments because 1 ng/L is far below the method detection limit of the machine (= 0.25 µg/L).
SE = standard error.

CONCLUSIONS

To our knowledge, the findings of the present study provide the first evidence of developmental guanylurea toxicity in ELS fish. The growth effects seen in the present study are similar to those of medaka exposed to the parent compound, metformin; however, these effects occurred at concentrations <0.25 µg/L (nominal concentrations as low as 1.0 ng/L), which is 3 orders of magnitude lower than the effect concentrations for metformin. Furthermore, concentrations of guanylurea shown to affect the growth of ELS fish are several orders of magnitude below those commonly measured in surface waters. Full-life cycle exposures to environmentally relevant concentrations of guanylurea alone and in a mixture with metformin appeared to impact the average body size of males more than females. However, small sample sizes preclude us from reaching statistical significance.

Because of the very low effect concentrations reported in the present study and the widespread use, distribution, and occurrence of guanylurea and metformin in surface waters, more research is urgently needed on the effects of these compounds in nontarget aquatic organisms. This is particularly true given the evidence we provide of developmental effects, which have the potential to cause effects at higher levels of organization.

Data accessibility—Data, associated metadata, and calculation tools are available from the corresponding author (erinussery@hotmail.com).

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Disclaimer—The views, discussions, conclusions, and future recommendations expressed in the present study are those of the authors alone and do not represent the views of any other agency or organization.

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