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RESEARCH ARTICLE

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Neuro- and hepatic toxicological profile of (*S*)-2,4-diaminobutanoic acid in embryonic, adolescent and adult zebrafish

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Abstract

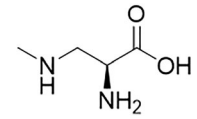
(*S*)-2,4-Diaminobutanoic acid (DABA) is a noncanonical amino acid often co-produced by cyanobacteria along with β-*N*-methylamino-L-alanine (BMAA) in algal blooms. Although BMAA is a well-established neurotoxin, the toxicity of DABA remains unclear. As part of our development of biocompatible materials, we wish to make use of DABA as both a building block and as the end-product of enzymatically induced depolymerization; however, if it is toxic at very low concentrations, this would not be possible. We examined the toxicity of DABA using both in vivo embryonic and adult zebrafish models. At higher sublethal concentrations (700 μM), the fish demonstrated early signs of cardiotoxicity. Adolescent zebrafish were able to tolerate a higher concentration. Post-mortem histological analysis of juvenile zebrafish showed no liver or brain abnormalities associated with hepato- or neurotoxicity. Combined, these results show that DABA exhibits no overt toxicity at concentrations (100-300 μM) within an order of magnitude of those envisioned for its application. This study further highlights the low cost and ease of using zebrafish as an early-stage toxicological screening tool.

KEYWORDS

cyanobacteria, DABA, diaminobutanoic acid, GABA, toxicity, zebrafish

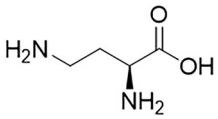
1 | INTRODUCTION

DABA is a noncanonical amino acid structurally related to ornithine and lysine in that is has a side-chain nitrogen (Figure 1). It is produced by cyanobacteria alongside β-*N*-methylamino-L-alanine (BMAA) (Al-Sammak, Hoagland, Cassada, & Snow, 2014; Fan, Qiu, Fan, & Li, 2015), a well-known neurotoxin (Al-Sammak, Rogers, & Hoagland, 2015; Cox et al., 2005; Cox, Davis, Mash, Metcalf, & Banack, 2016). As this is the major biological source of DABA and as DABA is always coexpressed with the highly toxic BMAA, determining the toxicity of DABA has not been a high priority. Nevertheless, the toxicity of DABA has remained a subject of interest and debate as different studies report substantial variability in their toxicity data (Arscott & Harper, 1963; Beart & Bilal, 1977; Bergenheim et al., 2006; Chen, Flory, & Koeppe, 1972; Johnston & Twitchin, 1977; Main & Rodgers, 2018; O'Neal, Chen, Reynolds, Meghal, & Koeppe, 1968). High, but localized concentrations of DABA selectively kill tumor cells but not healthy glial cells, and have been investigated as a potential co-therapy for glioblastoma (Bergenheim et al., 2006). Similar elevated concentrations of DABA have also been associated with liver toxicity and the development of brain abnormalities in rats (Chen et al., 1972; Johnston & Twitchin, 1977; Mushahwar & Koeppe, 1963; O'Neal et al., 1968) and chicks (Arscott & Harper, 1963), possibly through inhibiting reuptake of GABA (Beart & Bilal, 1977; Main & Rodgers, 2018). High concentrations of BMAA reduced the life span in *Drosophila* models, decreased



β-N-methylamino-L-alanine

BMAA



L-2,4-Diaminobutyric acid

DABA

FIGURE 1 Chemical structures of the known neurotoxin BMAA and its isomer DABA

locomotor function, decreased learning and memory, reduced fertility in females, and delayed neurological impairment in aged adults (Zhou, Escala, Papapetropoulos, & Zhai, 2010). All of these studies were done using relatively high concentrations (generally with 1 mM as the lowest investigated concentration) of BMAA or DABA. The toxicity of a low-dose exposure to DABA has not been systematically evaluated in vitro or in vivo. The toxicity of low concentrations of DABA are of interest to our groups as we propose using it as a component of a targeted, traceless, drug delivery capsule.

Targeted drug delivery is an ever-expanding field in the biomedical sciences (Arpicco et al., 2016; Bertrand, Wu, Xu, Kamaly, & Farokhzad, 2014; Noble, Stefanick, Ashley, Kiziltepe, & Bilgicer, 2014; Ulbrich, et al., 2016). Current methods have several drawbacks, and self-immolative materials may provide a solution to this problem by allowing for complete degradation to small molecules; however, current systems are not sufficiently biocompatible (Fan & Gillies, 2015; Fan et al., 2016; Fan et al., 2018). Self-immolative materials are compounds that depolymerize, unit-by-unit, when exposed to a particular stimulus. The polymer we are seeking to create generates DABA as a product and we seek to evaluate its toxicity and compatibility for this application in an animal model.

Zebrafish have emerged as a highly promising screening system for environmental toxicity (Hill, Howard, Strahle, & Cossins, 2003; Hill, Teraoka, Heideman, & Peterson, 2005; Ton, Lin, & Willett, 2006; Van den Bulck et al., 2011). For example, the related BMAA was recently tested on zebrafish to determine the impact of the compound on the development and progression of amyotrophic lateral sclerosis. At low BMAA levels, only zebrafish with a familial amyotrophic lateral sclerosis-sensitizing mutation were affected by BMAA, wild-type zebrafish remained unaffected (Powers, Kwok, Lovejoy, Lavin, & Sher, 2017). DABA toxicity has yet to be evaluated using zebrafish. Consequently, we report the effect of exposure to varying doses of DABA on zebrafish at different stages of maturation. In this paper, we describe our investigation into the toxicity of this material and address the suitability for its use in our biomedical self-immolative polymer technology.

2 | MATERIALS AND METHODS

2.1 | Synthesis of (*S*)-2,4-diaminobutanoic acid

Full synthetic details on the synthesis of DABA can be found in the accompanying supporting information. Samples of synthetic DABA are available for donation upon request for collaborative purposes, although the commercial cost has dropped more than 10-fold since we initiated this study.

2.2 | General experimental protocols

Solvents were purchased from Caledon Labs, Sigma-Aldrich or VWR Canada. Other chemicals were purchased from Sigma-Aldrich, AK Scientific, Oakwood Chemicals, Alfa Aesar or Acros Organics and were used without further purification unless otherwise noted. All heated reactions were conducted using oil baths on IKA RET Basic stir plates equipped with a P1000 temperature probes. Thin-layer chromatography was performed using EMD aluminum-backed silica 60 F254-coated plates and was visualized using either ultraviolet light (254 nm), $KMnO_4$, vanillin, Hanessian's stain or Dragendorff's stain. Column chromatography was carried out using a standard flash technique with silica (Siliaflash-P60, 230-400 mesh; Silicycle) using compressed air pressure. Standard work-up procedure for all reactions undergoing an aqueous wash involved back extraction of every aqueous phase, a drying of the combined organic phases with anhydrous magnesium sulfate, filtration either using a vacuum and a sintered-glass frit or gravity through glass wool, and concentration under reduced pressure on a rotary evaporator (Buchi or Synthware) at 40°C. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 300 or 500 MHz, and ^{13}C NMR spectra were obtained at 75 or 125 MHz on Bruker instruments. NMR chemical shifts (δ) are reported in ppm and are calibrated against solvent residual signals of $CHCl_3$ (δ 7.26), dimethyl sulfoxide-d5 (δ 2.50), HDO (δ 4.79) or methanol-d3 (δ 3.31).

2.3 | Cell culture

HepG2 liver cancer cells were purchased from the American Type Culture Collection: The Global Bioresource Center. The U251 glioma cell line was a kind gift from Dr. Rutka (The Hospital for Sick Children Research Institute, University of Toronto). HepG2 cells were cultured in Eagle minimum essential media supplemented with 10% fetal bovine serum and 30 000 units penicillin/30 000 μg streptomycin solution. U251 cells were cultured in DMEM media supplemented with 10% fetal bovine serum and 30 000 units penicillin/30 000 μg streptomycin solution. Cells were maintained under normoxic conditions (5% CO_2) at 37°C.

2.4 | MTT analysis

HepG2 and U251 cells were seeded at 8000 cells/well in 96-well plates. DABA was added at the indicated concentrations along with

fresh media daily for 4 days. On the fifth day, 0.6 mg/mL MTT reagent was added to the cells and incubated at 37°C for 3 hours. The quantity of viable cells was measured by recording the changes in absorbance at 570 nm using a plate reading spectrophotometer.

2.5 | Animal care and handling

Wild-type zebrafish (*Danio rerio*), AB strain, were handled in compliance with local animal care regulations and standard protocols for Canada and following the University of Windsor animal care protocol no. 16-04. The original colony was formed through breeding of zebrafish purchased from Pet Wise Pet Supplies store. Adults were housed in an Aquaneering zebrafish housing system. Adult fish were kept at 26°C, pH 7.6, dissolved oxygen 5.1 mg/L with a 14:10 light/dark cycle. Adult fish were bred according to standard protocols (Westerfield, 1993).

2.6 | Zebrafish treatment and analysis

Eggs were collected after fertilization and kept in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.0003% methylene blue) at 32°C in an incubator; pH 7 and under a 14:10 light/dark cycle (hours).

2.6.1 | (*S*)-2,4-Diaminobutanoic acid exposure

A stock solution of 55 mM of DABA in deionized water was diluted to the indicated measured concentrations (10, 50, 100, 300, 400, 500, 600, 700 μM, 1, 2, 3.5 and 4 mM) in system water. At 18 hours post-fertilization (hpf), eggs were placed in multiple 96-well plates, one egg per well. In total, 10 eggs per replicate (30 eggs per treatment total) were utilized to determine hatching ability. Eggs were treated with 100 μL of the indicated concentrations of DABA daily, and water and DABA was changed every 24 hours. The number of viable embryos and the number of unhatched eggs were counted and imaged using a Leica Stereoscope in this unblinded study. At 3 days post-fertilization, embryos were placed one fish per well in 96-well plates and treated with 100 μL of the indicated diluted concentrations of DABA daily. In total, 30 embryos per concentration were used with 10 embryos per replicate for each endpoint readout. Viability, health and heart beat were quantified and imaged. Heart beat was measured by removing each embryo from its well in the 96-well plate and placed on a slide with 40 μL of embryo water. Heart beat was monitored microscopically for 1-minute intervals and recorded. The pigmentation level was determined using ImageJ software through the creation of a histogram for each embryo at each concentration. The mean pixel level was determined as an average of the pigmentation intensity for each fish (30 fish per treatment were analyzed). At both 1 month and 3 months of age, 10 fish per replicate were retrieved from a 3 L stock tank and were placed in a smaller 1 L tank and treated with 500 mL of the indicated concentrations of DABA daily and their viability was assessed.

2.6.2 | Daily water changes

Water changes from 96-well plates were performed using a micropipettor, and then waste water was disposed of in a glass container labeled for cytotoxic waste. Water changes for adult fish were performed by removing the fish from one container and transferring them into a new container with 500 mL of fresh water dosed with the indicated concentration of DABA.

2.7 | Histological methods

Following the analysis of fish at 1 month, fish were humanely killed and then embedded in paraffin. Briefly, fixation of fish was done overnight in 4% paraformaldehyde in phosphate-buffered saline at 4°C. Fish were then washed in phosphate-buffered saline and embedded (embryo) or stored in 20% EDTA (pH 8.0) for 10 days before embedding. Paraffin embedding was carried out according to standard procedures, and 8 μm sections were cut. For histology, sections were dewaxed, dried and stained with hematoxylin and eosin (H&E). H&E was carried out according to the manufacturer's protocol: 3-minute incubation followed by 10-minute washing in running tap water.

2.8 | Spinning task assay

To assess 3-month-old adult zebrafish swimming endurance, we performed the spinning task assay as previously published with minor modifications (Blazina, Vianna, & Lara, 2013). We amended the protocol, lowering the rpm of rotation, to account for the smaller size and decreased swimming ability of our 3-month-old fish compared with either the >8-month fish Blazina and colleagues used, or the 5-month-old fish examined by Powers and coworkers (Powers et al., 2017). Fish were tested in a 1 L opaque beaker containing 800 mL of DABA diluted in system water to the required concentration. The water was heated to 26°C and placed on a hot plate stirrer at the indicated rpm (VWR; no. 12365-382). Zebrafish were acclimatized to the beaker for 10 minutes before testing. Three tests were performed in which zebrafish were monitored and timed as they swam against a current generated by a 9 × 50 mm bar spinning at 100 rpm. Swimming time was recorded using a stopwatch and was defined as the amount of time before the fish were swept up by the whirlpool current created by the stir bar.

2.9 | Statistical analysis

All experiments were performed in triplicate and repeated three times. All results are expressed as the mean ± standard error of the mean. Statistical analysis was done using the GraphPad Prism 5.01 software (GraphPad Software). Differences were compared using one- or two-way ANOVA followed by Tukey multiple comparison tests to compare differences among multiple groups. Differences with $P < .05$ were considered significant.

3 | RESULTS

3.1 | (*S*)-2,4-Diaminobutanoic acid does not affect human cancer cells at high concentrations

Most early-stage toxicological tests of potentially biomedically relevant materials involve easy to conduct high-throughput cell viability assays (Patrick, 2017). As DABA is known to influence liver and neural functions, we tested whether DABA could alter the metabolic activity of human liver (HepG2) and brain (U251) cancer cells. Cells were seeded in 96-well plates and treated over 4 days with DABA, followed by MTT analysis. Neither cell type showed any response to the addition of DABA (Figure 2); DABA appears neither to accelerate cancer cell growth nor to cause any cytotoxicity at these doses. To test the potential effects at the organ or organism-level, we moved to examine the effects in vivo using a zebrafish model.

3.2 | (*S*)-2,4-Diaminobutanoic acid has toxicological effects in zebrafish in vivo at high concentrations

We know from the work of others that DABA has deleterious effects at very high concentrations (>1 mM). To determine long-term health effects of DABA at lower concentrations on vertebrate viability and toxicity, we employed the *D. rerio* (zebrafish) in vivo model at various life stages with exposure periods of between 2 and 7 days. After 42 hours of treatment, the hatching ability of the embryos from their chorion had become affected at the higher concentrations (Figure 3A, 400-700 μM). Even after 3 days of treatment, approximately 90% of the embryos treated with 700 μM did not hatch from the chorion (Figure 3A).

As the concentration of DABA was increased, developmental abnormalities were seen, including curved spine, yolk sac edema and a separated portion of the yolk sac (Figure 3B and 3C). At a lower exposure concentration of 300 μM DABA, 60% of fish showed signs of pericardial edema and yolk sac edema, while both control fish and those treated with ≤200 μM of DABA showed no signs of edema (Figure 3B). At higher concentrations, the rate of abnormalities seen increased to 100% at 700 μM, including those who were manually unhatched from the chorion. This extremely sharp demarcation in the dose-response curve is unusual. Embryos at the lower concentrations, 10, 50, 100 and 200 μM and even most of those at 300 μM, developed normally after hatching with no apparent ill effects from exposure to DABA.

3.3 | (*S*)-2,4-Diaminobutanoic acid does not alter the pigmentation of zebrafish

Lack of pigmentation can be indicative of abnormal development, and overpigmentation can be associated with cancer (Lister, 2002). This makes it a useful screening tool for gross toxicity. Following hatching, treated zebrafish were analyzed for altered pigmentation through ImageJ analysis of pictures taken of 30 different fish per treatment. These measurements and calculations determined that there is no meaningful difference in pigmentation levels (<4% variance), or any observable trend, regardless of DABA dose at the tested concentrations (Figure S1; see Supporting Information).

3.4 | (*S*)-2,4-Diaminobutanoic acid affects viability of embryo and adult zebrafish

To determine viability after hatching, six groups of fish at 3 days post-fertilization were treated with increasing concentrations of DABA for up to 7 days. Fish were evaluated for viability as well as for being stressed. Stress was defined as either having trouble breathing, swimming or remaining upright. After 1 day of treatment, approximately 81% of the fish survived at 700 μM while no fish survived at 3.5 mM (Table 1). By the second day, no fish were alive at 2 mM, but no further deaths were observed at 700 μM (Table 1). At the end of treatment, 700 μM and 1 mM showed similar viabilities with only 25%-28% death, 3%-18% stressed and 56%-68% healthy (Table 1). However, at the lower concentration of 300 μM, few ill effects were observed, and the single observed death is within experimental error.

Next, to determine the toxicity on mature fish, populations aged from 1 month (juvenile) to 3 months old (adulthood), were treated with 500 mL of water with DABA concentrations ranging from 0 to 4 mM. The 1-month-old fish treated with 700 μM of DABA remained

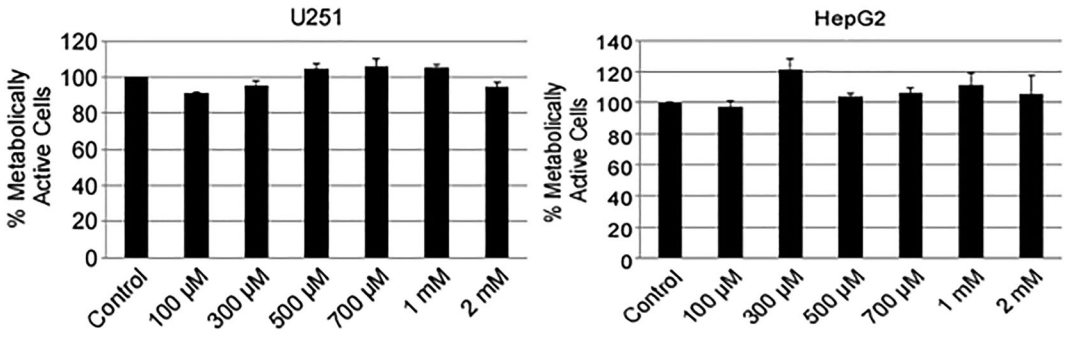


FIGURE 2 Human U251 and HepG2 cells do not change metabolic activity rate in vitro with DABA. U251 and HepG2 cells were treated with increasing concentrations of DABA and subjected to MTT assay. Error bars reflect SE between at least three experiments. One-way ANOVA was performed, and no statistical difference was found between any of the treatments; $P > .05$. DABA, (*S*)-2,4-diaminobutanoic acid

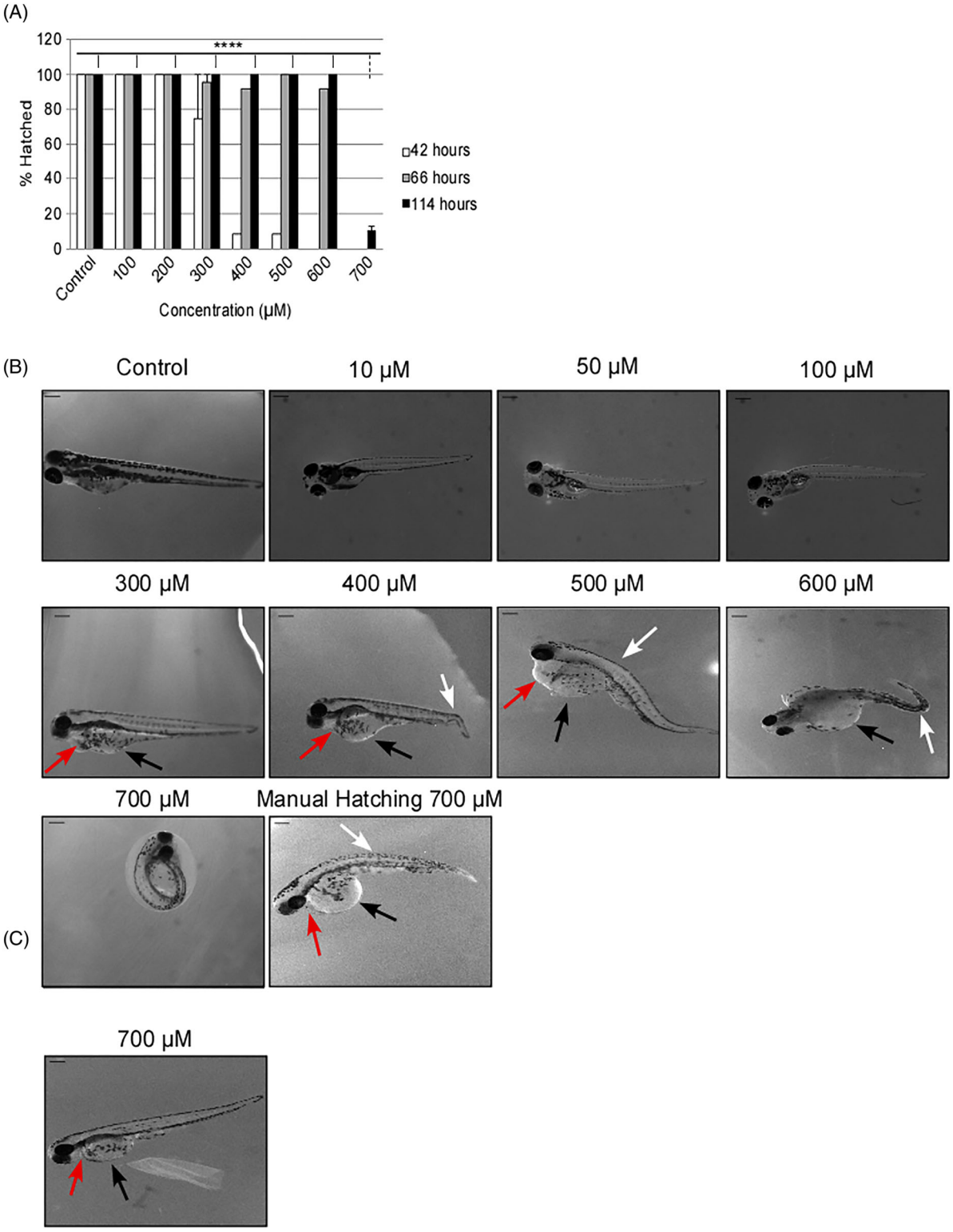


FIGURE 3 Variability in hatching ability and morphology of zebrafish embryos with increasing DABA concentrations. A, Zebrafish embryos were treated 18 h post-fertilization with increasing concentrations of DABA and monitored for hatching ability. Time refers to hours post-fertilization. Error bars reflect SE between at least three independent experiments, n = 30 fish/treatment. Two-way ANOVA was performed; ****P < .0001. Solid vertical line indicates the samples are identical to one another but different from the hashed line. B, After hatching, fish were incubated with DABA at the same concentration they had been exposed to in the embryo for an additional 72 h. Fish were imaged to identify developmental anatomical abnormalities. This is a separate experimental group from those used in the experiment described in (A). The images are typical of the fish at the labeled concentrations; pericardial edema (red arrow), yolk-sac edema (black arrow) and curved spine (white arrow) are indicated on figure. n = 30 fish/treatment, scale bar = 250 μm. C, Zebrafish after treatment with 700 μM DABA showing pericardial (red arrow) and yolk-sac edema (black arrow). Scale bar = 250 μm. DABA, (*S*)-2,4-diaminobutanoic acid

TABLE 1 Viability of embryonic fish is affected with increasing DABA concentrations

		Alive (%)	Stressed (%)	Dead (%)
Day 1	Control	100	0	0
	100 μM	100	0	0
	300 μM	92.4	7.6	0
	700 μM	81.5	8.1	10.3
	1 mM	56.1	26.8	17.1
	2 mM	21.7	0	78.3
	3.5 mM	0	0	100
Day 2	Control	100	0	0
	100 μM	100	0	0
	300 μM	98.0	2.0	0
	700 μM	73.6	14.9	11.5
	1 mM	73.2	9.8	17.1
	2 mM	0	0	100
Day 3	Control	100	0	0
	100 μM	100	0	0
	300 μM	100	0	0
	700 μM	80.4	6.9	12.7
	1 mM	73.2	4.9	22.0
Day 7	Control	100	0	0
	100 μM	100	0	0
	300 μM	83.1	13.0	3.9
	700 μM	56.6	18.2	25.3
	1 mM	67.8	4.9	27.3

DABA, (*S*)-2,4-diaminobutanoic acid.

Zebrafish embryos (3 days post-fertilization) were placed one embryo per well of a 96-well plate and treated with indicated DABA concentrations (100 μL per well). Concentrations of DABA were maintained both before and after hatching. Fish were monitored for viability, stress and death after each 24-h period. A stressed fish was defined as any fish that was having trouble breathing, swimming or remaining upright

viable over all the 3 days of treatment. The fish exposed to 1 mM of DABA showed 100% viability at 24 hours; however, no fish survived after 48 hours (Figure 4A). We examined both juvenile and adult fish brains and livers following treatment to determine whether we observed any physical abnormalities. No gross difference in liver or brain structure was seen between control and treated fish using H&E staining in any of the examined samples (representative images provided as Figure 4B). Neurotoxicity and hepatotoxicity are often associated with abnormalities in these organs during development.

The 3-month-old fish responded very differently. Within 15 minutes of being treated with 4 mM DABA, 0% was viable, after 45 minutes 0% viability was seen for 1 and 2 mM DABA, and after 1.5 hours 0% of fish was viable at 700 μM; however, 80% of fish were viable at 500 μM DABA (Table 2). After 24 hours, no fish remained viable in the presence of 500 μM; however, those exposed to DABA at either 100 or 300 μM remained 100% viable. We continued to monitor these two groups of fish for an additional 72 hours. These 3-month-old fish exposed to these lower levels of DABA did not suffer any deaths over the 4-day experiment. These viability data indicate that the ability of the zebrafish to tolerate DABA from embryo to adult changes and that adults tolerate a much lower concentration of DABA than the embryos, but that there is a dose that shows no increase in mortality. Again, there is a very sharp cut-off in viability above 300 μM. These data demonstrate that the ability of the fish to tolerate DABA changes from embryo to adult stage. Interestingly this occurs without direct gross effects to the cells of the liver or brain, suggesting that the mechanism of toxicity may not be due to hepatotoxicity or neurotoxicity as reported with higher concentrations of DABA.

3.5 | Zebrafish swimming ability affected with increasing (*S*)-2,4-diaminobutanoic acid concentration

To determine if a short exposure of DABA could have an effect on the swimming ability, endurance and motility of the zebrafish, we modified the established spinning task protocol (Blazina et al., 2013). To quantify endurance, 10 zebrafish were selected from each treatment group and tested in three separate trials. A current was generated from a 100 rpm stir bar in 800 mL of system water or increasing concentrations of DABA diluted in system water. Once the zebrafish were swept up by the current and lost their orientation, the stopwatch was stopped and the time was recorded. The control group and that subjected to the lowest DABA concentration (100 μM) had identical success swimming against the current, and their latency was longer than that of the fish subjected to higher DABA concentrations (300 and 500 μM) (Figure 5). These data support that short-term exposure of higher DABA concentrations impairs zebrafish motility.

3.6 | Heart beat affected after exposure to high levels of (*S*)-2,4-diaminobutanoic acid

Following treatment, potential sublethal effects of DABA were investigated by measuring the average heart beat of the zebrafish embryos at control (0), 100, 300 and 700 μM concentrations. The average heart beat in the studied populations of embryos did not significantly change between control and 100 or 300 μM, staying consistent at 120-130 beats per minute (bpm) (Figure 6). This heart rate is consistent with typical zebrafish embryonic heart-beat rates (De Luca et al., 2014). However, at 700 μM there is a slight, but not significant, increase in bpm after 72 hpf compared with control treated fish. After 96 hpf, we see a significant increase in bpm, followed by a significant decrease at 126 hpf (Figure 6).

4 | DISCUSSION AND CONCLUSION

Although not envisioned to be used as a drug itself, the biological impact and toxicity of DABA is of significant interest as it will form part of our drug delivery capsule. Our preliminary investigations indicate that the operational DABA concentration will be around 20 μM. Testing effects on cellular metabolism in vitro using human liver and brain cancer cells did not reveal any adverse effects at concentrations as high as 2 mM. This was encouraging, but required further testing using in vivo models. The related compound, BMAA has been shown to be incorporated into proteins in place of structurally similar L-serine (Dunlop, Cox, Banack, & Rodgers, 2013; Glover, Mash, & Murch,

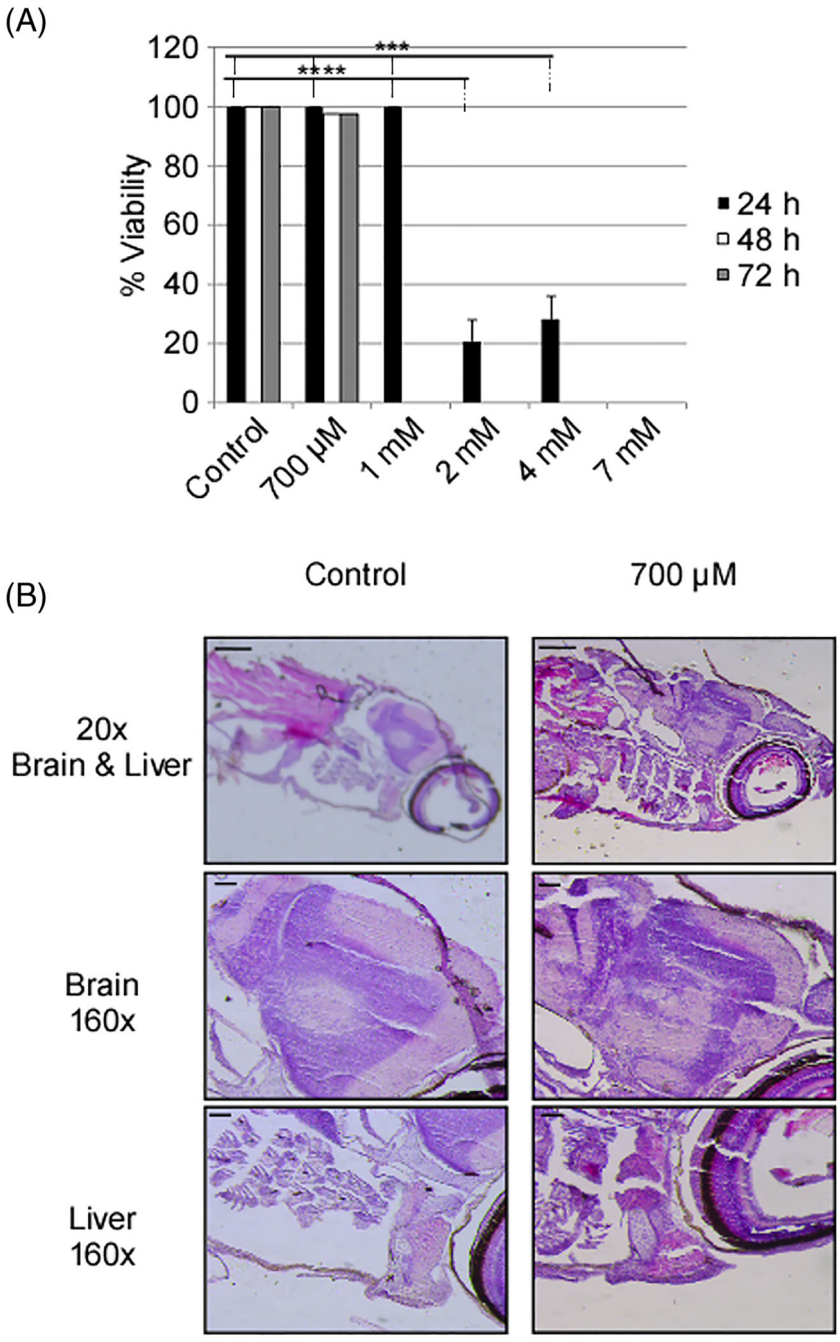


FIGURE 4 Juvenile and adult zebrafish are less tolerable to DABA. A, 1-month-old zebrafish were treated with increasing concentrations of DABA and monitored for viability. At the higher concentrations and longer exposure times, viability is 0% (represented by no visible bar). Error bars reflect SE between at least three experiments, n = 30 fish/treatment. One-way ANOVA was shown for the 24 h treatment (there is no difference between the control and 700 µM for the 48 and 72 h treatment groups); ***P < .0002, ****P < .0001. Solid vertical line indicates the samples are identical to one another but different from the hashed line. B, Juvenile zebrafish were subjected to hematoxylin and eosin staining following treatment. 20× image shows an overview of both the brain and liver, while a 160× zoom is provided to show detail for both the brain and liver areas. Scale bar (20×) = 1 mm, scale bar (160×) = 200 µm. DABA, (*S*)-2,4-diaminobutanoic acid

2014) resulting in cellular toxicity, but DABA has never been detected in proteins, and does not appear to work through this mechanism (Glover et al., 2014). Instead, it is reasonable to consider that toxicity might be due to its structural similarity to GABA. At high concentrations, DABA has been shown to interfere in an irreversible noncompetitive manner with carrier-mediated transport of GABA in rat synaptosomes (Levi, Rusca, & Raiteri, 1976) acting as a reuptake inhibitor; therefore, it is not unreasonable to believe that toxicity proceeds through a systems-level mechanism that would not be detectable using a traditional cell viability assay.

Testing systemic effects requires the use of an animal model, and the high-throughput nature of zebrafish with fast maturation, low cost and small size, made them highly appealing. Using this in vivo toxicity model, we have demonstrated that DABA affects the viability in both adult and embryonic zebrafish. Cardiotoxicity becomes a concern at concentrations of ≥300 µM but has no observable effect below this value. The ability of zebrafish to hatch from their chorion was affected at 700 µM DABA. Although beyond the scope of this study, it is possible that DABA interferes with the proteolytic enzymes required for the chorion softening for hatching to occur (Kim et al., 2004). Following hatching, morphological changes were seen in embryos treated with increasing concentrations of DABA above 300 µM. Developmental abnormalities were seen in the embryos, including curved spine, yolk sac edema and a separated portion of the yolk sac. Again, no effect is observed below this concentration threshold. The cause for these abnormalities is unknown; however, it is

TABLE 2 Viability of 3-month-old fish is affected with increased DABA

Concentration	Time	% Viable
Control (0 μM)	96 h	100 100
100 μM	24 h 96 h	100 100
300 μM	24 h 96 h	100 100
500 μM	45 min 1.5 h 24 h	100 80 0
700 μM	45 min 1.5 h	100 0
1 mM	15 min 45 min	100 0
2 mM	15 min 45 min	100 0
4 mM	15 min	0

DABA, (*S*)-2,4-diaminobutanoic acid.

Adult zebrafish (3 months old) were treated with increasing concentrations of 500 mL DABA and monitored from 0 to 96 h (every 15 min for the first 1.5 h, then at 24, 48 and 96 h). Data represent selected timepoints and illustrates the dose-dependency of adult zebrafish viability.

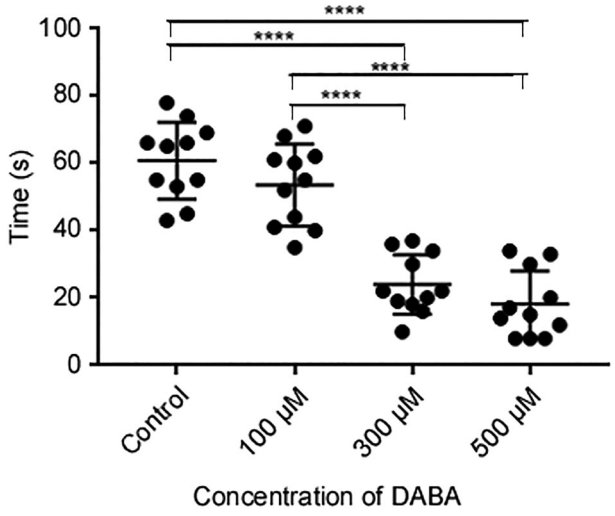


FIGURE 5 Swimming ability is altered in high DABA concentrations. 3-month-old zebrafish were treated with DABA and monitored for swimming ability and endurance by spinning task protocol. Latency of swimming against the current was recorded. Error bars reflect SE between at least three experiments, n = 10 fish/treatment. One-way ANOVA was performed; ****P < .0001. Solid lines indicate that the two samples are different from one another (control and 100 μM are statistically identical, as are 300 and 500 μM; however, both members of both pairs are distinct from both members of the other pair). DABA, (*S*)-2,4-diaminobutanoic acid

consistent with the observation of developmental abnormalities in other animal systems, such as the development of crooked toes when DABA was fed to chicks at high concentrations (Arscott & Harper, 1963). DABA clearly causes developmental abnormalities at high doses for young and embryonic organisms; however, we observe no ill effects whatsoever below a threshold dose (<300 μM). These concentrations (100-300 μM) are approximately an order of magnitude higher than our expected therapeutic concentrations. The toxicity profile of the DABA on developing and adult zebrafish is also essential to delineating the potential safety profile of this material. Interestingly, histology showed no gross structural abnormalities in the brain or liver of fish treated with DABA, which was shown to cause brain abnormalities and liver toxicity in rats, albeit at much higher concentrations (Mushahwar & Koeppe, 1963; O'Neal et al., 1968). Perhaps the lower dosages did not result in these abnormalities or they did not develop within this time frame. Behavioral analysis showed that zebrafish swimming ability and motility was affected even after short-term exposure to DABA above the threshold indicating the possible interference with the GABA reuptake that can alter cognitive ability. As far as we are aware, this is the first report identifying a lowest-observed-effect concentration for DABA toxicity in any animal system and we cannot find any reports using such low levels of the amino acid. We observe no differences from control at concentrations <300 μM: our lowest-observed-effect concentration for DABA in zebrafish is consequently 300 μM and this preliminary value is supported by the multiple independent measurements conducted.

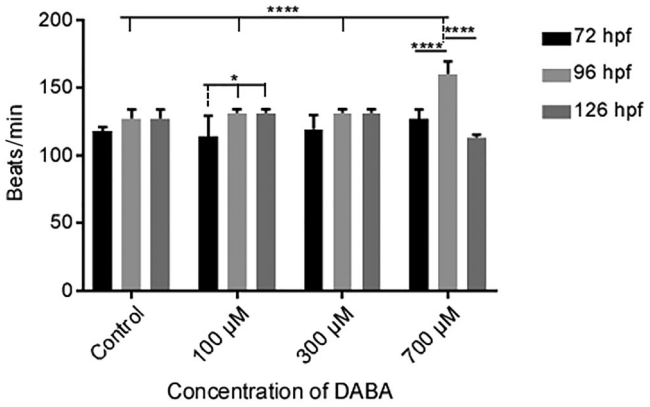


FIGURE 6 Heart beat is affected when exposed to highest DABA concentration. 72-hpf zebrafish were treated with increasing concentrations of DABA and heart beat was measured over 5 days. Error bars reflect SE between at least three experiments, n = 30 fish/treatment. One-way ANOVA was performed; *P < .01, ****P < .0001. Solid vertical line indicates the samples are identical to one another but different from the hashed line. DABA, (*S*)-2,4-diaminobutanoic acid; hpf, h post-fertilization

As the zebrafish were able to tolerate the threshold concentration of 300 μm, we were not surprised to see a steady heart rate across the lower concentrations (0-300 μM). However, as the heart rate of fish exposed to higher concentrations of DABA were elevated, it is possible that at these low micromolar concentrations, toxicity occurs through some form of metabolic stress. This observation is completely consistent with the suspected GABA receptor inhibition (DiMicco, Gale, Hamilton, & Gillis, 1979). The goal of this study was to determine whether DABA is toxic at 20 μM, which was the concentration of interest for our application. The effects are not observed at this low

level. Although beyond the scope of this study, we believe that this model could prove useful for examining the mechanism of toxicity. Known GABA receptor inhibitors could be screened to determine whether the same impacts on zebrafish viability, hatching ability, heart rate and behavior are observed as noted in this study. Further work from our group examining the toxicity will be necessary as the biomedical application development advances; particularly regarding the interactions of DABA with the GABA receptors and the two isoforms of glutamic acid decarboxylase (GAD65 and GAD67). However, the stage of the technology development is still too preliminary currently to justify these studies.

This project highlights the importance of integrating appropriate multi-organ models, such as the zebrafish, early in a toxicology screen. Nevertheless, as the effective concentrations expected to be used for our anticipated biomedical applications will be on the order of 20 μM, all the data collected in this study indicate that the compound deserves further development and investigation. Additional toxicity data will need to be gathered on the final formulation. This is a very early-stage toxicity screen for the material. It demonstrates both the insufficiency of using in vitro cell-based assays alone for suspected systems-based toxicity and that zebrafish are an inexpensive and very useful tool for early-stage toxicological evaluation of potential biomedically employed chemical materials before resources are spent developing technologies that are unacceptably toxic.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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